# Nitric Oxide-Releasing Nonsteroidal Anti-inflammatory Drugs: Novel Gastrointestinal-Sparing Drugs

Upul K. Bandarage* and David R. Janero

*NitroMed, Inc., 12, Oak Park Drive, Bedford, MA 01730, USA*

**Abstract:** Nonsteroidal anti-inflammatory drugs (NSAIDs) have unacceptable morbidity and mortality due to their gastrointestinal toxicity. Attempts so far to improve the safety profile of NSAIDs have met with limited clinical acceptance. Nitric oxide (NO) functions as an endogenous mediator of gastric mucosal health and defense. Recent medicinal chemistry approaches attempt to exploit the tissue-protective function of NO against NSAID-induced gastric injury. Both nitroxybutyl-ester and nitrosothiol NSAID derivatives have been synthesized. Profiling of these NO-donating NSAIDs in both the laboratory and the clinic suggests that they might offer a unique solution to the problem of NSAID-induced gastropathy without sacrificing the well-accepted pharmacological activity of these agents in the management of pain and inflammation.

## INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of pain, fever, and inflammation [1]. The worldwide NASAID market for both occasional and chronic users has been conservatively estimated at over 60 million people, and certain NSAIDs (aspirin, naproxen, ibuprofen) are among the most popular over-the-counter medications [2, 3]. Chronic NSAID therapy effectively reduces the symptoms of many painful arthritic syndromes, but invites adverse gastrointestinal (GI) complications ranging from stomach irritation to life-threatening GI ulceration, bleeding, and perforation to more serious small-bowel ulceration [4-6]. At the tissue level, the most common clinical manifestation of NSAID-related GI damage is a combination of gastroduodenal erosions and ulcerations often called NSAID-induced gastropathy [5, 6], affecting at least 25% of chronic NSAID patients. NSAID-induced gastropathy may limit long-term NSAID therapy and cause a significant financial burden to the healthcare system [5-9].

During the 1990's, several structurally-diverse compounds displaying gastroprotective properties in laboratory animal models were identified, but with limited clinical impact as concomitant therapy against NSAID-induced gastropathy [10-13]. The challenge still exists in the pharmaceutical industry to develop safer, effective anti-inflammatory agents with enhanced safety profiles. Recently, two strategies have emerged as state-of-the-art approaches to improve the NSAID safety profile: (a) selective inhibitors of the cyclooxygenase (COX) enzyme isoform, COX-2, induced in the setting of inflammation; and (b) NSAIDs capable of generating the radical biomediator and gastroprotective agent, nitric oxide (NO). COX-2 inhibitor chemistry, preclinical and clinical biology, and commercialization have been recently summarized elsewhere [14-16]. This review focuses on NO-releasing NSAIDs by first summarizing the pathogenesis of NSAID-induced gastropathy and the physiology of NO as a mediator of gastric homeostasis and GI tissue health. The latest developments in the area of NO-NSAID medicinal chemistry will then be detailed.

*Address corresponding to this author at NitroMed, Inc., 12, Oak Park Drive, Bedford, MA 01730, USA; Tel: (781) 685-9723; Fax: (781) 275-1127; E-mail: ubandarge@nitromed.com

## NSAID-INDUCED GASTRODUODENAL MUCOSAL INJURY

The mammalian GI system may be conceptualized as a segmentally differentiated tube ("alimentary canal") to optimize nutrient absorption and waste excretion from ingested foodstuffs [17, 18]. Along the GI tract, a mucosal lining functions as the dynamic interface between the deeper layers of the tissue wall and the lumen content. In the stomach, the mucosa and the surface layer of cells lining (the gastric epithelium) are interposed between the deeper, blood vessel-rich layers of the muscular stomach wall and the gastric content being digested within the strongly acidic stomach lumen. The mucosal lining of the GI tract, particularly in the stomach, is exposed continuously to potentially damaging agents such as acids, microbial toxins, bile, and digestive enzymes [18].

Several endogenous mechanisms help to maintain the integrity and restitution of the gastric mucosa and defend it from potential injury [19, 20]. The mucus gel layer secreted by mucosal cells acts at the gastric epithelium as a barrier to acidic (pH ≈ 2) gastric juice and noxious substances such as alcohol, bile acids, and digestive enzymes. The mucus gel layer thereby helps to maintain the tissue pH (≈ 7.2) of the cells in the stomach wall and reduce mechanical tissue trauma during food digestion [21]. A second major contributor to gastric protection is locally secreted bicarbonate, which serves to neutralize the acidic gastric juice and maintain organ acid/base balance [22]. A third critical element in gastric homeostasis is perfusion of the

1389-5575/01 $20.00+.00 

stomach wall with nutritive, oxygenated blood at a level sufficient to support normal cellular physiology and remove unnecessary, if not potentially damaging, substances from the tissue [23]. Finally, mucosal prostaglandins (PGs), support several gastric defense mechanisms by inhibiting stomach acid secretion, promoting mucus and bicarbonate secretion, and enhancing gastric mucosal blood flow [24]. PGs additionally exert a cytoprotective effect upon the gastric mucosa independent of their influence upon gastric acid secretion [24, 25].

The chronic use of NSAIDs compromises the mucosal defense system and elicits NSAID-induced gastropathy by multiple mechanisms involving local (topical) and systemic effects [9,19]. NSAID-induced topical mucosal damage may be caused directly by an ion-trapping mechanism. Most NSAIDs are weak organic acids ($pK_a \approx 3$-5) bearing a free carboxylic acid group under moderately acidic or neutral conditions. The lipophilicity of most NSAIDs allows them to diffuse readily through the gastric mucus and into gastric epithelial cells. The cytoplasmic pH (≈ 7.2) favors intracellular NSAID dissociation to water-soluble ionized forms, resulting in trapping of hydrogen ions. In this way, a significant NSAID concentration gradient is established across the epithelial-cell plasma membrane and causes back-diffusion of damaging, acidic gastric juice in an attempt to reduce the tissue load of free NSAID ions. This results in increased membrane permeability of gastric epithelial cells [9, 19]. Topical irritation is considered an important factor in establishing superficial stomach erosion, particularly in the corpus region of the stomach.

Systemic effects of NSAIDs appear to have a predominant role in the tissue pathologic response, mainly reflecting a reduction in the constitutive biosynthesis of PGs that serve as cytoprotective mediators in the GI system [1, 9, 19]. The principal therapeutic effects of NSAIDs reflect their inhibiton of COX enzymes catalyzing PG production. Most currently used NSAIDs nonselectively inhibit the two known COX isoforms, the constitutive COX-1 enzyme and the enzyme induced in settings of inflammation, COX-2, or have some COX-1 selectivity [1]. It has been suggested that the anti-inflammatory properties of NSAIDs are mediated through the inhibition of COX-2, whereas the simultaneous inhibition of COX-1 is responsible for adverse GI side-effectsconsequent to a reduction in the constitutive production of cytoprotective PGs. Inhibition of gastric PG (particularly $PGI_2$ and $PGE_2$) synthesis promotes stomach acid secretion, reduces bicarbonate and mucus production, and restricts mucosal blood flow—responses that counter gastric defense and could predispose stomach tissue to damage [25]. Enhanced adherence of activated neutrophils to the gastric vascular endothelium in regions of low mucosal blood flow may exacerbate and amplify tissue damage in a pro-inflammatory manner through the release of oxygen-derived free radicals and proteases [26]. There has been only limited market acceptance of therapy combining a NSAID with a PG-related cytoprotectant due to bothersome side effects and contraindications at optimal therapeutic doses of the prototype, misoprostol [12].

Pathogenesis of GI injury in any given case of NSAID-induced gastropathy likely reflects a multifactorial tissue insult from NSAID ion-trapping, NSAID-mediated topical irritation, and the deleterious consequences of reduced tissue PGs due to NSAID inhibition of COX-1 [1, 3, 5, 6, 19]. Whatever differences of opinion there may be about the exact pathogenesis of NSAID-induced gastric injury or even about the ultimate actions of NSAIDs that contribute to their therapeutic efficacy, increasing recognition is being given to NO as a critical endogenous mediator of gastric mucosal defense. As addressed in the next section, attempts are being made to harness NO for therapeutic benefit against NSAID-induced GI damage and its undesirable clinical symptoms.

## NO AS BIOLOGICAL MEDIATOR: GASTRIC MUCOSAL DEFENSE

Although first described in 1980 as an endothelium-derived vascular relaxing factor [27], NO is now recognized as a ubiquitous signaling molecule able to elicit a wide variety of biological responses. The details of NO biology are beyond the scope of this article and have been considered elsewhere [28-36]. Nonetheless, a few general concepts concerning the physiological chemistry of NO merit highlighting from the perspective of NO-related therapeutics.

NO is a highly reactive colorless gas, one of three inorganic nitrogen monoxide species chemically related to the nitrosating agent nitrosyl ($NO^+$) and the anion oxonitrate(1-) ($NO^-$) by one-electron redox chemistry [35].

$$NO^+ + e^- \rightarrow NO + e^- \rightarrow NO^-$$

The biology of NO largely reflects the formation and molecular reactivity of NO as a paramagnetic radical [36]. Cellular NO generation is enzymatically controlled. In mammals, NO is produced exclusively by the three distinct enzyme systems called nitric oxide synthase (NOS) (EC 1.14.13.39) from L- arginine [37-39]. Those three distinct NOS isoforms are thought to serve diverse functions, particularly in the nervous, cardiovascular, and immune systems. NOS I (also called neuronal NOS, nNOS) and NOS III (endothelial NOS, eNOS) are constitutive enzymes (cNOS isoforms) responsible for providing the relatively modest (nanomolar), steady-state levels of NO important for normal cell function and tissue protection against injurious insult. The nNOS isoform plays a role in neurotransmission, whereas eNOS in the vascular endothelium produces NO that acts as a vasodilatory regulator of blood pressure and vascular tone. The third NOS isoform, NOS II or iNOS, is not constitutively present in cells but appears to be expressed in prolonged inflammatory conditions. Local iNOS activation generates transient, high (micro-to-millimolar) NO levels responsible for the destruction of invading pathogens, as part of an overall inflammatory response.

NOS catalysis is dynamically regulated by a combination of cofactors and covalent enzyme modifications [37, 39]. Yet the distinctive chemical biology of the NO radical itself is the decisive determinant of its physiological impact [33, 35, 40]. In biological systems, where molecular oxygen and water are usually abundant, NO displays a short half-life of ≈ 2-3 sec because of its facile autooxidation to nitrite ($NO_2^-$).

$$2NO + O_2 \rightarrow 2NO_2$$

$$NO_2 + NO \rightarrow N_2O_3$$

$$N_2O_3 + H_2O \rightarrow 2HNO_2 \rightarrow 2NO_2^- + 2H^+$$

In the process, $NO_2$ and $N_2O_3$, are produced that appreciably extend the biological activity of NO through their ability to modify biomolecules. In contrast to these indirect routes of NO activity, heme proteins and superoxide anion radical ($O_2^{\cdot -}$) are directly responsible for important NO transformations that affect mammalian cell physiology. Generation of an iron-nitrosyl adduct from the avid reaction between NO and hydrated ferrous ion, $Fe_{aq}(II)$, is the basis of the classic pathway of NO-mediated signal transduction through guanylate cyclase activation and accounts for the activity of hemoglobin as a major sink for excess NO [34].

$$Fe_{aq}(II) + NO \rightarrow Fe_{aq}(II)\text{-}NO$$

Reaction of NO with superoxide ($O_2^{\cdot -}$) at physiological pH or lower can damage cells by consuming bio-regulatory NO and/or by producing peroxynitrite ($ONOO^-$) and its conjugate acid (ONOOH), oxidizing agents capable of destroying most biomolecules [28, 33].

$$NO + O_2^{\cdot -} \rightarrow ONOO^- + H^+ \rightarrow ONOOH$$

From the foregoing, NO and NO-derived metabolites can act either as physiological or pathological agents and may shift between these two roles, depending upon many peripheral factors such as the total amount of NO available for interaction with the target tissue, the rate-time parameters of NO formation, and the localization of the NO [40]. One physiological action of NO that has engendered increasing interest in the pharmaceutical industry is its cytoprotective effect, by which NO helps some tissues withstand injury from chemical and other insults. Although the exact mechanisms of NO-based tissue defense are often ill-defined [32, 35, 40], a tissue-protective action for NO in the GI system has definite therapeutic implications regarding NSAID-induced toxicity.

Both constitutive and inducible NOS isoforms are present in GI tissue and have been given particular study in the gastric mucosa [41-43]. NO produced endogenously by cNOS isoforms in GI tissue supports basic GI physiology [44]. The endogenous tissue NO generated constitutively by GI nNOS and eNOS appears to play a key role in the chronic maintenance of GI tissue integrity and in adaptive cytoprotection to injury stimuli, perhaps acting synergistically with other cytoprotective PGs [41, 45-47]. Two general lines of evidence support this conclusion. In the first, exogenously administered NOS inhibitors cause GI damage in rodents [46, 48]. Secondly, exogenously administered NO-releasing compounds (sodium nitroprusside, glyceryl trinitrate, *S*-nitroso-*N*-acetyl-penicillamine, and S-NO-glutathione) consistently reduce the severity of gastric mucosal damage induced by HCl, ethanol, and NSAIDs in rodents [46, 49-51]. Thus, both endogenous (i.e., tissue) and exogenous NO help maintain GI tissue health and protect it from adverse insult. As do PGs, NO supports or promotes several gastric defense mechanisms which are believed to be capable of minimizing chemical-induced GI injury by increasing mucus and bicarbonate secretion in the GI tract, increasing mucosal blood flow, and inhibiting the pro-inflammatory activities of neutrophils and platelets. Additionally, NO may reduce inflammation-associated oxidative stress by scavenging reactive oxygen species ($O_2^-$) which can adversely increase mucosal permeability and kill cells. Specifically, NO gastroprotection against the topical irritancy component of chemical insults such as ethanol and NSAIDs is believed to reflect NO-mediated vasodilation to increase GI blood flow, mucosal perfusion, and, hence, tissue washout [45-47, 50]. In these regards, NO plays an important role in the maintenance of adequate blood flow at the margin of an established ulcer, an effect that may help accelerate ulcer healing [52].

In summary, the biology of NO in the GI system includes key roles in the health, defense, and repair of the gastroduodenal mucosa. NO acts as a multifunctional gastroprotective mediator by influencing several aspects of gastric physiology, including mucus and bicarbonate secretion, blood flow in the GI wall, and tissue inflammatory responses.

## NITRIC OXIDE-GENERATING NSAIDs AS GI-SPARING DRUGS

Data that NO donors effectively reduce gastric mucosal damage and may facilitate GI healing following chemical insult [41, 45-51] have made NO a prime therapeutic focus for reducing NSAID induced gastropathy associated with chronic NSAID use. On a more theoretical level, the potential for prostaglandins and NO to act synergistically as gastroprotectants [41] could itself be used to rationalize development of NO-releasing NSAIDs in that they might compensate for NSAID-induced suppression of cytoprotective PGs synthesis [1, 6, 19, 25]. As first conceptualized by Wallace and colleagues [50], modern drug discovery has focused on one general approach in an attempt to commercialize the therapeutic potential of NO against NSAID-induced gastric damage: covalent modification of NSAIDs with NO-releasing moieties. The resulting molecules would potentially provide NSAID analgesic, antipyretic, and anti-inflammatory activities with NO-based gastroprotection and, as a result, show greater symptomatic benefit and an improved safety profile over the parent NSAID. For commercialization, such appropriately functionalized NSAIDs would likely be new chemical entities able to co-deliver both the NSAID and NO (Fig. **1**).

To this intent, NSAIDs have been coupled through an ester linkage to a NO-releasing moiety, yielding a "prodrug" susceptible to rapid in vivo hydrolysis, upon which the parent NSAID and NO are generated. As next discussed, two distinct chemical classes of NO-releasing NSAIDs have been synthesized and biologically evaluated: one incorporating a nitrate ($-ONO_2$) group as the NO-donor functionality, the other a *S*-nitrosothiol (-*S*-NO) group. In this review, the nitrate-bearing NSAIDs are termed NO-NSAIDs, whereas the nitrosothiol NSAID derivatives are termed SNO-NSAIDs.

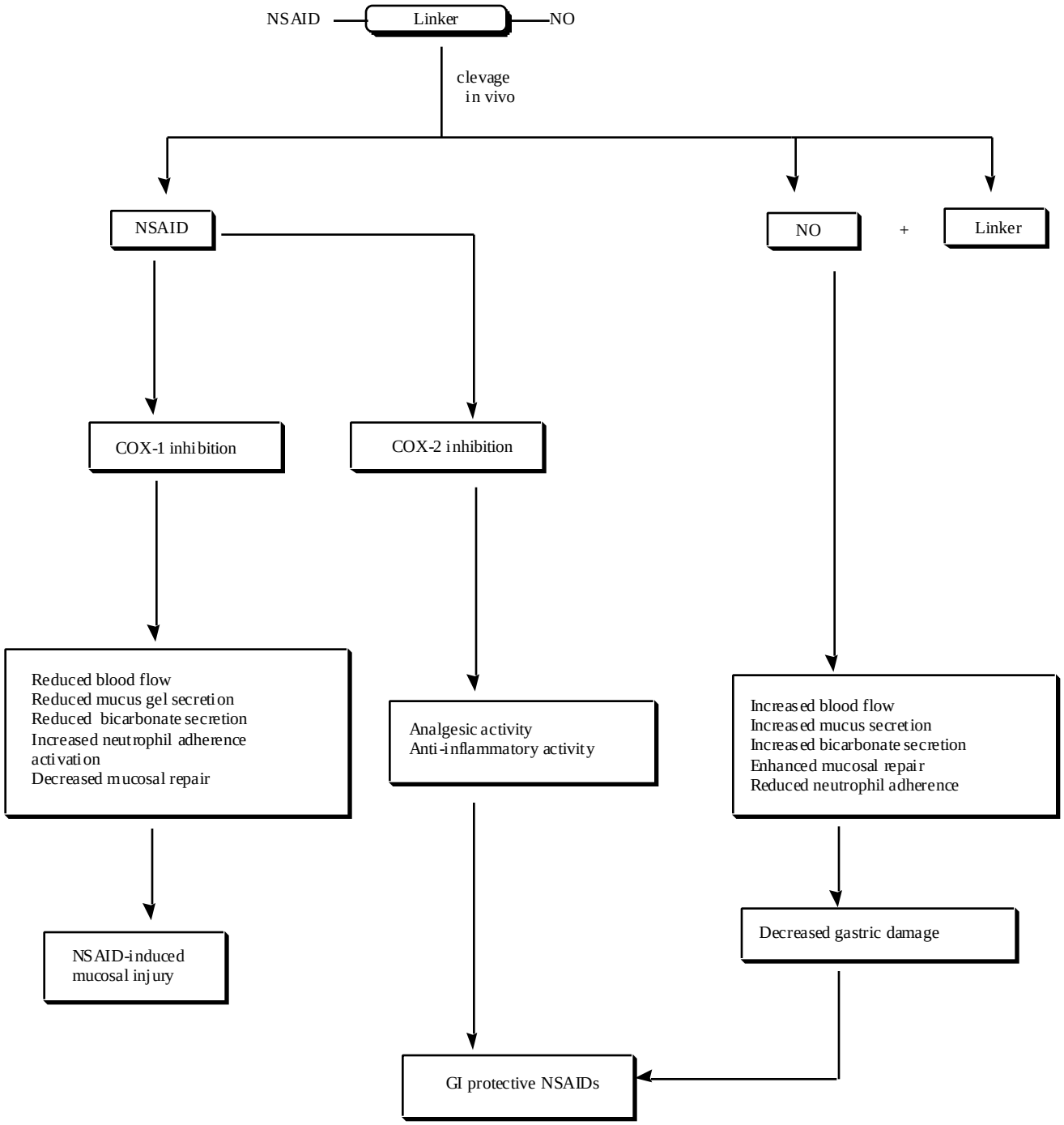


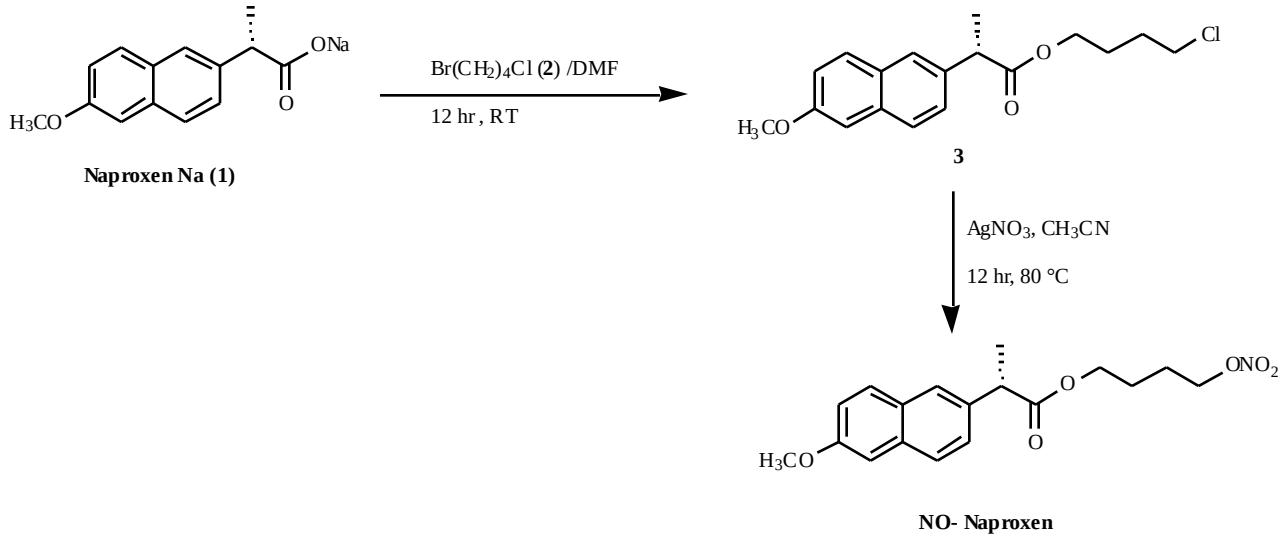

**Fig. (1).**

## NO-NSAIDs

The pharmaceutical company NicOx S.A. (Sophia Antipolis, France) has reported the synthesis and preclinical pharmacology of several NO-NSAIDs. By way of example, the reported synthesis of NO-naproxen is presented in (Scheme 1) [53, 54]. The sodium salt of naproxen (**1**) was treated with 4-chlorobutyl bromide (**2**) in DMF to produce naproxen chlorobutyl ester (**3**), which was subsequently nitrated with silver nitrate in acetonitrile at 80 °C to produce NO-naproxen in good yield. In a similar manner, the NO-releasing, nitrate-esters NO-aspirin, NO-diclofenac ("nitrofenac"), NO-fluribiprofen, and NO-ketoprofen have been synthesized from each respective parent NSAID [55, 56] (Fig. **2**). Similar to other organic nitrates, NO-NSAIDs would require reductive catabolism (presumably, enzyme-mediated) to serve as physiological NO source and may share a major therapeutic liability inherent to organic nitrates, development of tolerance during chronic treatment [57, 58].

## NO-Naproxen

Naproxen is commonly used world-wide for pain relief and as an anti-inflammatory agent [1]. Compared on a molar




**Scheme 1.**

basis to naproxen, NO-naproxen (Fig. **2**) administered orally at low mg/kg doses evidenced superior analgesic activity in an acetic acid-induced rodent model of writhing and comparable anti-inflammatory activity in the rat carrageenan-induced paw edema model [59]. Three hours after oral administration, NO-naproxen (58 or 116 mg/kg) caused less than 5% of the gastric (i.e., stomach) lesions elicited by equi-molar naproxen. As confirmed by histological examination, naproxen induced penetrating ulceration of the rat small intestine after twice-daily oral administration for 18 days, whereas equi-molar NO-naproxen was without obvious intestinal toxicity. The rats treated for 18 days with naproxen had a significantly (by some 40%) lower hematocrit than untreated controls, whereas the hematocrit in rats treated chronically with equi-molar NO-naproxen remained normal [59]. At oral doses in the low mg/kg range, markedly (≈ 60%) less naproxen was present in rat plasma following NO-naproxen administration than following an equimolar naproxen treatment. The relatively limited naproxen delivery from NO-naproxen did not appear to account for its reduced GI toxicity: in the rat, high-dose (i.e., 116 mg/kg) NO-naproxen resulted in a peak blood naproxen level far greater than that from administration of 30 mg/kg naproxen, but only the latter damaged gastric tissue. NO-naproxen was detected in the blood only at the highest administered dose (116 mg/kg). In this study, the possibility that differences in pharmacokinetics other than peak plasma naproxen level could have contributed to the GI-sparing effect of NO-naproxen was not explored, nor was the des-NO analog of NO-naproxen was studied as a control. The data suggest that NO-naproxen has a pharmacological profile at least equivalent to that of naproxen itself with an improved safety profile (less GI injury) in acute and chronic laboratory administration to rodents.

A subsequent study explored the effect of NO-naproxen on hypertension induced by chronic NOS inhibition [60]. To this intent, rats were fed the NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) in their drinking water over four weeks, causing elevated blood pressure and macroscopically visible damage to the gastric mucosa. Once-a-day oral co-administration of naproxen (10 mg/kg) over the four weeks potentiated the L-NAME-induced gastric damage and hypertension, whereas equimolar NO-naproxen (14.5

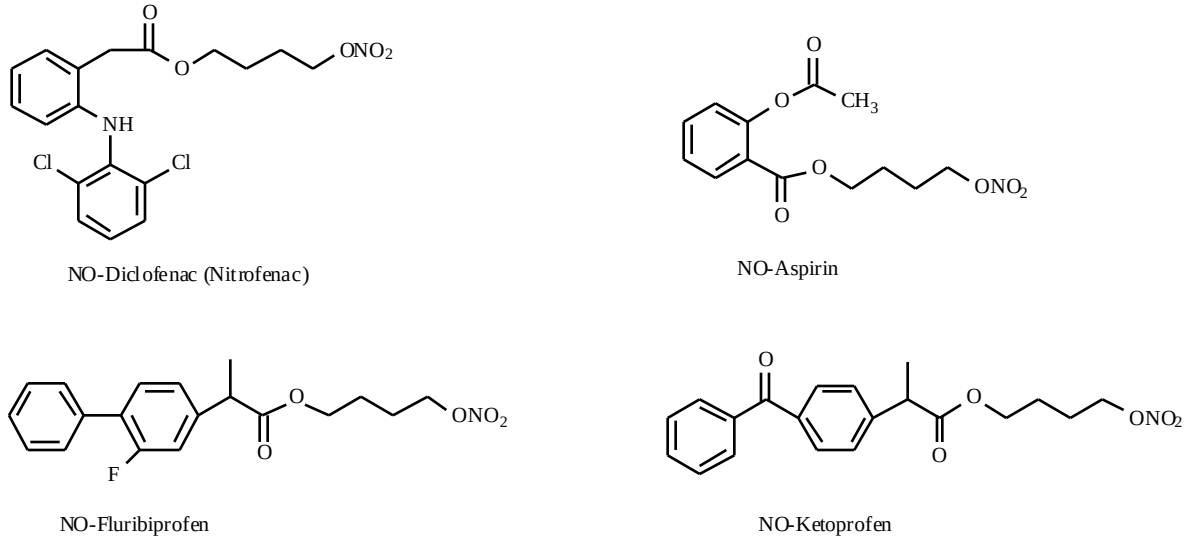



**Fig. (2).**

mg/kg) attenuated the gastric injury and hypertension caused by concomitant L-NAME treatment. In the absence of L-NAME, rats treated for four weeks with a daily oral dose of 14.5 mg/kg NO-naproxen exhibited no significant gastric damage, whereas equimolar naproxen (10 mg/kg) caused stomach hemorrhagic lesions. At these doses, both naproxen and NO-naproxen inhibited systemic COX activity by > 90%. This four-week rat study extends the safety profile of NO-naproxen vs. naproxen over time and suggests that NO-naproxen may respresent a safer alternative to naproxen in hypertensive patients. The blood-pressure data further implicate the NO-releasing moiety as an important component of the improved profile of this NSAID, despite the lack of comparative evaluation of the des-NO naproxen ester in this hypertension model.

NO–Naproxen, but not naproxen, markedly inhibited lipopolysaccharide-induced iNOS expression without affecting NOS enzyme activity in a cultured murine macrophage cell line [61], suggestive of a means by which the anti-inflammatory property of the parent NSAID could be augmented.

**NO-Aspirin**

Unlike the generally reversible nature of COX inhibition by most NSAIDs, aspirin acetylates COX and is thereby an irreversible COX inhibitor [62]. Despite the introduction of innumerable new drugs since 1899, aspirin (acetylsalicylic acid) has remained for over a century a venerable household remedy and the most widely administered analgesic [1]. Aspirin is also increasingly being used as an anti-thrombotic agent by virtue of its ability to inhibit thromboxane synthesis by platelet COX, and recent data have indeed demonstrated that chronic aspirin administration is prophylactic against thrombotic vascular disease [63]. Long-term aspirin use carries a risk of GI bleeding and development/exacerbation of gastric and duodenal ulcers [1].

Wallace and colleagues have studied the anti-thrombotic effects of NO-aspirin, a novel nitroxybutylester derivative of aspirin (Fig. **2**) [64]. NO-aspirin effectively inhibited *in vitro* human platelet aggregation and elevated platelet cGMP in a concentration-dependent manner, NO-aspirin being some 7-fold more potent an anti-aggregatory agent in this test than aspirin itself. The platelet effects of NO-aspirin were abrogated by a heme NO scavenger and were therefore considered to reflect bioactive NO release from NO-aspirin. Oral administration of aspirin to rats over the dose range 30-120 mg/kg caused extensive, dose-dependent hemorrhagic stomach lesions within 3 hours post-dosing. However, equimolar NO-aspirin (30-300 mg/kg oral dose) was without macroscopic GI toxcity [64]. Likewise, rats evidenced severe gastric damage following daily aspirin administration (100 mg /kg) for 14 days, whereas rats treated with equimolar NO-aspirin were free of either macroscopic or histologic stomach lesions [64]. Orally administered NO-aspirin (166 mg/kg) inhibited rat platelet aggregation *ex vivo* up to 3 h post-dosing as effectively as equimolar aspirin (100 mg/kg), but NO-aspirin did not inhibit platelet thromboxane synthesis or gastric prostaglandin synthesis, even after chronic administration [64]. NO-aspirin did not affect systemic arterial blood pressure when administered intravenously to the rat. Despite its marked anti-thrombotic effects, NO-aspirin was not readily converted to salicylate within 12 hours after oral administration to the rat: the amount of plasma salicylate from NO-aspirin (166 mg/kg dose) was only ≈ 27% that from an equimolar dose (100 mg/kg) of aspirin. Since NO-aspirin was much more active than aspirin as an inhibitor of platelet aggregation, this study suggests that NO-aspirin is unlikely to act purely as a pro-drug for salicylate. More data on the metabolism and pharmacokinetics of NO-aspirin are needed to support this conclusion. It also remains to be determined whether the gastroprotective property of NO-aspirin reflects its acute lack of stomach COX inhibition and/or local NO-related cytoprotection. Information on the metabolism, phramacokinetics, and GI effects of the des-NO analog of NO-aspirin may offer some insight into these issues. NO–Aspirin, but not aspirin, inhibited lipopolysaccharide-induced iNOS expression without influencing NOS enzyme activity in a cultured murine macrophage cell line [61], suggestive of a means by which aspirin's anti-inflammatory activity could be augmented.

Ukawa and colleagues [65] observed that gastric lesions induced by hyperthermic stress in the rat were worsened by subcutaneous administration of indomethacin (2 mg/kg) or aspirin (20 mg/kg), but were not affected by NO-aspirin (33 mg/kg) or the COX-2 inhibitor NS-398 (Taisho Pharmaceutical Co. Ltd., Tokyo, Japan) (10 mg/kg). At the equimolar doses specified, all four NSAIDs were equally effective anti-inflammatory agents in the rat carrageenan-induced paw edema test. However, all NSAIDs studied except NO-aspirin impaired gastric ulcer healing with repeated administration for up to 7 days, despite the ability of NO-aspirin to inhibit both COX-1 and COX-2. It is conceivable that NO released from NO-aspirin may have compensated for any potentially tissue-compromising effects of COX inhibition. Likewise, the finding that both NO-aspirin and NS-398 inhibited COX-2, but only NS-398 impaired gastric ulcer healing, suggests that NO released from NO-aspirin might promote the reparative response. In this regard, evaluation of the des-NO analog of NO-aspirin in this rat ulcer-healing model would be informative.

The molecular basis of NO-aspirin's GI-sparing effect has recently been investigated [66]. Data from this study confirmed previous results [64] on the limited bioavailability of NO-aspirin. Only NO-aspirin, and not aspirin itself, caused a time-dependent increase in plasma nitrate/nitrite. As previously demonstrated [64], the extent of acute gastric mucosal injury did not correlate with salicylate plasma level. NO-aspirin and NO donors such as sodium nitroprusside inhibited both mucosal apoptosis and caspase activation, and caspase inhibitors prevented aspirin-induced mucosal injury. The data suggest that prevention of gastric damage by NO-aspirin is due to the inhibition of gastric cysteine proteases critical to apoptosis. The enzymatic mechanism of caspase inhibition, the specific proteases(s) whose modulation may be necessary for gastric protection, and the potential involvement of a NO-related signal-transduction mechanism modulating gastric cell apoptosis remain to be determined.

The United States Food and Drug Administration (FDA) recently approved an Investigational New Drug application

in support of Phase-I clinical trials of NicOx NO-aspirin for the treatment of pain and inflammation [67]. Announcement has also been made by NicOx that the NO-aspirin derivative exhibited significant protective activity against acute myocardial infarction in preliminary human clinical studies and will thus be developed for other indications, including prevention and treatment of cardiovascular disorders [67].

**NO-Diclofenac (Nitrofenac)**

Although GI-toxic, diclofenac is a potent NSAID particularly useful with chronic administration against the painful disease of (rheumatic) arthritis [1]. In the 10-40 mg/kg oral dose range, the NicOx NO-releasing diclofenac derivative, nitrofenac (Fig. **2**), showed comparable anti-inflammatory activity to diclofenac in the standard carrageenan-induced rat paw edema model, and both agents inhibited gastric mucosal PG synthesis in the rat by ≥80% [68]. Intraperitoneal diclofenac (20 mg/kg) elicited a steady decline in gastric blood flow, reaching 50% of basal flow 60 min post-dosing, whereas equimolar nitrofenac did not affect gastric blood flow. At oral doses of 10-40 mg/kg, diclofenac caused macroscopic gastric damage within 5 hours after oral administration in rats, but equimolar nitrofenac elicited far less acute gastric injury. Penetrating gastric ulcers were observed in the rabbit after twice-daily administration of diclofenac (20 mg/kg) for 4 days. Nitrofenac-treated rabbits exhibited no macroscopically or histologically detectable stomach injury whatsoever [68]. These data allow the conclusion that esterification of dioclofenac with a nitroxybutyl moiety greatly reduced its GI toxicity and ulcerogenic potential without affecting its anti-inflammatory activity. In this work, the des-NO analog of nitrofenac was not profiled, and no data were provided on nitrofenac pharmacokinetics or metabolism.

A subsequent study examined whether nitrofenac has less intestinal toxicity than the parent NSAID, diclofenac, in healthy and colitic rats [69]. To this intent, healthy rats were given equimolar oral doses of diclofenac (10 mg/kg) or nitrofenac (15 mg/kg) twice daily for up to two weeks. All diclofenac-treated rats died prior to study completion and exhibited at autopsy massive small-intestinal ulcers and perforations. No mortality was observed in rats given nitrofenac, their only GI abnormality being diffusehyperemia in the small intestine. A similar difference was observed in a one-week study in colitic rats: diclofenac treatment (1-10 mg/kg, twice daily) resulted in dose-dependent mortality; only at the highest molar-equivalent dose (15 mg/kg) was limited (33%) mortality observed in the nitrofenac-treated rats. One-week diclofenac administration to colitic rats was associated with exacerbated intestinal injury and colonic perforation; nitrofenac did not increase colonic injury in colitic rats. These data demonstrate that nitrofenac has markedly reduced intestinal toxicity in both healthy and colitic rats as compared to diclofenac.

In addition to causing gastropathy, NSAIDs may adversely interfere with the natural healing response and scar tissue formation underlying GI ulcer repair [70]. The potential effect of diclofenac and nitrofenac on acid-induced gastric ulcer healing in the rat has been studied [71]. Seven days after induction of gastric ulcers with acetic acid, daily oral treatment with anti-inflammatory doses of diclofenac (5 mg/kg), nitrofenac (7.5 mg/kg, equimolar dose to diclofenac), or vehicle was started. In addition, the exogenous PG misoprostol (0.01 mg/rat), the COX-2 inhibitors nabumetone (75 mg/kg) and L745,337 (5 mg/kg), and the NO donor glyceryl trinitrate (0.1-10 mg/rat) were likewise tested for their potential effect on gastric ulcer healing. After a subsequent seven days, ulcer area was measured in all groups. Only nitrofenac and glyceryl trinitrite at ≥ 1.0 mg/rat significantly accelerated gastric ulcer healing, despite the finding that nitrofenac and diclofenac suppressed platelet COX-1 activity *in vivo* to a similar extent. Diclofenac, misoprostol, and two COX-2 inhibitors had no effect upon the natural healing response in this rat model. The authors, with support from data in rodent models showing that NO donors accelerate gastric ulcer healing and inhibition of endogenous NO synthesis impairs ulcer healing [72], conclude that nitrofenac is able to accelerate ulcer healing by virtue of its NO-donating property. Evaluation of the influence of the des-NOnitrofenac analog on ulcer healing would strengthen this conclusion.

**NO-Flurbiprofen and NO-Ketoprofen**

The NicOx nitroxybutyl ester derivatives of flurbiprofen and ketoprofen (Fig. **2**) have been synthesized and profiled biologically [73]. Both NO-flurbiprofen and NO-ketoprofen exhibited acute anti-inflammatory activity comparable to the respective parent NSAID in the carrageenan-induced paw edema model. Likewise, NO-flurbiprofen and NO-ketoprofen suppressed gastric PG synthesis to an extent comparable to inhibition from an equi-molar dose of the respective parent NSAID [73]. Each of these NO-NSAIDs caused significantly less gastric ulceration than did the respective parent NSAID, both acutely (5 hours post-dosing) and after twice-daily oral administration for one week. To assess whether direct contact with the gastric mucosa was necessary for NO-flurbiprofen to prevent gastric injury, the gastric mucosa of rats was evaluated histologically after systemic (subcuteanuous) administration of either NO-flurbiprofen or flurbiprofen (220 mg/kg) [73]. On average, the gastric mucosal damage in rats five hours after systemic NO-flurbiprofen administration was some 90% less than the stomach injury produced by equi-molar systemic flurbiprofen. Thus, the ability of NO-flurbiprofen to spare the gastric mucosa was not completely dependent upon direct contact of the compound with GI tissue, suggesting that NO-flurbiprofen was not acting solely as a "pro-drug." Further study of NO-flurbiprofen*in vivo* revealed that it was a more effective inhibitor of collagen-induced platelet aggregation than flurbiprofen [73, 74] and increased plasma nitrite/nitrate levels, consistent with NO release therefrom [74]. NO–Flurbiprofen and NO-ketoprofen, but not the respective parent NSAIDs, inhibited lipopolysaccharide-induced iNOS expression without influencing NOS enzyme activity in a cultured murine macrophage cell line [61].

In a mouse thromboembolism model, NO-flurbiprofen was a more potent inhibitor of collagen-induced platelet aggregation than flubiprofen [74]. *In vitro* studies with NO-flurbiprofen using washed human platelets further confirmed

data on NO-aspirin [64] by showing platelet-dependent NO release from NO-flurbiprofen [74]. Consequently, NO-flurbiprofen appears to have particular potential as an anti-thrombotic agent virtually devoid of gastrointestinal side-effects. NicOx has announced Phase-IIa clinical trials of a NO-flurbiprofen derivative for the treatment of a form of osteoporosis (Paget's disease) and has expressed interest in developing this compound for the treatment of urinary incontinence [67].

## SNO-NSAIDs

NitroMed, Inc., (Bedford, MA, U.S.A.) is currently developing SNO-NSAIDs, a novel class of nitrosothiol NSAID derivatives. In principle, this approach is similar to NicOx's NO-NSAID program in that existing NSAIDs have been functionalized to generate NO. But the NO-donor moiety in the SNO-NSAIDs is a *S*-nitrosothiol (-*S*-NO), not a nitrate (-$ONO_2$), and the linkers are distinct. Perhaps most critically, the biological chemistry of *S*-nitrosothiols is very different from that of organic nitrates, which rely upon intracellular metabolic transformation (i.e., enzyme-dependent reductive hydrolysis) to generate NO and express NO-related bioactivity [57]. Nitrate therapy carries with it the well-known liability of tolerance, by which the pharmacological effect of organic nitrates attenuates markedly, especially with high-dose exposure and/or steady use [58].

*S*-Nitrosothiol have gained increasing research and medical attention in recent years. Nitrosylation, the covelant attachment NO functionality to a thiol nucleophile, results in a *S*-nitrosothiol. Naturally occurring *S*-nitrosothiols, including *S*-NO-glutathione and *S*-NO-albumin, have been postulated to play a role in mammalian NO metabolism as NO storage sites, NO carriers, and intermediates in the pharmacology of organic nitrate vasodilators [75]. Although distinct enzyme systems have been identified that appear to elicit NO release from *S*-nitrosothiols (but not from organic nitrates) [76], their cellular importance, if any, has yet to be established [77]. Physiological NO release from *S*-nitrosothiols occurs in an enzyme-independent manner, possibly through metal ion-catalyzed *S*-nitrosothiol breakdown to NO and the corresponding disulfide [78]. Intramolecular determinants of NO release from *S*-nitrosothiols and modulators of *S*-nitrosothiol bioactivity include local redox status and the steric hinderance and electronic (i.e., inductive) effects upon the –*S*-NO group [79, 80]. In addition, *S*-nitrosothiols can participate in *S*-NO-thiol exchange (*S*-transnitrosation) reactions, in which a $NO^+$ moiety is transferred from the *S*-nitrosothiol to a thiol nucleophile in exchange for $H^+$ [81, 82]. Transnitrosation may also provide tissue protection by limiting the formation of toxic peroxynitrite ($ONOO^-$) [83]. Although formation of the $NO^+$ redox form of NO is considered thermodynamically and chemically unfavorable in an aqueous medium at physiological pH [84, 85], transnitrosation may represent a possible transfer pathway for nitrosative $NO^+$ equivalents *in vivo* [79, 81]. As such, transnitrosation is considered a candidate mechanism supporting the bioactivity of *S*-nitrosothiols in that it provides a potential route for influencing tissue (patho)physiology through the nitrosative modulation of thiol-containing biomolecules such as enzymes [28, 85]. The biological chemistry of *S*-nitrosothiols is thus both unique and very distinct from the metabolism of organic nitrates such as the NicOx NSAID nitroxybutyl esters.

The general medicinal chemistry approach applied by NitroMed, Inc., to design SNO-NSAIDs is illustrated in (Fig. **3**). An SNO-NSAID can be synthesized in two general ways: nitrosylating an NSAID derivatized with a sulfhydryl tether or esterifying a NSAID with a nitrosothiol tether. The former route is exemplified by the synthesis of ibuprofen derivative **NMI-172** (Scheme 2). Commercially available ibuprofen was reacted with oxalyl chloride in dichloromethane to produce acid chloride **4,** which was subsequently coupled with 3-methyl 3-thiobutanol (**5**) to produce ibuprofen sulfhydryl ester (**6**) in 65% yield [86]. Compound **6** was then nitrosylated with *tert*-butylnitrite (*t*-BuONO) in dichloromethane to obtain SNO-ibuprofen (**NMI-172**) as an oil in 65% yield (Scheme 2) [87]. In a similar fashion, an SNO-ketoprofen (**NMI-161**) was synthesized with an overall yield of 90% (Fig. **4**) [87].

Chronic and acute pharmacological profiling of **NMI-172** and **NMI-161** was carried out in rodents [87, 88]. After seven days, ibuprofen caused appreciable gastric lesions in the rat at a 145 μmol/kg daily oral dose. However, an equimolar dose of **NMI-172** did not elicit gastric lesions in the rat. Similarly, after four days, ketoprofen (39 μmol/kg/day) caused gastric lesions (5.8 mm) in the rat stomach, but the mean lesion score after dosing with equimolar **NMI-161** was almost four-fold lower (1.6 mm). Escalating oral doses of ibuprofen (48, 97, and 194 μmol/kg)

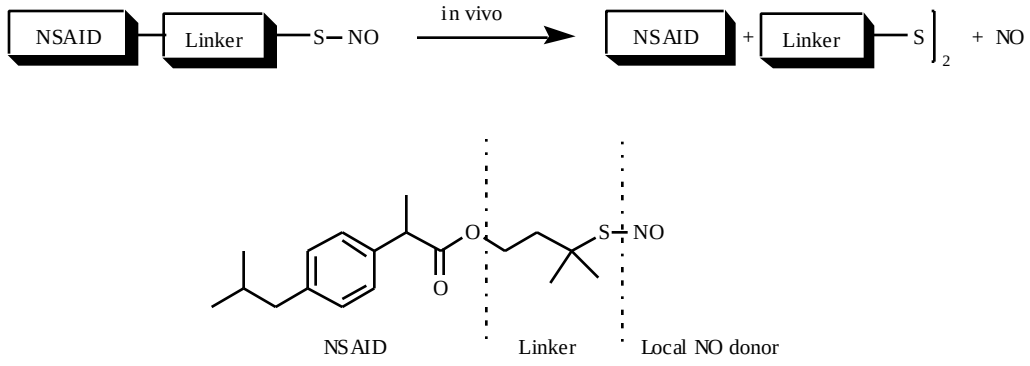
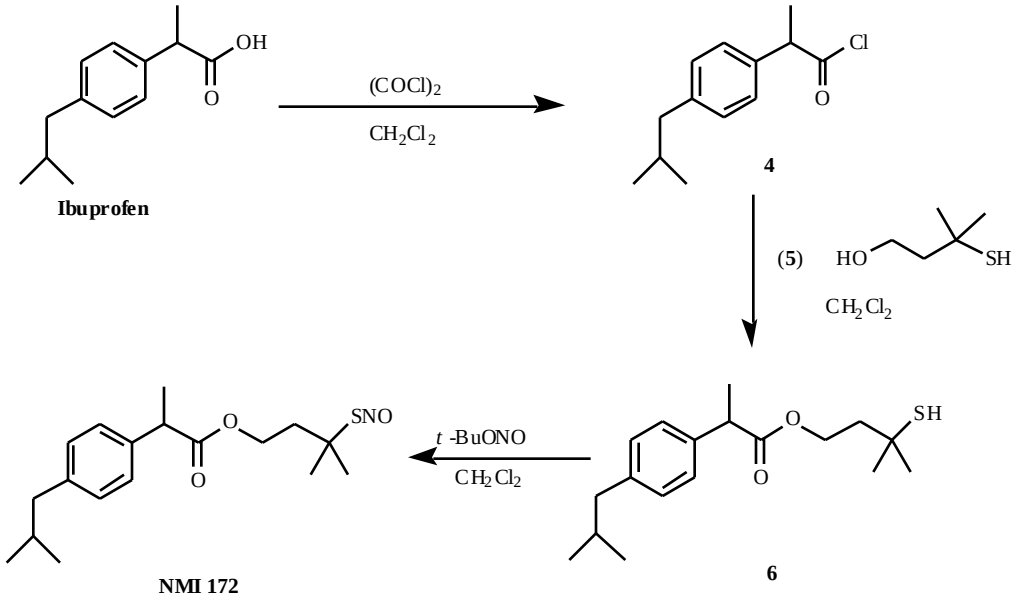
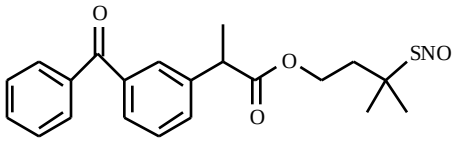



**Fig. (3).**




**Scheme 2.**

caused dose-related, acute gastric damage in rats, whereas **NMI-172** elicited far fewer lesions, even at the highest molar-equivalent dose. Both **NMI-172** and **NMI-161** had analgesic and anti-inflammatory activities comparable to their corresponding parent NSAID in the mouse writhing and rat paw-edema tests, respectively [87, 88].




**NMI 161**

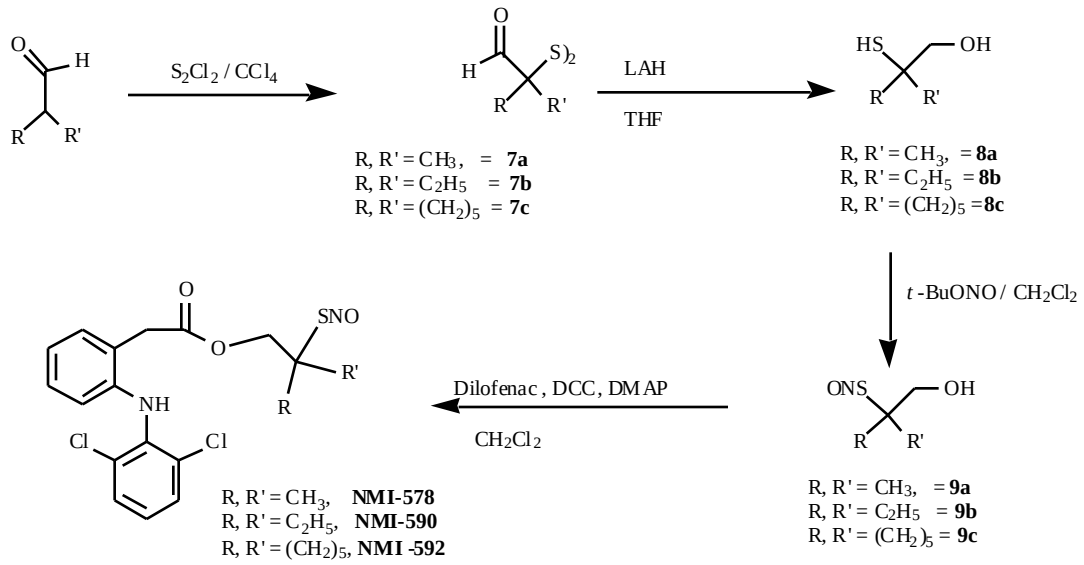
**Fig. (4).**

A potential complication to SNO-NSAID synthesis is the inherent chemical instability of some *S*-nitrosothiols under ambient temperature and light [89, 90]. Under these conditions, oily *S*-nitrosothiols are particularly susceptible to decomposition within a few hours to days, generating the corresponding disulfides and sulfhydryls (SH) as des-NO decomposition products (unpublished results). Tertiary *S*-nitrosothiols are relatively stable in comparison to primary and secondary *S*-nitrosothiols at ambient temperature [89, 90]. With an eye toward commercialization and acceptable shelf-life stability, these considerations led NitroMed, Inc., to focuss on SNO-NSAIDs that are crystalline solids bearing tertiary nitrosothiols as the NO-donor functionality. This rationale will be exemplified in the following account of the synthesis and biological characterization of SNO-diclofenac. The diclofenac derivatization further illustrates the general synthetic approach of esterifying an NSAID with a nitrosothiol as compared to nitrosylating an NSAID derivatized with a sulfhydryl tether (cf. Scheme 2).

A facile method for aliphatic tertiary thiol synthesis involves reacting an aliphatic aldehyde containing an enolizable proton with sulfur monochloride ($S_2Cl_2$) followed by reduction of resulting disulfide-dialdehyde to tertiary thiols [91, 92]. Commercially available aldehydes such as 2-methylpropanal, 2-ethylbutanal, and cyclohexane-carboxaldehyde were reacted with $S_2Cl_2$ in $CCl_4$ at 55°C to generate disulfides **7a-7c** which were reduced with LAH to afford thio-alcohols **8a-8c** in high yields (Scheme 3). Subsequently, each thio-alcohol was nitrosylated with *t*-BuONO in $CH_2Cl_2$ to give nitrosothiols **9a-9c** as green oils in moderate-to-good yields. Nitrosothiols **9a-9c** were coupled directly to diclofenac at 0 °C using DCC with DMAP in $CH_2Cl_2$ to produce the desired SNO-NSAID products, **NMI-578**, **NMI-590**, and **NMI-592**, in very high yield (Scheme 3) [93].

Additionally, nitrosylated tethers containing a tertiary amine have been synthesized and coupled to diclofenac with the idea that the amine would allow for subsequent salt formation (Scheme 4). Cyclohexanecarboxaldehyde was used to synthesize disulfide **7c**. Reductive amination of **7c** with primary amino-alcohols of varying chain lengths (n = 2, 3) formed secondary amino-disulfides **10a-b** in good yields**.** Methylation of **10a-b** was achieved using first aqueous formaldehyde to form cyclic intermediates **11a-b** followed by their cleavage with $NaBH_4$ in methanol or acetic acid to obtain **12a-b** in good yield. Reduction of the disulfide bonds of **12a-b** with LAH at room temperature afforded sulfhydryls **13a-b**, which were subsequently nitrosylated with *t*-BuONO in methanol-HCl to afford **14a-b**. Nitrosothiols (**14a-b**) were coupled to diclofenac at sub-ambient temperature to afford target SNO-diclofenac derivatives **NMI 346** and **NMI 377** in moderate-to-good yield (Scheme 4) [93].

**NMI-377, NMI-578, NMI-590, and NMI-592** were shelf-stable, crystalline solids, whereas **NMI-346** was a green oil convertible to an amorphous powder by HCl [93]. HPLC analyses indicated that crystalline nitrosothiol esters of diclofenac (**NMI-377**, **NMI-590**, **NMI-592**) were stable at ambient temperature in closed amber vials for over a year. Amorphous diclofenac nitrosothiol salts, such as **NMI-346**, decomposed to the corresponding sulfhydryl and disulfide




**Scheme 3.**

under the same storage conditions within 2-3 months. The oily free base of **NMI-346** decomposed much more rapidly [93]

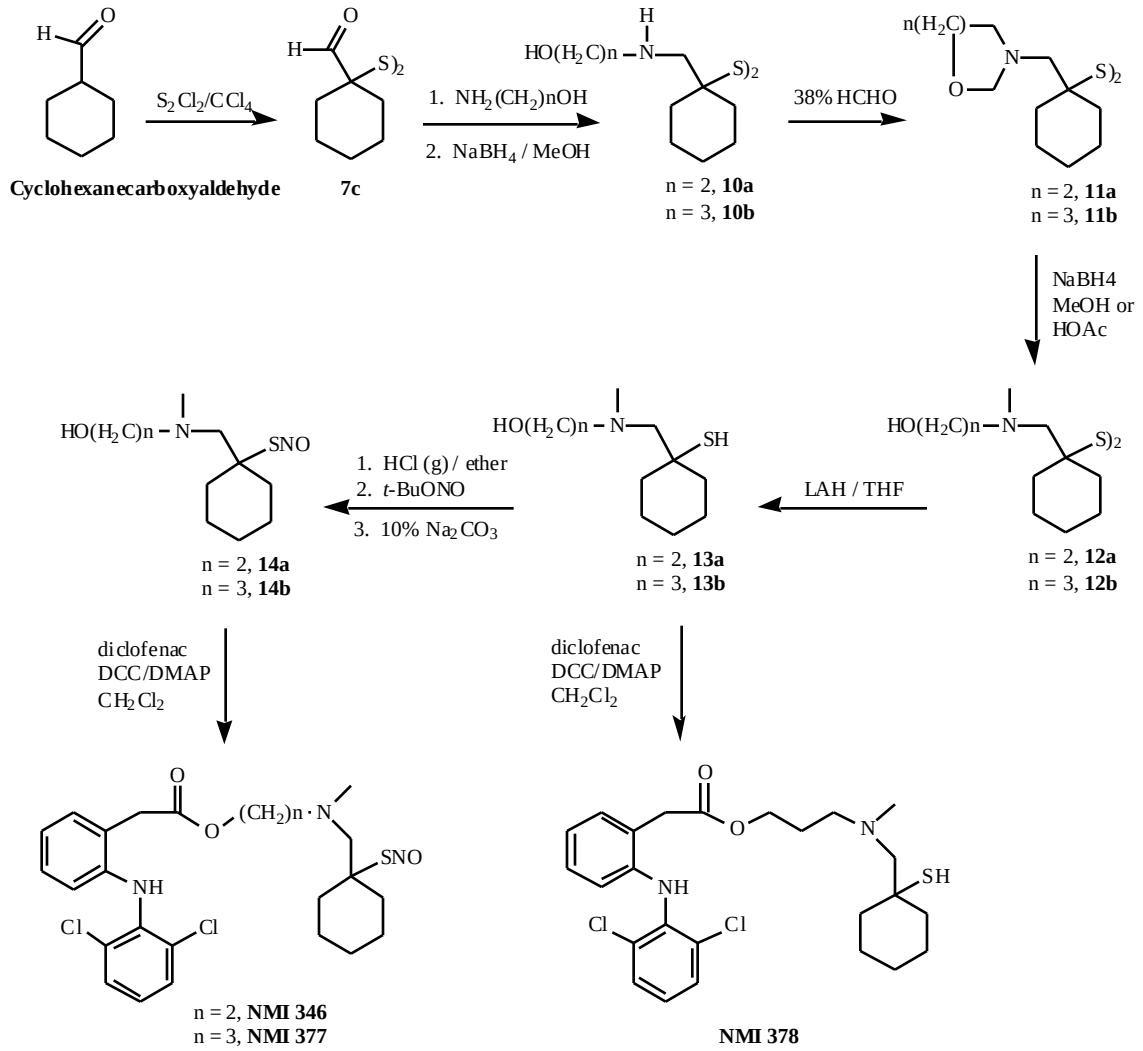
At equimolar oral doses, all four diclofenac SNO-NSAID derivatives showed analgesic and anti-inflammatory activities comparable to the parent NSAID, diclofenac [93-95]. For example, **NMI-590** and **NMI-377** inhibited phenyl benzylquinone-induced writhing in the mouse by 90% and 70%, respectively at an oral dose (100 µmol/kg) whereby diclofenac itself prevented writhing. **NMI-377** and diclofenac inhibited carragenan-induced rat-paw inflammation by 58% and 100% at an oral dose of 100 µmol/kg. Multiple-dose studies of **NMI-346** and **NMI-377** confirmed these acute results in showing that these diclofenac nitrosothiol derivatives have comparable analgesic and anti-inflammatory activities to diclofenac. After oral administration to mice and rats, the nitrosothiol diclofenac derivatives showed moderate to high (37%-80%) bioavailibility in that they resulted in 40-80% of the plasma diclofenac levels obtained from an equimolar dose of diclofenac itself with pharmacokinetic parameters similar to diclofenac as well. The rapid appearance of diclofenac in the blood after oral dosing with a nitrosothiol diclofenac derivative confirmed that the derivatives indeed hydrolyze in plasma post-absorption to produce parent NSAID [93-95].

Oral diclofenac (30-100 µmol/kg) caused significant stomach lesions in rats 18 hours post-dosing, whereas any of the diclofenac-based SNO-NSAIDs given at an equimolar dose induced negligible, if any, macroscopic gastric injury. An attempt was made to gain some pharmacological insight into the role of the NO moiety in the gastroprotection affordedby the nitrosothiol diclofenac derivatives. To this intent, GI lesion scores were compared 18 hours after a single oral dose 100 µmol/kg of diclofenac, **NMI-377**, or the des–NO analog of **NMI-377**, **NMI-378** (Scheme 4**).** The sulfhydryl **NMI-378** induced some 50% fewer gastric lesions than did diclofenac, but the nitrosothiol, **NMI-377**, induced no lesions at all [94]. Therefore, it is possible that a component of the gastroprotection afforded by **NMI-378** is NO-independent and may reflect, for instance, a "pro-drug effect"(i.e., suppression of local GI irritation) and/or GI cytoprotection by the free nucleophilic thiol group. Sulfhydryls (SH) with gastric-sparing properties have been identified [96].

## SUMMARY AND FUTURE PROSPECTS

Over sixty years have elapsed since the initial publication of clinical endoscopic data that the routine use of NSAIDs (i.e., aspirin) can seriously damage the human stomach [97]. Since that time, numerous new NSAID chemical forms and formulations have been designed, ostensibly with the aim of reducing NSAID GI toxicity, but few—if any—marketed have proven to be risk-free. Although NSAIDs are generally well tolerated, the large number of patients using NSAIDs to manage pain and inflammation, often over an extended period of time, makes NSAID GI toxicity a substantial patient risk and a costly healthcare and societal burden in terms of associated hospitalizations and morbidity, let alone mortality. A recent analysis [9] concludes that NSAID GI toxicity is largely a "silent epidemic" whose magnitude is just beginning to be appreciated by both physicians and patients. In 1997, for example, NSAID GI toxicity constituted the 15th most common cause of mortality in the United States, not counting deaths which could be ascribed to over-the-counter NSAID use [9]. Clearly, significant unmet medical needs involving substantial prescription and over-the-counter consumers would be served by a GI-sparing NSAID. This conclusion is substantiated by the conspicuous commercial success of COX-2 inhibitors, which are themselves not free of adverse GI events in the clinic [98].

A state-of-the-art approach currently used to address this medical need and capture the associated market share involves the exploitation of the biological activity of NO for therapeutic ends, i.e., using NO supplementation to improve the NSAID safety profile [31]. As reviewed herein, key factors auger well for the therapeutic and commercial success of this new, NO-based approach toward reducing the NSAID GI toxicity:




**Scheme 4.**

(a) the activity of endogenous (i.e., tissue) and exogenous NO as a critical biomediator of GI homeostasis, defense, and repair [41, 45-52]

(b) a wealth of preclinical and clinical information on NSAID pharmacology is known, as befitting a major, long-standing drug class [1-6, 9]

(c) the ability to transform chemically known NSAIDs into stable, proprietary NO-NSAID and SNO-NSAID derivatives [53-56, 87, 88]

(d) laboratory demonstration that NO-NSAIDs [50, 59-61, 64, 66, 68, 69, 71, 73, 74] and SNO-NSAIDs [88, 93-95] are effective, orally active anti-inflammatory and analgesic agents without significant GI or NO-related liabilities (i.e., systemic hypotension) in acute animal models

(e) clinical demonstration of the safety and overall tolerability of a NicOx NO-NSAID sufficient for USA-FDA approval as an Investigational New Drug Application in support of Phase-I clinical trials of NO-aspirin [67]

Extent data on NO-donor NSAIDs highlight some areas about which our current knowledge is merely observational at best and raise some interesting, if not provocative, questions demanding for further research. Organic nitrates and *S*-nitrosothiols have very different biological profiles in terms of the production of bioactive NO (or NO-equivalents) under physiological conditions [57, 58, 75-79, 81-85]. How distinct are these two forms of NO-donor when tethered to an NSAID with respect to decreasing the side-effect profile of that NSAID while remaining free of adverse, NO-dependent biological responses? In other words, to what extent is the chemical form of a NO-donor a decisive determinant of its ability to enhance the therapeutic profile of an NSAID? Or is the important factor of the ability of the NO itself, once delivered, to potentiate endogenous mechanisms of GI protection?

Several acute studies on both NO-NSAIDs and SNO-NSAIDs have concluded that the gastric-sparing property of these molecules does not reflect the pharmacokinetics with which the parent NSAID is generated therefrom; i.e., decreased bioavailability of irritant NSAID from the NO-donating derivative in comparison to an equimolar dose of the parent NSAID cannot account for the former's gastric tolerance [59, 93-95]. Largely correlative, inferential evidence

has been presented for a direct role of NO from NO-generating NSAIDs as a mediator of their reduced GI toxicity. Only one report has attempted to define the molecular events underlying the GI safety of NO-donating NSAIDs (specifically, NO-aspirin) and has identified as an acute injury pathway involving aspirin-induced activation of specific cysteine proteases (caspases) in the gastric mucosa [66]. In this study, the ability of NO-aspirin, a non-NSAID-based NO donor, and caspase inhibitors to ameliorate acute gastric tissue injury from aspirin invites several intriguing questions. Are there specific NO-sensitive targets or signal-transduction pathways that can be identified as determinants of gastric protection? Would the determinants be amenable to direct, targeted pharmacological intervention? If so, would such targeted molecules display therapeutic benefit (e.g., better efficacy, broader patient applicability, reduced risk) over NO itself? Could known NSAIDs be suitably derivatized to modulate the target without change in NSAID pharmacology? Might gene-based approaches be identified to modulate a specific, critical injury determinant, for example, a regulatory enzyme?

Information on these and other topics has potential clinical and therapeutic applications and would certainly aid our still limited understanding of the pathogenesis of NSAID-induced gastropathy. Even without the potential clinical and therapeutic insights such information might bring, emerging data on NSAID-NO-donor adducts holds promise that these molecules may emerge in the clinic as "bi-functional medicines" that bring a new level of safety to the routine management of pain and inflammation. Such drugs would also go a long way toward fulfilling the overall promise of NO "augmentation therapy" [31].

## ACKOWLEDGEMENTS

Authors wish to thank Drs. D.S. Garvey, J.D. Schroeder, G.L. Letts, R. Iyengar, J. K. Saha, S. W. Tam, R. R. Bandarage at NitroMed, Inc., and Prof. M. Wolfe at Boston University School of Medicine and Boston Medical Center for helpful comments and suggestions.

## ABBREVIATIONS

| | | |
|---|---|---|
| COX | = | Cyclooxygenase |
| DCC | = | Dicyclohexyl carbodiimide |
| DMAP | = | Dimethyl amino pyridine |
| DMF | = | Dimethylformamide |
| GI | = | Gastrointestinal |
| HOAc | = | Acetic acid |
| LAH | = | Lithium aluminum hydride |
| L-NAME | = | N-nitro-L-arginine methyl ester |
| MeOH | = | Methanol |
| NO | = | Nitric oxide |
| NOS | = | Nitric oxide synthase |
| NSAID | = | Non-steroidal anti-inflammatory drugs |
| PG | = | Prostaglandin |
| RT | = | Room temperature |
| *t* –BuONO | = | Tertiary butylnitrite |
| THF | = | Tetrahydrofuran |

## REFERENCES

[1] Insel, P.A. Analgesic-antipyretic and antiinflammatory agents and drugs employed in the treatment of gout. In: The Pharmacological Basis of Therapeutics, 9th Ed. Hardman,J.G.; Limberd, L.E.; Molinoff, P.B.; Ruddon, R.W.; Gilman, A.G. eds, McGraw-Hill: New York, **1996**. pp. 617-657.

[2] Garner, A. *Scand. J. Gastroenterol.* **1992**, *27* (suppl. 193), 83.

[3] Lichtenstein, D.R.; Syngal, S.; Wolfe, M.M. *Arthritis Rheum.,* **1995**, *38*, 5.

[4] Cash, J.M.; Klippel, J.H. *New Engl. J. Med.,* **1994**, *330*, 1368.

[5] Davies, N.M.; Wallace, J.L. *J. Gastroenterol.,* **1997**, *32*, 127.

[6] Wallace, J. *Gastroenterol.*, **1997**, *112*, 1000.

[7] Shorrock, C.J.; Langman, M.J. *Dig. Dis.,* **1995**, 13 (suppl. 1), 3.

[8] Singh, G.; Triadafilopoulus, G.J. *Rheumatol.,* **1999**, *26*, (suppl.), 18.

[9] Wolfe, M.M., Lichtenstein, D.R., and Singh, G. 1999. *New Engl. J. Med.,* **1999**, *340*, 1888.

[10] LaCorte, R.; Caselli, M.; Castellino, G.; Bajocchi, G.; Trotta, F. *Drug Saf.,* **1999**, *20*, 527.

[11] Huang, J.; Hunt, R.H. *Inflammopharmacology,* **1996**, *4*, 17.

[12] Ares, J.J.; Outt, P.E. *Curr. Pharmaceut. Design,* **1998**, *4*, 17.

[13] Friedel, H.A.; Langtry, H.D.; Buckley, M.M. *Drugs,* **1993**, *45*, 131.

[14] Prasit, P.; Wang, Z.; Brideau, C.; Chan, C.C.; Charleson, S.; Cromlish, W.; Ethier, D.; Evans, J.F.; Ford Hutchinson, A.W.; Gauthier, J.Y.; Gordon, R.; Guay, J.; Gresser M.; Kargman, S.; Kennedy, B.; Leblanc, Y.; Leger, S.; Mancini, J.; O'Neill, G.P.; Ouellet, M.; Percival, M.D.; Perrier, H.; Riendeau, D.; Rodger, I.; Zamboni, R. *et al.* **1999**, *Bioorg. Med. Chem. Lett., 9*, 1773.

[15] Hawkey, C.J. *Lancet.*, **1999**, *353*, 307.

[16] Fung, H.B.; Kirschenbaum, H.L. *Clin, Ther.*, **1999**, *21*, 1131.

[17] Ross, M.H.; Romrell, L.J.; Kaye, G.I. *Digestive System II:Esophagus and Gastrointestinal Tract. In: Histology*, 3rd Edition, Williams and Wilkins, Baltimore. **1995**, pp. 440-495.

[18] Redel, C.A.; Zwiener, R.J. 1998. *Anatomy and anomalies of the stomach and duodenum. In: Gastrointestinal and Liver Disease*, 6th Ed., Volume 1. M. Feldman, B.F.; Scharschmidt, M.H. Sleisenger, eds. W.B. Saunders Company: Philadelphia, **1998**, pp. 557-571.

[19] Schoen, R.T.; Vender, R.J. *Am. J. Med.*, **1989**, *86*, 449.

[20] Podolsky, D.K. *Am. J. Physiol.*, **1999**, *277*, G495.

[21] Kaunitz, J.D. *Keio. J. Med.*, **1999**, *48*, 63.

[22] Allen, A.; Garner, A. *Gut.*, **1980**, *21*, 249.

[23] Hui, W.W.; Chen, B.W.; Cho, C.H.; Luk, C.T.; Lam, S.K. *Digestion*, **1991**, *48*, 113.

[24] Miller, T.A. *Am. J. Physiol.*, **1983**, *245*, G601.

[25] Mitchell, J.A.; Warner, T.D. *Br. J. Pharmacol.*, **1999**, *128*, 1121.

[26] Wallace, J.L.; Keenan, C.M.; Granger, D.N. *Am. J. Physiol.*, **1990**, *259*, G462.

[27] Furchgott, R.F.; Zawadski, J.V. 1980. *Nature*, **1980**, *288*, 373.

[28] Ewing, J.F.; Janero, D.R.; Grinnell, T.A.; Schroeder, J.D.; Garvey, D.S. *Arch. Biochem. Biophys.*, **1997**, *343*, 131.

[29] Ewing, J.F.; Young, D.V.; Janero, D.R.; Garvey, D.S.; Grinnell, T.A. *J. Pharmacol. Exptl. Ther.*, **1997**, *283*, 947.

[30] Ewing, J.F.; Janero, D.R. *Free Radical Biol. Med.*, **1998**, *25*, 621.

[31] Janero, D.R. *Free Radical Biol. Med.*, **2000**, *25*, 1495.

[32] Murad, F. *Recent Prog. Horm. Res.*, **1998**, *53*, 43.

[33] Eiserich, J.P.; Patel, R.P.; O'Donnell, V.B. *Mol. Aspects Med.*, **1998**,*19*, 221.

[34] Denninger, J.W.; Marletta, M.A. *Biochim. Biophys. Acta*, **1999**, *1411*, 334.

[35] Patel, R.P.; Mc Andrew, J.; Sellak, H.; White, C.R.; Jo, H., Freeman, B.A.; Darley Usmar, V.M. *Biochim. Biophys. Acta*, **1999**, *1411*, 385.

[36] Fleming, I.; Busse, R. *J. Mol. Cell. Cardiol.*, **1999**, *31*, 5.

[37] Marletta, M.A.; Hurshman, A.R.; Rusche, K.M. *Curr. Opin. Chem. Biol.*, **1998**, *2*, 656.

[38] Boucher, J.L.; Moali, C.; Tenu, J.P. *Cell. Mol. Life Sci.*, **1999**, *55*, 1015.

[39] Stuehr, D.J. *Biochim. Biophys. Acta*, **1999**, *1411*, 217.

[40] Wink, D.A.; Mitchell, J.B. *Free Radical Biol. Med.*, **1998**, *25*, 434.

[41] Ko, J.K.; Cho, C.H. *Inflamm. Res.*, **1999**, *48*, 471.

[42] Castaneda, A.A.; Denning, J.W.; Chang, L.; Mercer, D.W. *J. Surg. Res.*, **1999**, *84*, 174.

[43] Asfaha, S.; Bell, C.J.; Wallace, J.L.; MacNaughton, W.K. *Am. J. Physiol.*, **1999**, *276*, G703.

[44] Jarvinen, M.K.; Wollmann, W.J.; Powrozek, T.A.; Schultz, J.A.; Powley, T.L. *Anat. Embryol.*. **1999**, *199*, 99.

[45] Kubes, P.; Wallace, J.L. *Med. Inflamm.*, **1995**, *4*, 397.

[46] Alican, I.; Kubes, P. *Am. J. Physiol.* **1996**, *270*, G225.

[47] Elliott, S.N.; Wallace, J.L. *J. Gastroenterol.* **1998**, *33*, 792.

[48] Whittle B.J.R.; Lopez-Belmonte, J.; Moncada, S. *Br. J. Pharmacol.*, **1990**, *99*, 607.

[49] Masuda, E.; Kawano, S.; Nagano, K.; Tsuji, S.; Takei, Y.; Tsujii, M.; Moshita, M.; Tomoki, M.; Kobayashi, I.; Nakama, A.; Fusamoto, H.; Kumido, T. *Gastroenterol.*, **1995**, *108*, 58.

[50] Reuter, B.; Wallace, J.L. A therapeutic application of nitric oxide: GI-sparing NSAIDs. In: *Nitric Oxide*: *A Modulator of Cell-Cell Interactions in the Microcirculation*. (P. Kubes, ed.) R.G. Landes Company, **1995**, pp. 157-168.

[51] Tam, S.W.; Saha, J.K.; Garvey, D.S.; Schroeder, J.D.; Shelekhin, T.E.; Janero, D.R.; Chen, L.; Glavin, A.; Letts, L.G. *Inflammopharmacology*, **2000**, *8*, 81.

[52] Konturek, S.J.; Brzozowski, T.; Majka, J.; Pytko-Polonczyk, J.; Stachura, J. *Eur. J. Pharmacol.*, **1993**, *239*, 215.

[53] Del Soldato, P. *US Patent*, **1998**, 5780495.

[54] Del Soldato, P. *US Patent*, **1997**, 5700947

[55] Arena, B. *WO Patent*, **1994**, WO 94/12463.

[56] Del Soldato, P. *EP Patent*, **1995**, EP 0722434B1.

[57] Chung, S.H.; Fung, H.L. *J. Pharmacol. Exptl. Ther.*, **1990**, *253*, 614.

[58] Elkaym, U. *Ann. Int. Med.*, **1991**, *114*, 667.

[59] Davies, N.M.; Roseth, A.G.; Appleyard, C.B.; McKnight, W.; Del Soldato, P.; Calignano A.; Cirino, G.; Wallace, J.L. *Aliment. Pharamacol. Ther.*, **1997**, *11*, 69.

[60] Muscara, M.N.; McKnight, W.; Del Soldato, P.; Wallace, J.L. *Pharmacol. Lett.*, **1998**, *62*, 235.

[61] Cirino, G.; Wheeler-Jones, C.P.D.; Wallace, J.L.; Del Soldato, P.; Baydoun, A.R. *Br. J. Pharmacol.*, **1996**, *117*, 1421.

[62] Lecomte, M.; Laneuville, O.; Ji. C.; DeWitt, D.L.; Smith, W.L. *J. Biol. Chem.*, **1994**, *269*, 13027.

[63] Elwood, P.C.; Hughes, C.; O'Brien, J.R. *Postgrad. Med. J.*, **1998**, *74*, 587.

[64] Wallace, J.R.; McKnight, W.; Del Soldato, P.; Baydoun, A.R.; Cirino, G. *J. Clin. Invest.*, **1995**, *96*, 2711.

[65] Ukawa, H.; Yamakuni, H.; Kato, S.; Takeuchi, K. *Digest. Dis. Sci.*, **1998**, *43*, 2003.

[66] Fiorucci, S.; Antonelli, E.; Santucci, L.; Morelli, O.; Miglietti, M.; Federici, B.; Mannucci, R.; Del Soldato, P.; Morelli, A. *Gastroenterol.*, **1999**, *116*, 1089.

[67] Etheridge, J. *Bioworld International*, **2000**. *11*, 20.

[68] Wallace, J.L.; Reuter, B.; Cicala, C.; McKnight, W.; Grisham, M.; Cirino, G. *Eur. J. Pharmacol.*, **1994**, *257*, 249.

[69] Reuter, B.K.; Cirino, G.; Wallace, J.L. *Pharmacol. Lett.*, **1994**, *55*, PL 1.

[70] Levi, S.; Goodlad, R.A.; Lee, C.Y.; Stamp, G.; Walport, M.J.; Wright, N.A.; Hodgson, H.J.F. *Lancet.*, **1990**, *336*, 840.

[71] Elliot, S.N.; McKnight, W.; Cirino, G.; Wallace, J.L. *Gastroenterol.*, **1995**, *109*, 524.

[72] Konturek, S.J.; Brzozowski, T.; Majka, J.; Pytko-Polonczyk, J.; Stachura, J. *Eur. J. Pharmacol.*, **1993**, *239*, 215.

[73] Wallace, J.L.; Reuter, B.; Cicala, C.; McKnight, W.; Grisham, M.B.; Cirino, G. *Gastroenterol.*, **1994**, *107*, 173.

[74] Cirino, G.; Cicala, C.; Mancuso, F.; Baydoun, A.R.; Wallace, J.L. *Thrombosis Res.*, **1995**, *79*, 73.

[75] Girard, P.; Potier, P. *FEBS Lett.*, **1993**, *320*, 7.

[76] Nikitovic, D.; Holmgren, A. *J. Biol. Chem.*, **1996**, *271*, 19180.

[77] Gordge, M.P.; Addis, P.; Noronha-Dutra, A.A.; Hothersall, J.S. *Biochem. Pharmacol.*, **1998**, *55*, 657.

[78] Singh, R.J.; Hogg, N.; Joseph, J.; Kalyanaraman, B. *J. Biol. Chem.*, **1996**, *271*, 18596.

[79] Kashiba-Iwatsuki, M., Kitoh, K., Kasahara, E., Yu, H., Nisikawa, M., Matsuo, M., and Inoue, M. *J. Biochem.*, **1997**, *122*, 1208.

[80] Young, D.V.; Serebryanik, D.; Bandarage, R.; Richardson, S.; Janero, D.R.; Garvey, D.S.; Tam, S.W.; Letts, L.G. *Mol. Biol. Cell.*, **1999**. *10*, 38a.

[81] Meyer, D.J.; Kramer, H.; Ozer, N.; Coles, B.; Ketterer, B. *FEBS Lett.*, **1994**, *345*, 177.

[82] Stamler, J.S.; Singel, D.J.; Loscalzo, J. *Science*, **1992**, *258*, 1898.

[83] Stamler, J.S.; Simon, D.I.; Osborne, J.A.; Mullins, M.E.; Jaraki, O.; Micheal, T.; Singel, D.J.; Loscalzo, J. *Proc. Natl. Acad. Sci. USA.*, **1992**, *89*, 444.

[84] Koppenol, W.H. *Meth. Enzymol.*, **1996**, *268*, 7.

[85] Keshive, M.; Singh, S.; Wishnok, J.S.; Tannenbaum, S.R.; Deen, W.M. *Chem. Res. Toxicol.*, **1996**, *9*, 988.

[86] Field, K.L. *et al., J Med. Chem.*, **1971**, *14*, 868.

[87] Garvey, D.S.; Letts, L.G.; Renfroe, B.H.; Tam, S.W.; *US Patent*, **1997**, 5703073.

[88] Garvey, D.S.; Richardson S.K.; Schroeder, J.D.; Shelekhin, T.E.; Marek, P.; Saha, J.K.; Tam, S.W.; Letts, L.G. **1997**. *214th ACS National Meeting (Med Chem), Las Vegas,NE, August 1997 (MEDI # 36).*

[89] Williams, D.L.H. *Chem. Soc. Rev.*, **1983**, *14*, 171.

[90] Field, L.; Dilts, R.; Ravichandran, R.; Lenhert, G.; Carnahan, G.E. *JCS Chem. Commun.*, **1978**, *6*, 249.

[91] Roy, B.; du Moulinet, D.; Hardemare, A.; Fontecave, M. *J. Org. Chem.*, **1994**, *59*, 7019.

[92] Stec, W.J.; Karwowski, B.; Boczkowska. M.; Guga, P.; Koziolkiewicz, M.; Sochacki, M.; Wieczorek, M. W.; Blaszczyk, J. *J. Am. Chem. Soc.*, **1998**, *120*, 7156.

[93] Bandarage, U.K.; Fang, X.; Garvey, D.S.; Janero, D.R.; Letts, L.G.; Mercer, G.J.; Saha, J.K.; Schroeder, J.D.; Tam, S.W.; *J. Med. Chem.*, **2000**, *43*, 4005.

[94] Bandarage, U.K.; Saha, J.K.; Schroeder, J.D.; Garvey, D.S.; Mercer, G.J.; Chen, L.; Glavin, A.; Janero, D.R.; Letts, L.G.; Tam, S.W. *218th ACS National Meeting (Med Chem), New Orleans,LA, August* **1999**, *(MEDI # 81).*

[95] Saha, J.K.; Bandarage, U.K.; Janero, D.R.; Chen, L.; Glavin, A.; Garvey, D.S.; Schroeder, J.D.; Letts, L.G.; Tam, S.W.; *Gastroentero.*, **1999**, *116*, A 298,

[96] Szabo, S.; Trier, J.S.; Frankel, P.W. *Science*, **1981**, *214*, 200.

[97] Douthwaite, A.H.; Lintott, G.A.M. *Lancet*, **1938**, *2*, 1222.

[98] Langman, M.J., Jensen, D.M.; Watson, D.J.; Harper, S.E.; Zhao, P.-L.; Hui, Q.; Bolognese, J.A.; Simon, T.J. *J. Am. Med. Assoc.*, **1999**, *282*, 1929.