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# Anti-Inflammatory Activity of a New Class of Nitric Oxide Synthase Inhibitors That Release Nitric Oxide

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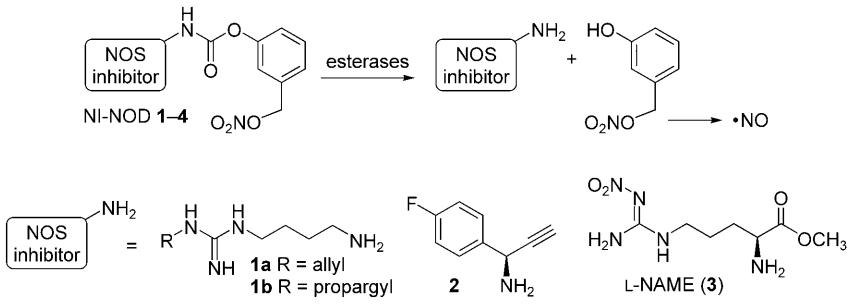
*Nitric oxide (NO) is a gaseous mediator that exerts key regulatory functions in mammalian cells. Low levels of NO exert homeostatic functions and counteract inflammation, whereas high amounts of NO cause tissue destruction and cellular death. Herein we describe a new class of nitric oxide synthase (NOS) inhibitor NO-donating drugs (NI-NODs). Human endothelial cells and human monocyte-based activity screening showed that NI-NODs inhibit IL-1β production, modulate $PGE_2$ production, and protect against apoptosis. In a rodent model of colitis, NI-NOD**1** and NI-NOD**2** potently decreased inflammation. These data show that NI-NODs are effective in both in vitro and in vivo models of inflammation, mimicking the positive effects of low levels of NO and suppressing NOS-induced NO production.*

## Introduction

Inflammation is a complex and multifactorial condition for which a number of mediators have been identified. Interestingly, some of the key features of inflammation converge on a common biological end point, namely the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway. It is widely accepted that, in physiological conditions, low levels of NO generated by endothelial NO synthase (eNOS) in the vascular bed exert homeostatic functions.[1–3] However, in inflammation eNOS becomes phosphorylated and generates high amounts of NO. The shift to a more active enzyme is triggered by a number of proinflammatory mediators, including shear stress, vascular endothelium growth factor (VEGF), and autacoids generated locally at the site of inflammation.[1] In addition to phosphorylated eNOS, high levels of NO are generated by the inducible isoform of NOS (iNOS). NO generated by the phosphorylated eNOS and iNOS contributes to inflammation by increasing vascular permeability and angiogenesis as well as formation of peroxynitrites and hydroxyl radicals that exert a major role in amplifying the tissue injury.[4–6] The identification of these pathways has led to the hypothesis that NOS inhibition might be beneficial in inflammation.[7] However, previous studies with iNOS inhibitors have failed to convincingly support this notion,[8] because of side effects linked to increased blood pressure that become evident after administration of these agents.[9]

Inflammatory bowel disease (IBD) is a family of chronic and destructive inflammatory disorders in which dysregulation of the innate immunity plays a mechanistic role. Human and animal studies have shown that, in these inflammatory conditions, a dysregulated intestinal immunity leads to eNOS phosphorylation and induction of iNOS.[10] High levels of NO generated in the inflamed intestine by these enzymes plays a mechanistic role in tissue destruction.[6–7] However, iNOS inhibition has led to controversial results, and iNOS deficient mice are not protected against colitis development.[11] In contrast, we have previously shown that modification of intestinal selective anti-inflammatory drugs, such as 5-aminosalicylic acid (5-ASA/mesalamine), by the addition of a NO-releasing moiety greatly increase the therapeutic activity of this agent.[12–14] Although

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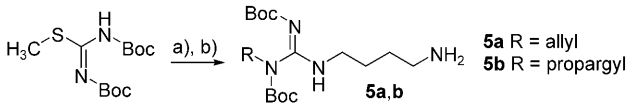
the mechanism of action of NO-donating mesalamine remains unclear, we have shown that administering it to colitic mice causes strong downregulation of the expression of proinflammatory cytokines such as interleukin (IL)-1β.[12]

As inhibition of high output NOSs could be beneficial in inflamed tissues, provided that low levels of NO are maintained, we have designed a new class of agents that couple an iNOS inhibitor scaffold with an NO-delivery moiety. The addition of a 3-(nitroxymethyl)phenyl moiety to the scaffold of nonselective NOS inhibitors, results in new chemical entities, the NOS inhibitor-NO donating drugs (NI-NODs), that might be beneficial in states of dysregulated production of NO.

## Results and Discussion

### Synthesis of NI-NODs

1-Amino-4-[$N^3$-allyl-$N^3$,$N^2$-bis(*tert*-butoxycarbonyl)guanidino]butane **5a** and 1-amino-4-[$N^3$-propargyl-$N^3$,$N^2$-bis(*tert*-butoxycarbonyl)guanidino]butane **5b** have been described previously as polyamine oxidase (PAO) and iNOS inhibitors ($K_i$: 20 and 15 μM, respectively). Compound **1a** is a weak iNOS inhibitor, but it has no activity toward eNOS.[15]




**Scheme 1.** a) R-OH, $Ph_3P$, DIAD, dry THF, 0 °C→reflux, overnight: **4a** 98%, **4b** 97%; b) 1,4-diaminobutane, THF, 50 °C, 1 h: **5a** 80%, **5b** 79%.

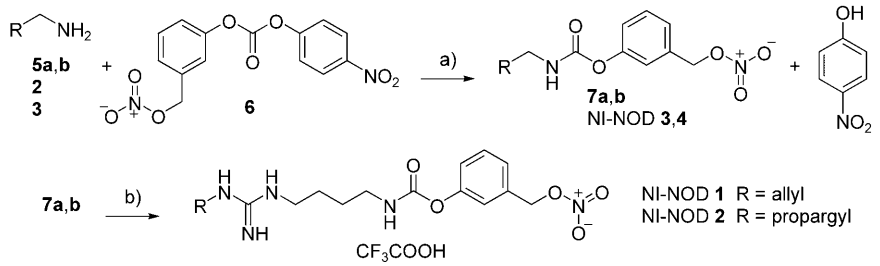
(*R*)-1-(4-fluorophenyl)prop-2-yn-1-amine **2** is an analogue of deprenyl-(*N*-1-phenylisopropyl-*N*-methyl-2-propynylamine) (LB), a drug used in the treatment of Parkinson's disease because of its anti-neurodegenerative action and antiapoptotic activity toward different neurotoxic endotoxins, resulting in the protection of mitochondria against oxidative stress. It has been shown that propargylamine inhibits neuronal NOS (nNOS, $IC_{50}$ = 108.8 μM, Botta et al., unpublished results). Nitro-L-arginine methyl ester (L-NAME) **3**, is a nonselective inhibitor of nNOS (bovine, $K_i$ = 15 nM), eNOS (human, $K_i$ = 39 nM), and iNOS (murine, $K_i$ = 4.4 μM).

The synthesis of compounds **5a** and **5b** was carried out following the procedure used for the preparation of aminoalkylguanidines.[15] Alkylation of *N*,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea with an appropriate alkyl alcohol using a Mitsunobu protocol gave 98% yield of **4a** and 97% for **4b**.[16] These in turn were treated with an excess of 1,4-diaminobutane to afford **5a** and **5b** in good yield (80 and 79%, respectively; Scheme 1).

The 3-(nitrooxymethyl)phenyl moiety was incorporated into amino acid **3**, agmatino (**5a** and **5b**) and phenylpropargylamino (**2**) compounds by carbamate linkage. The synthesis of all molecules was accomplished by following a common synthetic pathway in which compounds **5a**, **5b**, **2**, and **3**, containing a free primary amino group, were coupled with the asymmetric carbonate **6** by nucleophilic displacement (Scheme 2).

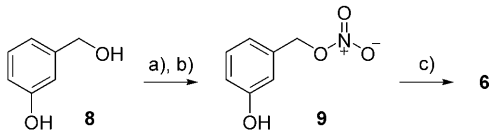
3-hydroxybenzyl bromide **8** was prepared by bromination of *m*-hydroxybenzyl alcohol and, after fast purification on silica gel under vacuum, was treated with $AgNO_3$[17] to give compound **9**. This in turn was coupled with 4-nitrophenyl chloroformate to yield compound **6** (Scheme 3).

The amino/guanidinium protecting group present in compounds **7a** and **7b**, was cleaved in mild acidic conditions with trifluoroacetic acid to afford target compounds NI-NOD**1** and NI-NOD**2** in quantitative yields as trifluoroacetic salts (Scheme 2).[18] During the cleavage no benzyl ester or phenyl carbamate hydrolysis was observed.



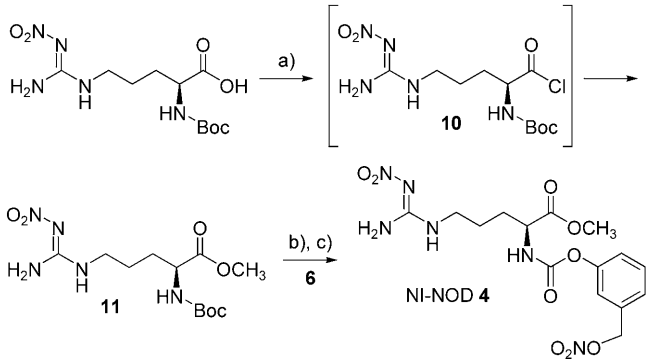

**Scheme 2.** a) Dry $CH_3CN$, RT, 8 h: **7a** 45%, **7b** 43%; b) 10% TFA in dry $CH_2Cl_2$, RT, 12 h, quantitative yield.

$N^{\omega}$-nitroarginine methyl ester (L-NAME) was easily prepared by conversion of $N^{\alpha}$-Boc-$N^{\omega}$-nitroarginine compound **11** with DMF–$SOCl_2$. Quantitative conversion was accomplished in only six minutes by microwave irradiation (Scheme 4).[19] The intermediate **10** is generated either in DMF or in dioxane that, as non-nucleophilic solvents, are compatible with the synthesis of other esters as well as the methyl ester. It is worth pointing out that the mildness of the chloroacylation conditions of this protocol needs only a catalytic amount of DMF and no base as scavenger. Deprotection of compound **11** with trifluoroacetic and subsequent desalination with triethylamine gave L-NAME (**3**) that in turn was treated with carbamate **6** to afford the target compound NI-NOD**4** in an overall yield of 53% (Scheme 4). The synthesis of enantiomerically pure compound NI-NOD**3** was carried out by coupling the enantiomerically pure propargylamine **2** previously prepared by us, with carbonate **6.**[20]




**Scheme 3.** a) $PBr_3$, dry Py, dry $CH_3CN$, −10 °C→RT, 12 h: **8** 98%; b) $AgNO_3$, dry $CH_3CN$, RT, 12 h: **9** 80%; c) 4-nitrophenylchloroformate, $Et_3N$, dry $CH_2Cl_2$, 0 °C→RT, 16 h: **6** 70%.




**Scheme 4.** a) DMF–$SOCl_2$, dry MeOH, MW (100 W), $t = 2 \times 3$ min, $P = 200$ psi, $T_{max} = 100\,^{\circ}C$, 95%; b) 10% TFA in dry $CH_2Cl_2$; c) $Et_3N$, dry $CH_3CN$, RT, 24 h, 53%.

### Effects of NI-NODs on NOS activity

We have previously demonstrated that HUVECs express eNOS spontaneously and iNOS expression can be induced by incubating HUVECs with LPS and IL-1β.[21] In HUVECs, all NI-NODs, apart from NI-NOD**4**, caused a significant decrease of L-citrulline production at the dose of 100 μM, an index of NOS activation, indicating that NI-NOD**1**, NI-NOD**2**, and NI-NOD**3** are NOS inhibitors (Figure 1 A). In contrast, NI-NOD**4** did not cause any change of L-citrulline production indicating that this compound does not behave as a NOS inhibitor.

### Effect of NI-NODs on NO release

Prolonged NO release is necessary for an effective pharmacological activity for NO donors drugs. For this assay human umbilical endothelial cells (HUVECs) were used.[21] As shown in Figure 1 B and C, NI-NOD**1** and NI-NOD**3** (100 μM) increased nitrite/nitrate ($NO_2/NO_3$) release in both medium alone and in cell supernatants, indicating that NI-NODs not only induce NO formation, but also release NO spontaneously. Treating cells with NI-NOD**1** and NI-NOD**3** resulted in a prolonged NO release, as demonstrated by the increased $NO_2/NO_3$ concentration after 24 h for both compounds. Interestingly, the ability of the two compounds to release NO was not affected by the addition of L-NAME (200 μM) into the incubation mixture, which supports the notion that NO release by these compounds does not require NOS. Moreover, NI-NODs also release NO spontaneously, indicating that this process does not require an enzyme-dependent pathway. The demonstration that NI-NOD**1** and NI-NOD**3** generate high levels of NO is consistent with previous observations made by incubating cells with a 3-(nitroxymethyl)phenyl moiety. This moiety releases low levels of NO, resulting in low but sustained formation of cGMP in endothelial cells over a period of 24 h. This mode of NO generation is different from that of other NO donors such as 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (DETA-NO)[22–23] that causes a short burst of NO, in high concentration leading to formation of peroxynitrites and hydroxyl radicals that might be toxic to cells.

Despite the fact that SNAP, another NO donor, releases NO with different kinetics than NI-NODs, resulting in a prolonged (hours) increase in cGMP concentration. The biological effects of the 3-(nitroxymethyl)phenyl moiety are only partially reproduced by SNAP. We have demonstrated that the NO moiety releases NO intracellularly, and the intracellular compartments involved are likely to be the same as those that the scaffold of the parent molecule targets. In the case of NOS inhibitors, it is known that NOS is localized in specific compartments, such as the caveolae in the cell membrane. We have previously shown that a 3-(nitroxymethyl)phenyl derivative of aspirin (NCX-4016) releases NO by inducing "hot spots" of activity near the plasma membrane. This mechanism of release is different from that of DETA-NO which is not selective for any cell compartment and causes a generalized time- and concentration-dependent increase of cytosolic fluorescence.

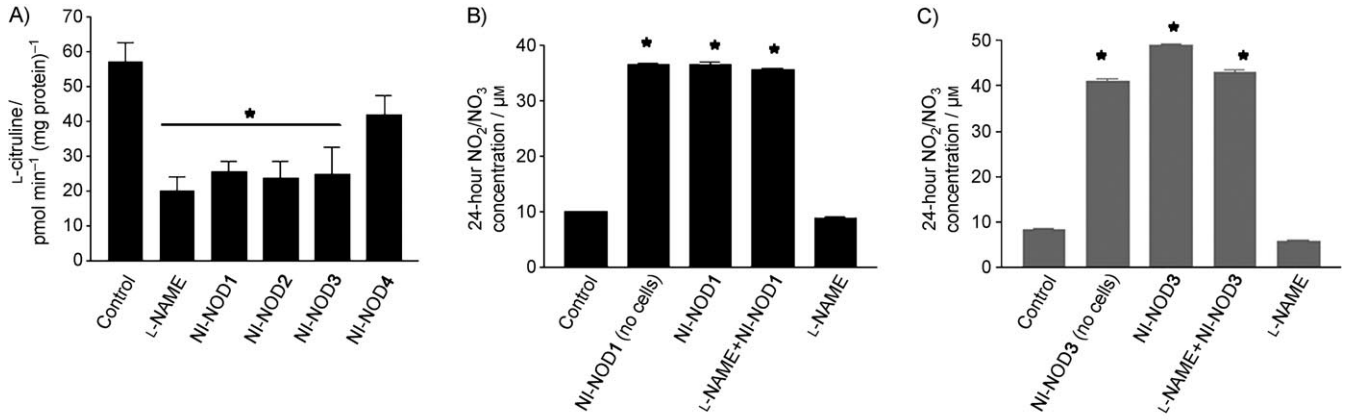



**Figure 1.** A) In HUVECs, NI-NOD**1**, **2**, and **3** (100 μM) cause a significant decrease in L-citrulline production, demonstrating that all these compounds act as NOS inhibitors, whereas NI-NOD**4** has no effect. B) and C) Treating HUVECs and LPS/IL-1β-primed HUVECs with NI-NOD**1** and NI-NOD**3** (100 μM) resulted in a prolonged nitrite/nitrate ($NO_2/NO_3$) release as demonstrated by the increased $NO_2/NO_3$ concentration after 24 h for both compounds; NI-NOD**3** released NO approximately twice as much and as fast as NI-NOD**1**, and this action may explain the more potent activity of NI-NOD**3** on IL-β production and apoptotic activity relative to NI-NOD**1**; $^*p < 0.05$; the concentration of L-NAME was 200 μM.

### Effects of NI-NODs on IL-1β and $PGE_2$ production

IL-1β, a macrophage derived cytokine, has a central role in mounting immune response and inflammation. Some of the more promising experimental anti-inflammatory compounds are natural analogues of IL-1β which inhibit multiple protein kinases which participate in the regulation of IL-1β and iNOS induction, whereas selective and nonselective COX inhibitors (that is, aspirin) do not prevent IL-1β and iNOS induction.[24] NI-NOD**1** and NI-NOD**2** (100 μM) decreased IL-1β release by LPS-primed macrophages by ~40%, whereas NI-NOD**4** (100 μM) had no activity, however NI-NOD**3** (100 μM) decreased IL-1β release by ~90% (Figure 2A). As all NI-NODs, except NI-NOD**4**, act as NO donors and NOS inhibitors, this effect was likely due to this dual mode of action of NI-NODs and was not reproduced by DETA-NO which simply donates NO.

Exposure of macrophages to IL-1β induces COX-2 expression and significantly enhances prostaglandin $PGE_2$ generation. Aspirin and salicylic acid are potent inhibitors of COX-1, and it is well recognized that COX inhibition is the main mechanism of action of nonsteroidal anti-inflammatory drugs (NSAIDs).

Exposure of macrophages to LPS/IL-1β induced COX-2 expression significantly enhanced generation of $PGE_2$ (Figure 2B). NI-NOD**1** (100 μM) effectively decreased $PGE_2$ generation by ~10% ($p < 0.05$ versus IL1-LPS). The addition of L-NAME (200 μM) had no effect on $PGE_2$ release (Figure 2B).

These data suggest that neither endogenously formed NO nor NOS inhibition is involved in NI-NOD1-induced inhibition of $PGE_2$ generation. Thus, *N*-allyl-*N*′-(4-aminobutyl) guanidine **1a** from NI-NOD**1** (see Experimental Section) could be the mainly responsible for its modulatory effect on $PGE_2$ generation, making NI-NOD**1** an attractive starting point for future refinement.

### Effects of NI-NODs on endothelial protection

The endothelium plays a pivotal role in regulating recruitment of blood-borne cells during inflammation and immune surveillance. Alteration of endothelial function may contribute to inflammatory and degenerative disorders. NO has been demonstrated to be involved in the regulation of apoptosis[21,25] and was shown to induce both apoptosis and necrosis as well as protect from apoptosis in different cell types.[21,25]

We have therefore examined the effect of NI-NODs on a mitochondria-dependent model of apoptosis in HUVECs. None of the NI-NODs induced apoptosis or necrosis when incubated with HUVECs alone (data not shown).

However, exposure of staurosporine-treated HUVECs to NI-NOD**3** (100 μM) significantly decreased the number of apoptotic cells (57.6% reduction, $p < 0.05$, 3 h incubation) without improving the transition from apoptosis to necrosis (data not shown), and similar results were also obtained with NI-NOD**4** (44.6% reduction over 3 h).

Remarkably both NI-NOD**3** and NI-NOD**4** (100 μM) postponed the progression to apoptosis in cells that were exposed to staurosporine for 24 h (48.4 and 43.4% reduction, respectively; Figure 3). NI-NOD**1** did not protect HUVECs against the apoptotic action of staurosporine. NI-NOD**2**, a propargyl agmatine derivative, did not protect staurosporine-treated HUVECs (12.2 and 8.5% less after 3 and 24 h, respectively; Figure 3).

### Effect of NI-NODs on mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_m$) is a measure of mitochondrial function.[26] NO was previously shown to cause mitochondrial hyperpolarization, an effect mediated by the transitory inhibition of cell respiration and oxygen consump-

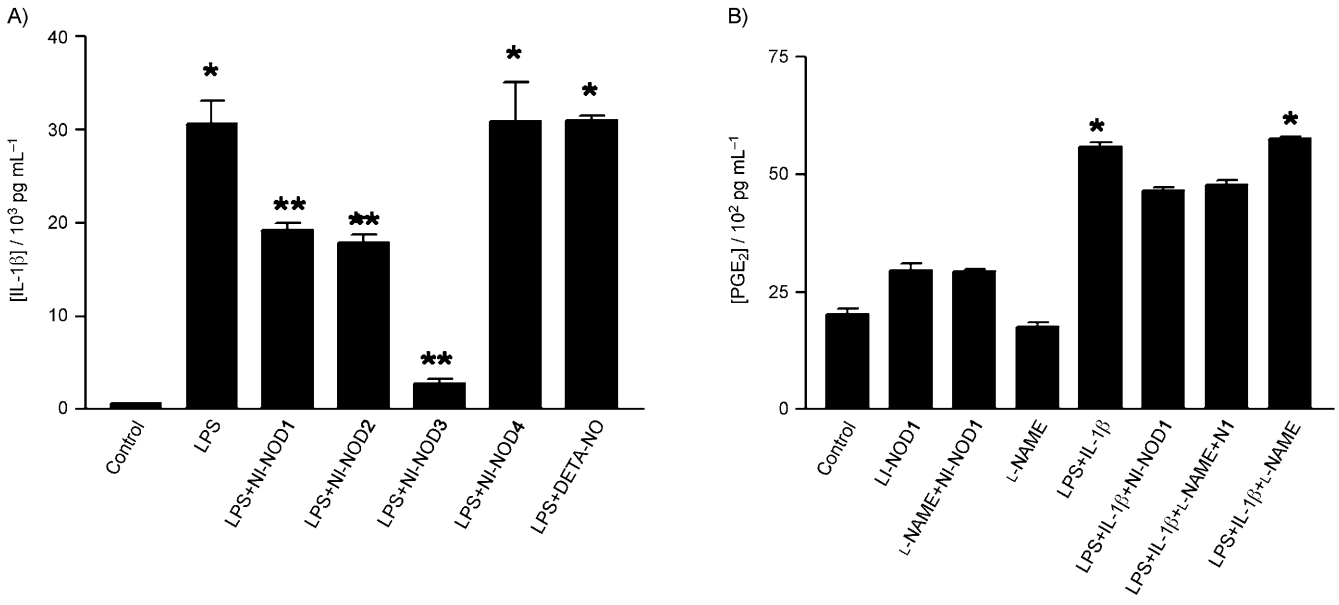



**Figure 2.** A) LPS (10 μg mL$^{-1}$) induces a significant increase of IL-1β production from human monocytes that is inhibited by NI-NOD**1** and NI-NOD**2** (100 μM) and almost completely suppressed by NI-NOD**3** (100 μM), whereas NI-NOD**4** (100 μM) and DETA-NO (100 μM) have no effect. B) In HUVECs, NI-NOD**1** led to a slight increase in $PGE_2$ concentration that was not affected by the addition of L-NAME (200 μM), whereas after the addition of LPS (10 μg mL$^{-1}$) plus IL-1β (10 μg mL$^{-1}$) to cells, NI-NOD**1** caused a significant decrease in $PGE_2$ concentration; *$p < 0.05$.

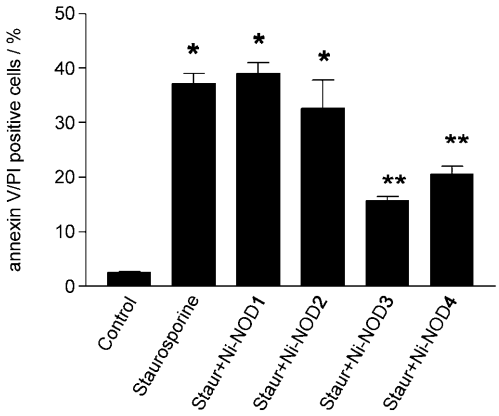



**Figure 3.** Both NI-NOD**3** and NI-NOD**4** at the same dose (100 μM) retard the progression to apoptosis after 24 h (48.4 and 43.4% less, respectively). NI-NOD**1** (100 μM) does not protect HUVECs against the apoptotic action of staurosporine, as it shows a slight increase of apoptotic cell rate (5.4% more after 3 h and 5.1% more after 24 h). In contrast, NI-NOD**2** (100 μM), a propargyl agmatine derivative, does not protect cells from staurosporine-induced apoptosis (12.2 and 8.5% less after 3 and 24 h, respectively); $*p < 0.05$ versus control; $**p < 0.05$ versus staurosporine.

tion. Consistent with the fact that NI-NOD**1** and NI-NOD**3** release NO, we found that exposure of HUVECs to these two agents results in prolonged increase of $\Delta\Psi_{m.}$ (Figure 4). $\Delta\Psi_m$ hyperpolarization induced by NI-NOD**1** (100 μM) (+25%) was slightly more pronounced than that induced by NI-NOD**3** (100 μM) (+8%). These effects were resistant to the addition of L-NAME (200 μM) in the incubation medium.

### Effect of NI-NODs on TNBS-induced colitis in vivo

As an in vitro study revealed that NI-NOD**1**, NI-NOD**2**, and NI-NOD**3** generated NO protects endothelial cells from apoptosis and inhibits IL-1β release from cells of innate immunity, we investigated whether these in vitro features could be exploited for treating against development of colon inflammation in mice administered with trinitrosulfonic acid (TNBS).[27] Colitis induced by TNBS is a well-validated model of colon inflammation that shares similarities with Crohn's disease, an immune-mediated disorder of small and large intestine in which a cytokine-driven production of NO plays a mechanistic role in tissue destruction. 5-ASA is commonly used for treating colitis induced by TNBS. As shown in Figure 5A, TNBS-induced colitis caused a severe weight loss that was only partially reversed by 5-ASA, L-NAME, and NI-NOD**2** ($*p < 0.05$ versus control). In contrast, NI-NOD**1**-treated animals gained significant weight when compared with colitis mice ($p < 0.05$ versus TNBS-treated animals). Exposure to TNBS also decreased stool consistency, a marker of bowel inflammation, which was not reversed by L-NAME and 5-ASA. In contrast, NI-NOD**1** and NI-NOD**2** increased stool consistency when compared with TNBS alone (Figure 5B). Finally, the analysis of macroscopic inflammatory score revealed that only NI-NOD**2** significant ameliorated the inflammation induced by TNBS (Figure 5C). Similar results were obtained with NI-NOD**1** and NI-NOD**3** when administered orally (data not shown).

## Conclusions

NI-NODs are a new class of anti-inflammatory drugs targeting NO homeostasis. Although the mechanism of action of NI-NOD needs to be further investigated, they showed strong biological activity on some of the pivotal steps involved in inflammation induction, both in the in vitro and in the in vivo experiments. All NI-NODs, except NI-NOD**4**, are demonstrated to be NOS inhibitors, NI-NOD**1** and NI-NOD**3** release significant amounts of $NO_2/NO_3$ both spontaneously and when added to HUVECs. Moreover, NI-NOD**1** and NI-NOD**3** significantly inhibit IL-1β release and $PGE_2$ production, and determine endothelial protection. NI-NOD**1** and NI-NOD**2** maintain their anti-inflammatory activity in vivo in a rodent model of colitis, suggesting that this new class of compounds is an attractive starting point for future refinement.

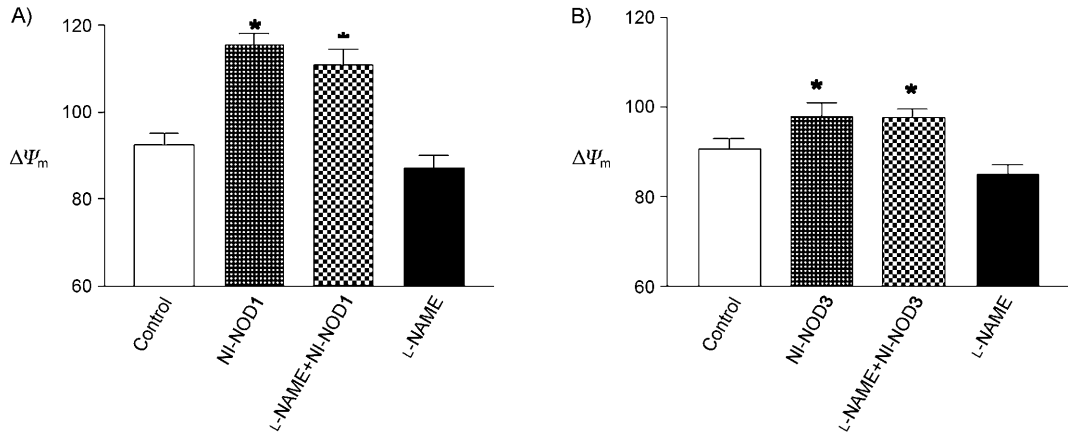



**Figure 4.** NI-NOD**1**- and NI-NOD**3**-induced $\Delta\Psi_m$ hyperpolarization (measured as red fluorescence intensity) is maintained over the time investigated (24 h). A) $\Delta\Psi_m$ hyperpolarization induced by NI-NOD**1** (100 μM) is more pronounced than by B) NI-NOD**3** (100 μM). L-NAME (200 μM) has no effect on the hyperpolarization caused by either NI-NOD**1** or NI-NOD**3**, and alone had no effect on $\Delta\Psi_m$; $*p < 0.05$.

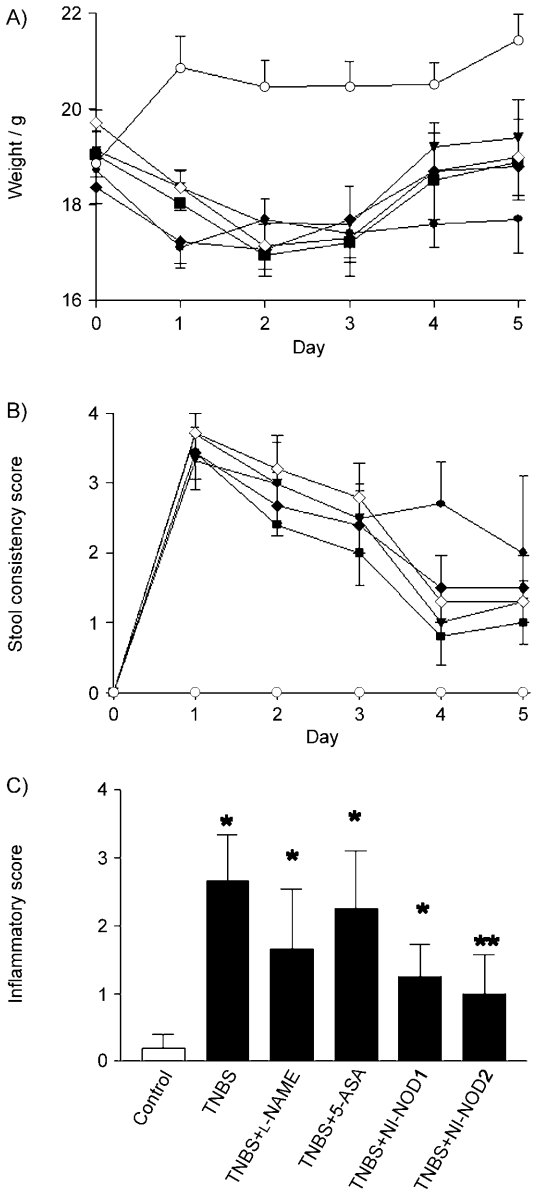



**Figure 5.** A) TNBS-induced colitis causes severe weight loss that is only partially reversed by 5-ASA (◆, 50 mg kg$^{-1}$ day$^{-1}$ for 4 days i.r.), L-NAME (▼, 10 mg kg$^{-1}$ day$^{-1}$ for 4 days i.r.), and NI-NOD**2** (◇, 18 mg kg day$^{-1}$ for 4 days i.r.). In contrast, NI-NOD**1**-treated animals (■, 18 mg kg$^{-1}$ day$^{-1}$ for 4 days i.r.) gain significant weight in comparison with colitic mice (*$p < 0.05$ versus control (○); **$p < 0.05$ versus TNBS-treated animals (●)). B) TNBS causes a marked decrease in stool consistency that is reversed by neither L-NAME (▼) nor 5-ASA (◆). In contrast, NI-NOD**1** (■, 18 mg kg$^{-1}$ day$^{-1}$ for 4 days i.r.) and NI-NOD**2** (◇, 18 mg kg$^{-1}$ day$^{-1}$ for 4 days i.r.) induce a significant increase in stool consistency in comparison with TNBS alone (*$p < 0.05$ versus control (○); **$p < 0.05$ versus TNBS-treated animals (●)). C) NI-NOD**1** (18 mg kg$^{-1}$ day$^{-1}$ for 4 days i.r.) does not cause a significant amelioration of the macroscopic inflammatory score, whereas NI-NOD**2** (18 mg kg$^{-1}$ day$^{-1}$ for 4 days i.r.) significantly ameliorates TNBS-induced inflammation (*$p < 0.05$ versus control; **$p < 0.5$ versus TNBS-treated animals).

## Experimental Section

### Chemistry

**Materials**. Reagents were obtained from commercial suppliers and used without further purification. Merck silica gel 60 was used for flash chromatography (230–400 mesh). Uncorrected melting points were taken on a Gallenkamp apparatus. Microanalyses were performed on a PerkinElmer 240C Elemental Analyzer. All compounds were analyzed for C, H, and N, and the results obtained were within ±0.4% of calculated values. Optical rotations were measured on AA-5 Optical Activity polarimeter. The IR spectra were recorded on a PerkinElmer 1600 FTIR. The $^1$H NMR spectra were recorded at 200 MHz. Chemical shifts are reported relative to $CDCl_3$ at $\delta = 7.24$ ppm and tetramethylsilane at $\delta = 0.00$ ppm. Low-resolution mass spectrometric data were recorded with an electron beam of 70 eV.

**1-Amino-4-[$N^3$-alkyl-$N^3$,$N^2$-bis(*tert*-butoxycarbonyl)guanidino]butane (5a,b).** *General procedure.* A solution of *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*-(alkyl)-*S*-methylisothiourea (10 mmol) in THF (25 mL) was added dropwise to a stirred solution of 1,4-diaminobutane (2.29 g, 26 mmol) in THF/$H_2O$ (40 mL, 20:1, *v*/*v*). After stirring for 1 h at 50 °C, the reaction mixture was concentrated under vacuum and the residue was partitioned between $CHCl_3$ and 10% aqueous $NaHCO_3$. The organic layer was dried and evaporated. The residue was purified by flash chromatography ($CH_2Cl_2$/EtOAc 5:5 to 2:8) to give **5a** (80%), **5b** (79%) as a colorless oil.

**1-Amino-4-[$N^3$-allyl-$N^3$,$N^2$-bis(*tert*-butoxycarbonyl)guanidino]butane (5a).** $^1$H NMR ($CDCl_3$): $\delta = 8.18$ (br s, NH), 5.81–5.72 (m, 1H), 5.23–5.03 (m, 2H), 4.17 (d, $J = 6.7$ Hz, 2H), 3.19–3.15 (m, 2H), 2.66–2.62 (m, 2H), 1.92–1.51 (m, 4H), 1.40 (s, 9H), 1.38 ppm (s, 9H); MS (ESI): $m/z = 393.1$ $[M+Na]^+$, 371.1 $[M+H]^+$; Anal. calcd for $C_{18}H_{34}N_4O_4$: C 58.35, H 9.25, N 15.12, found: C 58.11, H 9.17, N 15.32.

**1-Amino-4-[$N^3$-propargyl-$N^3$,$N^2$-bis(*tert*-butoxycarbonyl)guanidino]butane (5b).** $^1$H NMR ($CDCl_3$): $\delta = 8.10$ (br s, NH), 4.373 (s, 2H), 3.23–3.18 (m, 2H), 2.64–2.60 (m, 2H), 2.14–2.13 (m, 1H), 1.61–1.52 (m, 4H), 1.38 (s, 9H), 1.37 ppm (s, 9H); MS (ESI): $m/z = 369.1$ $[M+H]^+$; Anal. calcd for $C_{18}H_{32}N_4O_4$: C 58.67, H 8.75, N 15.21, found: C 58.84, H 8.52, N 15.46.

**3-Hydroxybenzyl bromide (8)**. A solution of $PBr_3$ (3.534 mL, 37.60 mmol) and dry pyridine (1.514 mL) in dry $CH_3CN$ (21 mL) was added slowly to a stirred solution of 3-hydroxybenzyl alcohol (10.738 g, 86.5 mmol) and dry pyridine (515 μL) in dry $CH_3CN$ (32 mL) cooled at −10 °C, and the reaction mixture was stirred under argon atmosphere at −10 °C. After 1 h the reaction mixture was warmed to room temperature and stirred overnight, the solvent was evaporated, and the residue was purified by flash chromatography (petroleum ether/EtOAc, 7:3) affording **8** as a solid, 6.891 g (98%). $^1$H NMR ($CDCl_3$): $\delta = 7.22–7.14$ (m, 1H), 6.96–6.92 (m, 1H), 6.87–6.75 (m, 2H), 5.21 (br s, 1H), 4.40 ppm (s, 2H); MS (ESI): $m/z = 188.03$ $[M+H]^+$; Anal. calcd for $C_7H_7BrO$: C 44.95, H 3.77, Br 42.72, found: C 45.09, H 3.57, Br 42.88.

**3-Hydroxybenzyl alcohol nitro ester (9).** Silver nitrate (1.64 g, 9.66 mmol) was added to a solution of **8** (8.05 mmol) in dry $CH_3CN$ (20 mL) and the reaction mixture, protected from light, was stirred at room temperature for 12 h. The solvent was removed under vacuum and the residue was dissolved in $CH_2Cl_2$ (10 mL). The mixture was transferred to a separating funnel and washed with water (1×3 mL), dried ($Na_2SO_4$), filtered, and evaporated under reduced pressure to leave a colorless solid. The residue, purified by flash chromatography (80% petroleum ether, 20% EtOAc), afforded pure **9** as an oil, 1.088 g (80%). $^1$H NMR ($CDCl_3$): $\delta = 7.27–7.19$ (m, 1H), 6.94–6.84 (m, 3H), 6.5 (br s, 1H, OH), 5.32 ppm (s, 2H); MS (ESI): $m/z = 170.04$ $[M+H]^+$; Anal. calcd for $C_7H_7NO_4$: C 49.71, H 4.17, N 8.28, found: C 49.95, H 4.34, N 8.20.

**3-Nitrooxymethylphenyl 4-nitrophenyl carbonate (6).** *p*-Nitrobenzyl chloroformate (2.147 g,10.65 mmol) was added to a stirred solution of **9** (1.80 g, 10.65 mmol) and $Et_3N$ (1.482 mL) in dry $CH_2Cl_2$ (30 mL), and the reaction mixture, protected from light, was stirred at room temperature for 2 h. The solvent was removed under vacuum and the residue was purified by flash chromatography (100% $CH_2Cl_2$) affording **6** as a yellow oil, 2.49 g (70%). $^1$H NMR ($CDCl_3$): $\delta = 8.21–8.11$ (m, 2H), 7.43–7.38 (m, 3H), 7.26–7.22 (m, 3H), 5.37 ppm (s, 2H); MS (ESI): $m/z = 335.5$ $[M+H]^+$; Anal. calcd for $C_{14}H_{10}N_2O_8$: C 50.31, H 3.02, N 8.38, found: C 50.46, H 3.21, N 8.18.

**General procedure for the preparation *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*-(alkyl)-*S*-methylisothioureas 4a,b using a Mitsunobu protocol**. $Ph_3P$ (15 mmol) and the appropriate alcohol (13 mmol) were added to a stirred solution of *N*,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (10 mmol) in dry THF. The reaction mixture was first cooled at 0 °C and DIAD (15 mmol) was added dropwise, then the reaction mixture was stirred at reflux overnight. The crude mixture was concentrated and diluted with $H_2O$ and $CH_2Cl_2$, the aqueous layer was extracted twice with $CH_2Cl_2$, and the combined organic phases were washed with brine and dried over $Na_2SO_4$. The solvent was evaporated under vacuum and the crude mixture was purified by flash chromatography (90% petroleum ether, 10% EtOAc).

***N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*-(allyl)-*S*-methylisothiourea (4a).** Yield 82%. $^1$H NMR ($CDCl_3$): $\delta = 5.76–5.68$ (m, 1H), 5.17–5.00 (m, 2H), 3.97 (d, $J = 6$ Hz, 2H), 2.23 (s, 3H), 1.37 (s, 9H), 1.33 ppm (s, 9H); MS (ESI): $m/z = 682.9$ $[2M+Na]^+$, 369.0 $[M+K]^+$, 353.0 $[M+Na]^+$, 331.0 $[M+H]^+$; Anal. calcd for $C_{15}H_{26}N_2O_4S$: C 54.52, H 7.93, N 8.48, S 9.70, found: C 54.72, H 7.81, N 8.28, S 9.80.

***N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*-(propargyl)-*S*-methylisothiourea (4b)**. Yield 96%. $^1$H NMR ($CDCl_3$): $\delta = 4.22$ (s, 2H), 2.34 (s, 3H), 2.24 (s, 1H), 1.43 ppm (s, 18H); MS (ESI): $m/z = 681.1$ $[2M+Na]^+$, 367.0 $[M+K]^+$, 351.1 $[M+Na]^+$, 328.9 $[M+H]^+$; Anal. calcd for $C_{15}H_{24}N_2O_4S$: C 54.86, H 7.37, N 8.53, S 9.76, found: C 54.61, H 7.60, N 8.47, S 9.66

***N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*$^{\omega}$-nitro-L-arginine methyl ester (11)**. *Procedure A*. Trimethylsilyldiazomethane was added dropwise to a stirred solution of *N*$^{\alpha}$-(*tert*-butoxycarbonyl)-*N*$^{\omega}$-nitro-L-arginine (50 mg, 0.16 mmol) in MeOH (4 mL) until a deep yellow color persisted. *Procedure B*. *N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*$^{\omega}$-nitro-L-arginine (50 mg, 0.16 mmol) was dissolved in anhydrous dioxane (3 mL) and the reaction was heated until a clear solution was obtained, then the reaction mixture was allowed to reach room temperature. Thionyl chloride (3.8 μL, 0.31 mmol) in dry *N*,*N*-dimethylformamide (DMF, 0.015 mL) was added to that solution and the reaction mixture was stirred for 2 h, then MeOH (4 mL) was added and the reaction mixture was heated at reflux and stirred for 4 h. *Procedure C*. Thionyl chloride (3.8 μL, 0.31 mmol) in dry DMF (0.015 mL) was added to a solution of *N*$^{\alpha}$-(*tert*-butoxycarbonyl)-*N*$^{\omega}$-nitro-L-arginine (50 mg, 0.16 mmol) in anhydrous MeOH (4 mL) and the reaction mixture was heated at reflux and stirred for 4 h. *Procedure D*. Thionyl chloride (3.8 μL, 0.31 mmol) in dry DMF (0.015 mL) was added to a solution of *N*$^{\alpha}$-(*tert*-butoxycarbonyl)-*N*$^{\omega}$-nitro-L-arginine (50 mg, 0.16 mmol) in anhydrous MeOH (4 mL) and the reaction mixture was heated by microwave irradiation for 3 min twice (power: 100 W, $t = 3$ min, $P_{max} = 200$ psi, $T_{max} = 100$ °C).

**Work-up**. The reaction mixture was concentrated under reduced pressure, diluted with water and then extracted with $CH_2Cl_2$. The combined organic solution was dried ($Na_2SO_4$), filtered, and concentrated under reduced pressure to obtain the pure compound **11** (57–62 mg, 70–75% with Procedures A–B–C and 78 mg, 95% with procedure D). $^1$H NMR ($CDCl_3$): $\delta = 7.65$ (br s, 1H), 5.57 (br, 1H), 4.38–4.20 (m, 1H), 3.73 (s, 3H, $COCH_3$), 3.58–3.38 (m, 1H), 3.37–3.26 (m, 1H), 1.88–1.65 (m, 4H), 1.40 ppm (s, 9H); MS (ESI): $m/z = 332.4$ $[M+H]^+$; Anal. calcd for $C_{12}H_{23}N_5O_6$: C 43.24, H 6.95, N 21.01, found: C 43.12, H 6.84, N 21.12.

**3-Nitrooxymethylphenyl-4-nitrophenylcarbamate derivatives (7a,b and NI-NOD3).** *General procedure*. A solution of **5a,b** or **2** (0.55 mmol) and 3-Nitrooxymethylphenyl 4-nitrophenyl carbonate **6** (231 mg, 0.69 mmol) in dry $CH_3CN$ (15 mL), was stirred at room temperature protected from light for the required time. The solvent was removed under reduced pressure and the residue dissolved in $CH_2Cl_2$ (10 mL). The mixture was transferred to a separating funnel and washed with water (3 mL) and brine. The organic layer was separated, dried ($Na_2SO_4$), filtered, concentrated under reduced pressure, and purified by flash chromatography, using hexane/$Et_2O$ 1:1 as eluents followed by $Et_2O$, to afford pure **7a,b** and NI-NOD**3**.

**1-[((3-Nitrooxymethyl)phenoxycarbonyl)amino]-4-[*N*$^3$-allyl-*N*$^3$,*N*$^2$-bis(*tert*-butoxycarbonyl)guanidino]butane (7a)**. Reaction time: 8 h. Brown oil. Yield: 45%. $^1$H NMR ($CDCl_3$): $\delta = 7.61$ (br s, NH), 7.35–7.30 (m, 1H), 7.18–7.09 (m, 3H), 5.91–5.74 (m, 1H), 5.48–5.44 (br s, NH), 5.39 (s, 2H), 5.18–5.07 (m, 2H), 4.91 (d, $J = 6.22$ Hz, 2H), 3.22–3.20 (m, 4H), 1.60–1.59 (m, 4H), 1.44 (s, 9H), 1.41 ppm (s, 9H); MS (ESI): $m/z = 587.9$ $[M+Na]^+$, 565.9 $[M+H]^+$; Anal. calcd for $C_{26}H_{39}N_5O_9$: C 55.21, H 6.95, N 12.38, found: C 55.32, H 6.83, N 12.47.

**1-[((3-Nitrooxymethyl)phenoxycarbonyl)amino]-4-[*N*$^3$-propargyl-*N*$^3$,*N*$^2$-bis(*tert*-butoxycarbonyl)guanidino]butane (7b).** Reaction time: 8 h. Brown oil. Yield: 43%. $^1$H NMR ($CDCl_3$): $\delta = 7.35–7.32$ (m, 1H), 7.19–7.11 (m, 3H), 5.38 (s, 2H), 5.28 (br s, NH), 4.42 (s, 2H), 3.33–3.24 (m, 4H), 2.23 (s, 1H), 1.70–1.64 (m, 4H), 1.47 (s, 9H), 1.46 ppm (s, 9H); MS (ESI): $m/z = 586.0$ $[M+Na]^+$, 564.0 $[M+H]^+$; Anal. calcd for $C_{26}H_{37}N_5O_9$: C 55.41, H 6.62, N 12.43, found: C 55.52, H 6.50, N 12.37.

**(*R*)-3-(3-Fluorophenyl)-3-[((3-Nitrooxymethyl)phenoxycarbonyl]-propynyl amine (NI-NOD3).** Reaction time: 96 h. White solid. Yield: 45%; mp: 106 °C; $[\alpha]_D^{23} = +24.3$ ($c = 1.0$ in $CHCl_3$); $^1$H NMR ($CDCl_3$): $\delta = 7.55–54$ (m, 2H), 741–7.34 (m, 2H), 7.24–7.02 (m, 4H), 5.73 (s br, 1H, NH), 5.7 (d, $J = 1.9$ Hz, 1H), 5.39 (s, 2H), 2.58 ppm (d, 1H, $J = 1.9$); MS (ESI): $m/z = 367.1$ $[M+Na]^+$; Anal. calcd for $C_{17}H_{13}FN_2O_5$: C 59.30, H 3.81, N 8.14, found: C 59.21, H 3.68, N 8.30.

**1-[((3-Nitrooxymethyl)phenoxycarbonyl)amino]-4-(*N*$^3$-alkyl- guanidino)butane trifluoroacetic salt (NI-NOD1 and NI-NOD2).** *General procedure*. A solution of trifluoroacetic acid (3.2 mL) in dry $CH_2Cl_2$ (8.8 mL) was added to a solution of **7a,b** (0.55 mmol) in anhydrous $CH_2Cl_2$ (20 mL) under argon and the reaction was stirred at room temperature for 12 h. Trifluoroacetic acid was removed by means of co-evaporation with $CH_2Cl_2$ (5 mL × 10) to afford pure compounds NI-NOD**1** and NI-NOD**2** in quantitative yield.

**1-[((3-Nitrooxymethyl)phenoxycarbonyl)amino]-4-(*N*$^3$-allyl- guanidino)butane trifluoroacetic salt (NI-NOD1).** Brown oil. $^1$H NMR ($CD_3COCD_3$): $\delta = 8.111$ (NH, br s), 7.45–7.15 (m, 4H), 6.99 (NH, br s), 5.91–5.79 (m, 1H), 5.56 (s, 2H), 5.30–5.15 (m, 2H), 3.92 (s, 2H), 3.36–3.35 (m, 2H), 3.25–3.23 (m, 2H), 1.71–1.65 ppm (m, 4H); MS (ESI): $m/z = 366.1$ $[M+H]^+$; Anal. calcd for $C_{18}H_{24}F_3N_5O_7$: C 45.10, H 5.05, N 14.61, found: C 45.12, H 5.04, N 14.53.

**1-[((3-Nitrooxymethyl)phenoxycarbonyl)amino]4-[*N*$^3$-propargyl-guanidino)butane trifluoroacetic salt (NI-NOD2).** Brown oil. $^1$H NMR ($CD_3COCD_3$): $\delta = 8.14$ (br s, NH), 7.45–7.13 (m, 4H), 5.54 (s,

2H), 4.12 (s, 2H), 3.35–3.23 (m, 4H), 2.83 (s, 1H), 1.94–1.68 ppm (m, 4H); MS (ESI): $m/z = 364.1$ $[M+H]^+$; Anal. calcd for $C_{18}H_{22}F_3N_5O_7$: C 45.29, H 4.64, N 14.67, found: C 45.46, H 4.44, N 14.80.

**$N^{\alpha}$-[(3-Nitrooxymethyl)phenoxycarbonyl]-$N^{\omega}$-nitro-L-arginine methyl ester (NI-NOD4).** A solution of trifluoroacetic acid (0.1 mL) in dry $CH_2Cl_2$ (0.9 mL) was added to **11** (26, 4 mg, 0.074 mmol) dissolved in anhydrous $CH_2Cl_2$ (1 mL) under argon and the reaction was stirred at room temperature for 4 h. Trifluoroacetic acid was removed by co-evaporation with $CH_2Cl_2$ (5 mL×10). The residue was dissolved in dry $CH_3CN$, triethylamine (0.015 mL, 0.074 mmol) was added, and the reaction mixture was stirred at room temperature for 1 h. Compound **6** (24.8 mg, 0.074 mmol) was added and the reaction mixture was stirred at room temperature for a further 24 h. The solvent was removed under vacuum and the residue dissolved in $CH_2Cl_2$ (10 mL). The mixture was transferred to a separating funnel and washed with water (3 mL) and brine. The organic layer was separated, dried ($Na_2SO_4$), filtered, and the solvent was removed by evaporation under reduced pressure. Flash chromatography, using hexane/$Et_2O$ 1:1 followed by $Et_2O$ as eluents, afforded pure NI-NOD**4** (17 mg, 53%). $[\alpha]_D^{23} = -17.12$ ($c = 1.0$ in $CHCl_3$); $^1$H NMR ($CDCl_3$): $\delta = 7.40$–$7.09$ (m, 4H), 6.04 (br, 1H, NH), 5.38 (s, 2H), 4.40–4.38 (m, 1H), 3.76 (s, 3H), 3.45–3.27 (m, 2H), 1.97–1.72 ppm (m, 4H); MS (ESI): $m/z = 427.2$ $[M+H]^+$; Anal. calcd for $C_{15}H_{20}N_6O_9$: C 42.06, H 4.71, N 19.62, found: C 42.21, H 4.62, N 19.50.

### In vitro studies

**Human umbilical endothelial cells (HUVECs).** Primary cultures of HUVECs were from Istituto Zooprofilattico of Brescia (Brescia, Italy). HUVECs were grown in endothelial basal medium supplemented with bovine brain extract (12 μg mL$^{-1}$), human epithelial growth factor (10 ng mL$^{-1}$), hydrocortisone (1 μg mL$^{-1}$), penicillin (100 U mL$^{-1}$), streptomycin (100 μg mL$^{-1}$) and gentamicin (5 μg mL$^{-1}$), and 2% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% $CO_2$. Cells were used between passages 2–5. Cells (~$10^5$ per well) were treated with L-NAME (200 μM), NI-NOD**1**–**3** (100 μM), and cell activation was obtained by LPS (10 μg mL$^{-1}$) plus IL-1β (10 ng mL$^{-1}$). The $NO_2/NO_3$ concentrations in cell supernatants were measured using a commercially available enzyme immunoassay kit (Cayman Chemical, Michigan, USA). $PGE_2$ levels were measured in cell supernatants using a commercially available enzyme immunoassay system (Amersham Pharmacia Biotech, Buckinghamshire, UK). $PGE_2$ concentrations were calculated from the standard curve and results expressed as pg mL$^{-1}$. The kit was specific and showed negligible cross-reactivity with several other eicosanoids (data furnished by the manufacturer).

**Monocyte isolation.** Human PBMCs were obtained from normal individual donors from the Blood Bank Service of Perugia University Hospital. PBMCs were isolated by density gradient centrifugation (400×g for 30 min at room temperature) using Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden). The major band, containing the mononuclear cells, was harvested with a typical yield of 1.5–2×$10^8$ cells per isolation. PBMC were washed by centrifugation three times using RPMI 1640 (Life Technologies Italia Srl, Milan, Italy) supplemented with 10% fetal calf serum, L-glutamine, penicillin (100 U μL$^{-1}$), streptomycin (100 U μL$^{-1}$), and gentamicin (10 μg mL$^{-1}$); 2×$10^7$ cells were placed into each 75 cm$^2$ tissue culture flask (Corning, NY, USA) and incubated for 1 h at 37 °C in an atmosphere containing 5% $CO_2$ and 95% air. At the end of incubation, nonadherent cells were removed by washing, and the cell layers (monocytes) were collected and incubated for 24 h in 24-well plates at a concentration of 1×$10^6$ mL$^{-1}$ and stimulated as indicated.

**L-Citrulline measurement.** The effect of NI-NOD compounds on L-citrulline production was detected in HUVECs by using a commercial ELISA kit (NOSdetect Assay Kit, (ALX-850–006) Alexis Biochemical, San Diego, USA).

**Cytokine ELISA measurement.** IL-1β concentrations in cell supernatants were measured using a commercial ELISA kit (Endogen, and R&D Systems, Minneapolis, MN) using the standard procedure recommended by the manufacturers. Cytokine concentrations were calculated from the standard curves using the GraphPad Prism software (GraphPad Software, San Diego, CA) and results expressed as pg mL$^{-1}$. Each kit was specific and showed negligible cross-reactivity with several other cytokines (data furnished by the manufacturer).

**Mitochondrial potential.** To measure $\Delta\Psi_m$ after exposure of cells to different agents, HUVECs (5×$10^5$ mL$^{-1}$) were incubated with JC-1 (5 μg mL$^{-1}$). This cyanine dye accumulates in the mitochondrial matrix under the influence of the $\Delta\Psi_m$, and forms J aggregates that have characteristic absorption and emission spectra. After incubation for 20 min at room temperature in the dark, cells were washed once with PBS; cell suspensions were prepared for flow cytometry and the 488 nm line of an argon ion laser was used for excitation. Orange emitted fluorescence was collected through 585/542 nm (FL2) bandpass filters. Flow cytometry was performed with an Epics XL instrument (Coulter-Beckman, Milan, Italy). After eliminating small (that is, noncellular) debris, 50 000 events were collected for each analysis. Results are expressed as the mean aggregate fluorescence alone (red).

**Detection of apoptosis.** HUVEC cells were incubated for 24 h as indicated and the apoptotic cells were detected by flow cytometry after staining with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (PI) by using a commercially available kit (Annexin V–FITC Kit, Immunotech, Marseille, France) as described previously.[27] Cells were considered apoptotic when they were annexin V-positive and PI-negative. Staining of cells by PI was an indicator of the loss of plasma membrane integrity.

### In vivo studies

Six- to eight-week-old male BALB/c mice (20–22.5 g) were obtained from Charles River Breeding Laboratories. Animal committees at the University of Perugia and the University of Calgary approved all protocols according to governmental guidelines. Colitis was induced in mice as described.[27] Fasted mice were anesthetized, and 0.5 mg of TNBS (Sigma) in 0.1 mL 50% EtOH was administered into the colon lumen by catheter; control mice received 50% EtOH alone. Animals were monitored daily for loss of body mass and survival. At the end of the studies, surviving animals were killed, blood samples were collected by cardiac puncture, and colons were excised, weighed, and evaluated for macroscopic damage.

**Drugs.** The day after TNBS induction, drugs were administered intrarectally (i.r.) at the following doses: L-NAME 10 mg kg$^{-1}$, 5-ASA 50 mg kg$^{-1}$, NI-NOD**1** 18 mg kg$^{-1}$, NI-NOD**2** 18 mg kg$^{-1}$ once a day for four days.

**Macroscopic grading of colitis.** Colons were examined with a dissecting microscope (magnification, ×5) and graded for macroscopic lesions on a scale from 0 to 10 based on criteria for inflammation, such as hyperemia, thickening of the bowel, and the extent of ulceration.[27]

### Data analysis

All values are expressed as mean ± SEM. Variation among data sets was tested with ANOVA, and significance was tested with unpaired t tests, with a Bonferroni modification for comparison of more than two groups of data. Differences were considered significant at $p < 0.05$.

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