

Enhancing the pharmacodynamic profile of a class of selective COX-2 inhibiting nitric oxide donors



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ABSTRACT

We report herein the development, synthesis, physicochemical and pharmacological characterization of a novel class of pharmacodynamic hybrids that selectively inhibit cyclooxygenase-2 (COX-2) isoform and present suitable nitric oxide releasing properties. The replacement of the ester moiety with the amide group gave access to *in vivo* more stable and active derivatives that highlighted outstanding pharmacological properties. In particular, the glycine derivative proved to be extremely active in suppressing hyperalgesia and edema.

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) (Fig. 1) have been used for decades to treat pain and inflammation in rheuma-

Abbreviations: NSAIDs, nonsteroidal anti-inflammatory drug; (t)NSAIDs, traditional nonsteroidal anti-inflammatory drug; GI, gastrointestinal; CV, cardiovascular; COX-2, cyclooxygenase 2; RA, rheumatoid arthritis; OA, osteoarthritis; coxibs, cyclooxygenase-2 inhibitors; NO, nitric oxide; CINODs, cyclooxygenase-inhibiting nitric oxide donor; NOBA, NO releasing moiety (nitroxy)butanol; cNOS, constitutive nitric oxide synthases; SAR, structure relationship studies; EDCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMAP, dimethylaminopyridine; HOBT, 1-hydroxybenzotriazole; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; CBz, carboxybenzyl; Boc, *tert*-butyloxycarbonyl; ODO, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; AA, arachidonic acid; HWB, human whole blood; SGF, simulated gastric fluid; PBS, phosphate buffer solution; FBS, fetal bovine serum; RIA, radioimmunoassay; PGE₂, prostaglandin E₂; LPS, lipopolysaccharide; DMEM, Dulbecco's modified eagle's medium; TX, thromboxane; ipl, intraplantar; ip, intraperitoneal; E_{max}, maximal vasorelaxing response.

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tologic disorders and other conditions. Long-term therapies with traditional (t)NSAIDs are associated to mild-to-severe side effects, especially gastrointestinal (GI), renal and cardiovascular (CV) ones, which can reduce the compliance of the patients.^{1–3} Albeit the development of selective cyclooxygenases-2 (COX-2) inhibitors (coxibs) has given access to more effective drugs for the treatment of severe symptoms related to chronic diseases (rheumatoid arthritis, RA, and osteoarthritis, OA) with reduced GI side effects, the widely documented cardiovascular toxicity of these molecules 'shrank' their administration to a narrower population.^{4–7}

'Molecular hybrids' characterized by a nitric oxide (NO) releasing moiety have been explored by several research groups for improving the pharmacological profile of the parent drugs.^{8,9} Indeed, at physiological concentrations the 'gasotransmitter' NO is a fundamental modulator of the homeostasis in many systems, particularly active in the cardiovascular one, with its powerful vasodilating and antiplatelet activities. Furthermore, NO is known to be 'cardioprotective' against myocardial ischemia/reperfusion injury.^{10–12} The pioneering 'strategy' adopted by NicOx led to the development of the first candidate of the COX Inhibiting Nitric

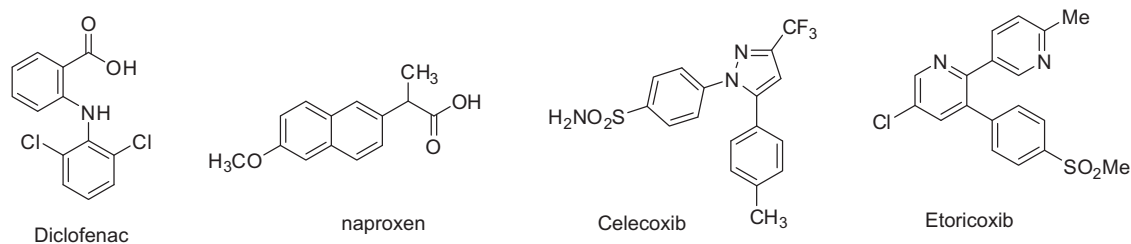


Figure 1. Structure of some selective and non selective COX-2 inhibitors.

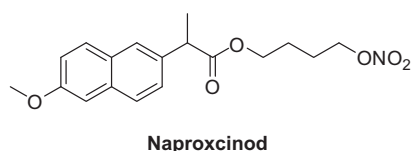


Figure 2. Chemical structure of naproxcinod.

Oxide Donors (CINODs) family (naproxcinod, AZD3582, Fig. 2), which completed phase III trials (osteoarthritis of the knee and hip).^{13–18} Interestingly, though the GI side-effects associated to the administration of naproxcinod appeared slightly reduced when compared to naproxen, a safer CV outcome was highlighted from these studies.^{17,18} In addition to naproxcinod, many other examples have been reported regarding ‘NSAIDs hybridization’, even though very few of them concern the hybridization of selective COX-2 structures.^{19–24}

In this context, our research was focused on the development of a class of CINODs endowed with a diarylpyrrole scaffold^{25–28} and our first report consisted of the synthesis of a first generation of diarylpyrrolacetic esters showing very promising COX-2 inhibition and nitric oxide donating properties (Fig. 3).²⁶ The chemical manipulation of this first generation gave access to a class of more soluble derivatives (Fig. 3).²⁷

However, the chemical and enzymatic liability of these esters was speculated to be responsible of a potential gap between in vitro and in vivo pharmacological profiles as discussed-above for naproxcinod. Therefore, we focused our efforts on nitro-

oxyalkyl ethers which were designed with the aim of preventing the hydrolysis of the side chain (Fig. 3).^{28,29} However, despite their enhanced stabilities, the nitro-oxyalkyl ethers were characterized by low solubility.

To complete the picture, the aim of this work is to generate compounds endowed with greater stability and solubility as well as improved pharmacokinetic properties. We report herein the development of three different subclasses of compounds generated through replacement of the ester moiety with the amide group. In particular, we first explored a subclass of ‘linear’ acetic amides (1a–d), through the replacement of the ester moiety of the first generation of diarylpyrrolacetic esters with the amide group. Then, with the aim of enhancing the solubility of the series, we have prepared two subclasses of compounds decorated with either a free carboxylic substituent (‘serine/homoserine’ acetic amides 2a–d) or with a free amino group (glycine amides 3a–b) (Fig. 4).

Nowadays, evidences account for protective roles of NO (generated by the constitutive nitric oxide synthases (cNOS) isoform) in the joint, thus suggesting that mild NO-donors could be an asset in arthritis treatment. Accordingly, we speculated that an increased penetration of our compounds in the cartilage could be an additional target. Due to the presence of an amino group which is protonated at physiological pH, the glycine derivatives (3) were expected to better penetrate into the cartilage. Indeed, positively charged compounds are reported to better diffuse into the cartilage matrix due to electrostatic interaction with the negatively charged GAG-sulphates.^{30–32}

The substitution pattern of compounds was based on the structure activity relationship (SAR) knowledge acquired during the

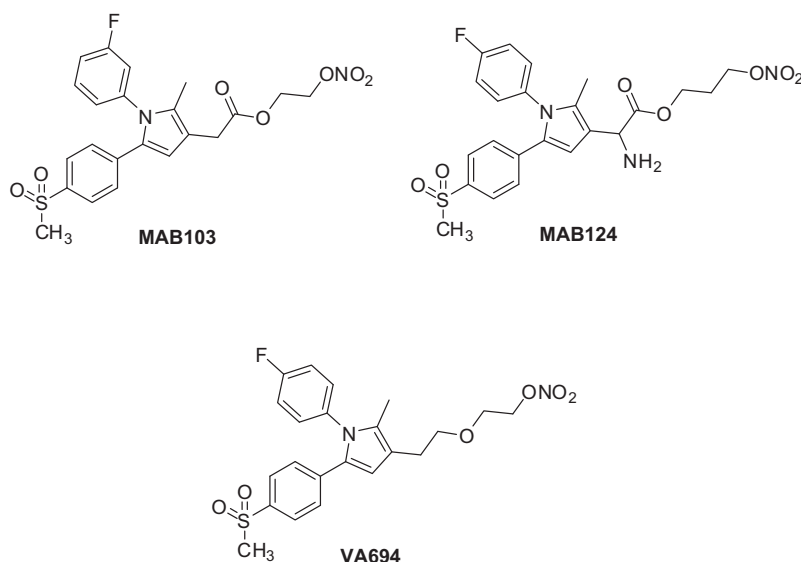


Figure 3. Representative chemical structures of compounds previously generated.

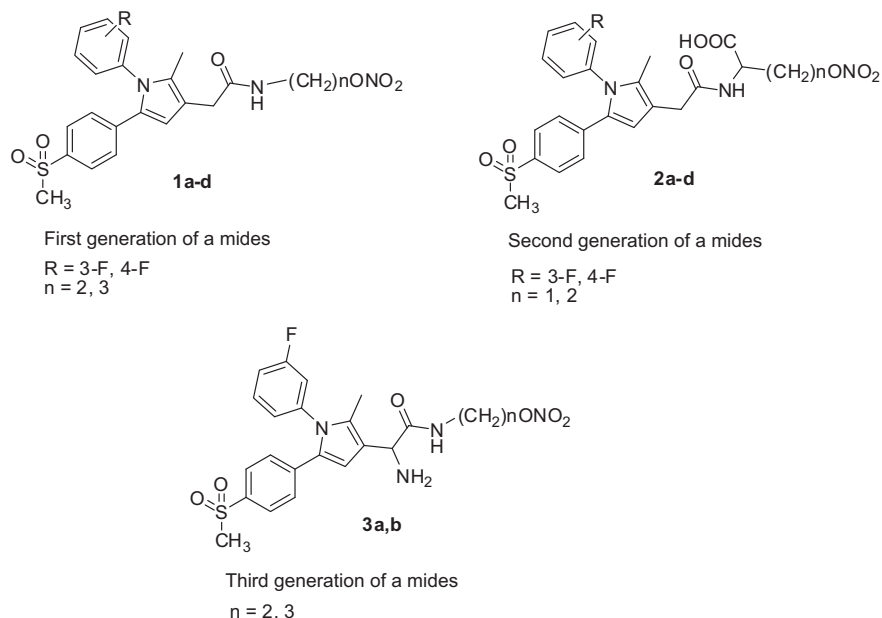


Figure 4. Chemical structures of amides **1a-d**, **2a-d** and **3a,b**.

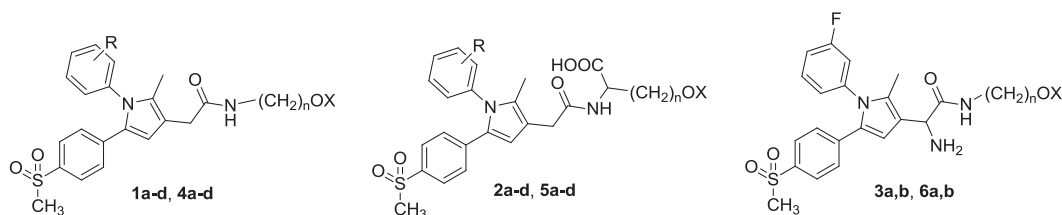
studies of the previous series,^{25–28} thus fluorine substituted (*meta* or *para*) compounds were initially explored. The phenyl ring in C5 of the central core, is substituted with the methanesulfonyl group which was proven to contribute to selectivity towards COX-2.^{33–40} Since these nitro-esters can be metabolized to the corresponding alcohols which in turn could be COX-2 inhibitors, these possible metabolites (hydroxy-derivatives) (**4a-d**, **5a-d**, and **6a-b**) were synthesized and tested for COX-2 inhibition (**Table 1**).

2. Results and discussion

2.1. Chemistry

Compounds **1a-d** and **4a-d** were obtained as shown in **Scheme 1**. Access to the acetic acid derivatives **7a,b** was achieved as previously reported.^{25–27,33–39}

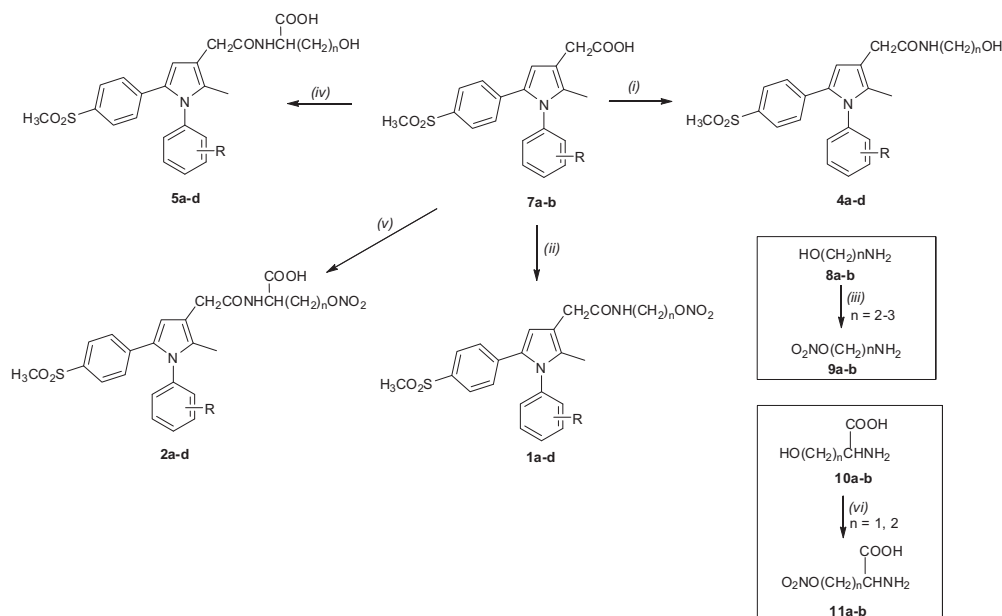
Table 1
In vitro COXs inhibition and selectivity for compounds **1a-d**, **2a-d**, **3a,b**, **4a-d**, **5a-d** and **6a,b**



Compd	R	X	n	% Inhib COX-1 (10 μM)	% Inhib COX-2 (10 μM)	IC ₅₀ (COX-1) ^a (μM)	IC ₅₀ (COX-2) ^a (μM)	COX-1/COX-2 ^b (S.I.)
1a	3-F	NO ₂	2	0	95	>10	0.300	>33
1b	3-F	NO ₂	3	0	100	>10	0.250	>40
1c	4-F	NO ₂	2	9	100	>10	0.249	>42
1d	4-F	NO ₂	3	0	100	>10	0.310	>32
4a	3-F	H	2	0	95	>10	0.290	>34
4b	3-F	H	3	17	100	>10	0.045	>222
4c	4-F	H	2	0	100	>10	0.300	>33
4d	4-F	H	3	21	100	>10	0.068	>147
2a	3-F	NO ₂	1	0	100	>10	0.140	>71
2b	3-F	NO ₂	2	27	43	>10	ND	>6
2c	4-F	NO ₂	1	0	100	>10	0.310	>32
2d	4-F	NO ₂	2	6	89	>10	1.600	>6.2
5a	3-F	H	1	23	100	>10	0.068	>167
5b	3-F	H	2	16	79	>10	ND	>10
5c	4-F	H	1	23	100	>10	0.160	>62.5
5d	4-F	H	2	19	89	>10	0.086	>16
3a	—	NO ₂	2	0	90	>10	0.054	>185
3b	—	NO ₂	3	0	86	>10	0.140	>71
6a	—	H	2	0	25	>10	>10	ND
6b	—	H	3	0	83	>10	0.047	>213

^a Results are expressed as the mean (n = 3 experiments) of the percentage inhibition of PGE₂ production by test compounds with respect to control samples and the IC₅₀ values were calculated by GraphPad Instat program; data fit was obtained using the sigmoidal dose–response equation (variable slope) (GraphPad software).

^b In vitro COX-2 selectivity index [IC₅₀ (COX-1)/IC₅₀(COX-2)].



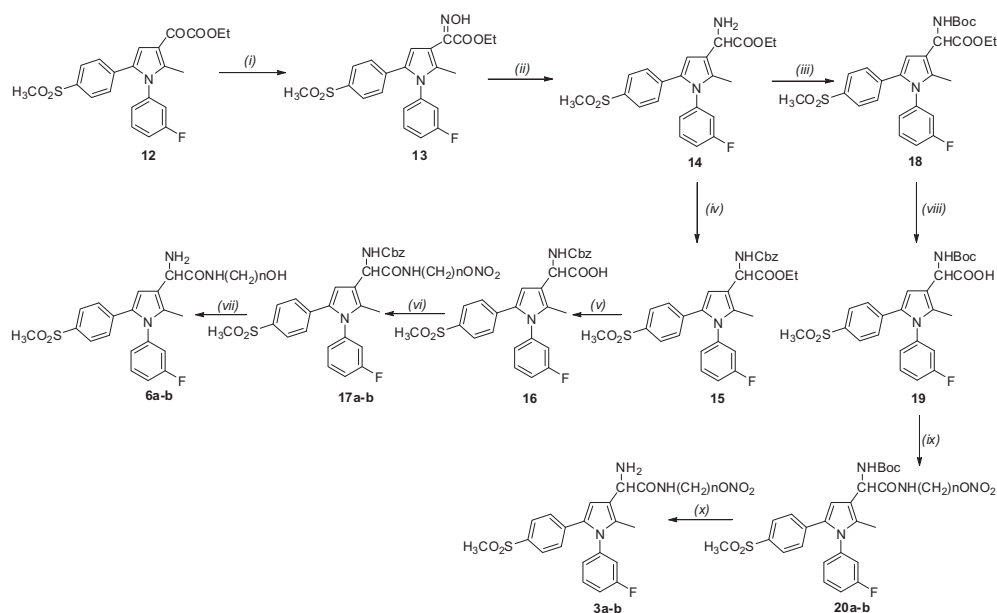
Scheme 1. Synthesis of acetic acid derivatives bearing a non-ionisable side chain. Reagents and conditions: (i) **8a-b**, HOBt, EDCI, TEA, DCM, rt, 12 h, (ii) **9a-b**, EDCI, DMAP, TEA, DCM, rt, 12 h; (iii) HNO₃ fuming, DCM, 1 h, then (CH₃CO)₂O 45 min; (iv) **10a-b**, pyBOP, TEA, THF, rt, 4 h; (v) **11a-b**, pyBOP, TEA, THF, rt, 4 h; (vi) HNO₃ fuming, DCM, 1 h, then (CH₃CO)₂O, 45 min.

The coupling stage which led to the nitro-oxy compounds **1a-d** was afforded by means of EDCI as activating agent and DMAP as covalent nucleophilic catalyst, then the active species was reacted with the nitro-oxyalkyl amines **9a,b**. The nitro-oxyalkyl amine **9a,b** were obtained via nitration of amino alcohols **8a,b** by employing fuming nitric acid in dichloromethane and then precipitated as nitrated salts after addition of acetic anhydride. The hydroxyl derivatives **4a-d** were obtained via EDCI/HOBt coupling, in order to discriminate the nucleophilicity of the oxygen and the nitrogen.

Compounds **2a-d** and **5a-d** were obtained as depicted in Scheme 1. Activation of the acid derivative with PyBOP and

subsequent reaction with the aminoacids (**10a,b** and **11a,b**) gave access to the final molecule (hydroxy or nitro-oxy derivative). Compounds **11a,b** were obtained following the same nitrating procedure adopted for compounds **9a,b**. The used amino acids (**10a,b**) were racemic serine and homoserine.

Glycine derivatives **3a,b** and **6a,b** were prepared using a common route to derivatives **1** and **2** and then following two different strategies for the generation of nitro-oxy and hydroxy compounds (Scheme 2). The installation of the amino functionality was achieved via oxime formation and then its reduction gave the glycine ethyl ester (**14**). A subsequent protection using two different



Scheme 2. Synthesis of acetic acid derivatives bearing an amino acid side chain. Reagents and conditions: (i) NH₂OH, NaOAc, ethanol/1,4-dioxan (1:1 v/v), 90 °C, 40 h; (ii) Zn, HCOOH, 0 °C, 1 h, then, rt, 19 h; (iii) (Boc)₂O, rt, 18 h; (iv) CbzCl, Na₂CO₃ DCM, rt, 4 h; (v) NaOH, MeOH, rt, 17 h; (vi) **9a-b**, EDCI, DMAP, TEA, DCM, rt, 12 h; (vii) Pd/c, NH₄COO, isopropanol, MW, 80 °C, 5 min, 150 W, 170 psi; (viii) NaOH, MeOH, rt, 17 h; (ix) **9a-b**, EDCI, DMAP, TEA, DCM, rt, 12 h, (x) TFA, DCM, 0 °C then MW, 60 °C, 40 min, 150 W, 170 psi.

protecting groups (Cbz for **15**, Boc for **18**), hydrolysis of the ethyl ester and coupling gave access to compounds **17a,b** and **20a,b**. A final deprotection step yielded the expected nitro-oxy and hydroxy compounds **3a,b** and **6a,b**, respectively.

2.2. Pharmacological activity

2.2.1. COX-2 inhibition in J774 cell line in vitro

Linear derivatives (**1** and **4**) displayed an average inhibition towards COX-2 which ranges from 0.045 to 0.310 μM . Surprisingly hydroxyl-derivatives **4b** and **4d** showed significant COX-2 inhibition. This may be due to a non-specific cooperation between the N1 substitution and the hydroxyl chain allowing very strong and effective interactions with the enzyme. As far as the serine/homoserine derivatives are concerned, results show, in general, that the homoserine derivatives are more active than the corresponding serines. In particular, the best inhibition is obtained when a hydroxyl-derivative is allowed to interact with the enzyme (**5a** and **5c**). With respect to the glycine compounds **3a,b** and **6a,b**, nitro-oxy-derivative **3a** and hydroxyl-derivative **6b** showed the best activities.

In general, outstanding COX-2 inhibitory potencies and high selectivity values were obtained within the linear derivatives and the glycine compounds (**3a**, **5a**, **5c**, and **6b**), demonstrating that the negative effect obtained in vivo with the previous class of glycine esters was probably due to a mismatch-interaction rather than to the presence of the amino group itself (Table 1).²⁷

2.2.2. Writhes reduction in the acetic acid-induced abdominal constrictions in mice

All the nitro-oxy-derivatives have been tested for evaluating their analgesic effects in the writhes reduction test. All the tested compounds highlighted a very good and dose-dependent activity in reducing the abdominal writhes induced by acetic acid (Table 2). In particular, nitro-oxy-derivative **1c** and its corresponding hydroxyl-derivative **4c**, serine hydroxyl-derivative **5c** and glycine nitro-oxy-derivative **3a** are endowed with a higher extent of writhes reduction compared to the other compounds. In particular, compounds **1c** and **3a** were the best ones displaying a 77% and 79% of reduction, respectively, when dosed at 20 mg/kg. Noteworthy the introduction of the amino moiety (which is associated to higher solubility as discussed-below) gave rise to a compound (**3a**) with outstanding properties. Derivative **3a** was even active at 3 mg/kg with a reduction of writhes around 23%; the reduction extent increased with the dose and reached 56% and 79% when administered at 10 mg/kg and 20 mg/kg, respectively.

2.2.3. Reduction of hyperalgesia and edema in the carrageenan model

The most active nitro-oxy compounds **1c** and **3a** were selected for a further assessment of their in vivo profile through the carrageenan induced edema and hyperalgesia. Surprisingly, the data on the carrageenan induced inflammation showed that **1c** is associated with a good but not outstanding activity. Indeed, dosing the compound at 40 mg/kg the maximum activity was reached after 60 min with a reduction of hyperalgesia of 60%. A negligible activity (10%) was found when testing the compound for its anti-edemigenic properties. On the other hand, compound **3a** proved to be fast and highly active, with a reduction of hyperalgesia of 80% after 30 min. The activity was maintained for 60 min (70%) and after 90 min a satisfactory activity (50%) was still displayed. After 2 h, some analgesic activity could be still detected (26%). An anti-edemigenic effect was observed with the administration of this compound at 20 mg/kg, with the edema volume reduced of about 76% (Table 3).

2.2.4. Ex vivo nitric oxide releasing properties

The potential NO-mediated vasorelaxing effects of all the nitro-oxy compounds were tested on rat aortic rings. Naproxcinod was used as a reference NO-releasing agent. The compounds showed variable NO-dependent vasorelaxing properties. In particular, naproxcinod showed a high vasorelaxing efficacy (about 70%) and a potency value slightly lower than the μM level. While compound **1a** showed low vasorelaxing effect, compounds **1b** and **1c** proved high vasorelaxing efficacy, with potency spanning in the μM order of magnitude. Derivatives **2a–d** produced a modest vasorelaxing effect. The introduction of the amide functionality within the glycine skeleton, did not compromise the NO-mediated vasorelaxing effects. Indeed, compounds **3a** and **3b** exhibited high levels of vasorelaxing efficacy as well as μM levels of potency. The vasorelaxing activity of all the tested compounds were significantly inhibited by ODQ, inhibitor of guanylate cyclase, indicating the implication of the NO-cGMP pathway (Table 4).

2.2.5. In vitro nitric oxide releasing properties

The kinetics of NO-release for **1c**, **3a** and naproxcinod were also evaluated. In particular, the time-dependent progressive increase of the concentrations of inorganic nitrites and nitrates (i.e. the stable metabolites of NO), after incubation of the tested compounds (1 mM) in rat liver homogenates (with appropriate co-factors), was amperometrically detected. Two hours after incubation of **1c** the nitrite concentration was $27.0 \pm 12.1 \mu\text{M}$ and the nitrate concentration was $38.3 \pm 4.3 \mu\text{M}$ for **1c**, while **3a** showed

Table 2
Writhes reduction for compounds **1a–d**, **4c**, **2a–d**, **5c**, and **3a,b**

Compound	n° Mice	Dose po mg/kg ⁻¹	n° Writhes after 30 min	Writhes reduction (%)
CMC	43	—	32.6 ± 2.1	—
1a	10	10	26.8 ± 2.1 ⁺	18
	10	20	20.6 ± 3.0 ⁺	37
1b	10	40	14.7 ± 2.1 ⁺	55
	9	10	33.2 ± 3.5	0
	9	20	29.8 ± 3.6	8.6
1c	10	40	22.9 ± 2.2 ⁺	30
	9	10	12.2 ± 2.3 ⁺	63
	9	20	7.4 ± 1.3 ⁺	77
1d	10	40	8.1 ± 1.2 ⁺	75
	8	3	27.2 ± 2.7	17
	8	10	20.4 ± 2.2 ⁺	37
4c	8	20	21.6 ± 2.8 ⁺	34
	10	10	20.7 ± 3.5 ⁺	37
	10	20	12.6 ± 2.7 ⁺	61
2a	10	10	28.3 ± 2.5	12
	10	20	17.1 ± 3.1 ⁺	47
	10	40	16.4 ± 2.9 ⁺	50
2b	8	20	29.2 ± 2.4	10
	10	40	25.2 ± 3.3 ⁺	23
2c	8	10	33.1 ± 3.5	0
	9	20	23.2 ± 2.3 ⁺	29
2d	10	40	16.3 ± 3.2 ⁺	50
	10	3	33.7 ± 3.0	0
5c	10	10	22.8 ± 2.7 ⁺	30
	11	20	14.9 ± 2.8 ⁺	54
	14	10	23.7 ± 3.0	27
3a	15	20	13.4 ± 2.8 ⁺	59
	15	40	12.9 ± 2.1 ⁺	60
	13	3	25.1 ± 2.3 ⁺	23
3b	10	10	14.2 ± 2.9 ⁺	56
	13	20	6.7 ± 2.0 ⁺	79
	9	10	30.5 ± 2.6	6
Celecoxib	10	20	21.4 ± 3.7 ⁺	34
	10	40	18.8 ± 3.1 ⁺	42
Celecoxib	13	10	13.5 ± 3.0 ⁺	59

⁺ $P < 0.01$.

⁺⁺ $P < 0.05$ in comparison with CMC treated animals.

42.9 ± 6.6 μM and 56.1 ± 16.0 μM for nitrites and nitrates, respectively. The corresponding concentration/time curves (Fig. 5) indicate that both the compounds are endowed with slow NO-releasing rate, which is thought to be a fundamental feature for the development of well-balanced hybrids. Naproxcinod exhibited a different behavior. Indeed, two hours after incubation of naproxcinod, the highest concentration of NO_x was detected (367.7 ± 11.4 μM); noteworthy, the concentration of NO_x was largely due to the amount of nitrates released (340.1 ± 5.0 μM), while a lower concentration of nitrites was detected (27.6 ± 6.2 μM), which was almost comparable with those detected for **1c** and **3a**. A similar feature of naproxcinod was already observed in human liver fractions.⁴¹ Such a lower ratio nitrites/NO_x would suggest that only a small part of the nitro-oxy group of naproxcinod is converted to NO (and in turn, to nitrites and nitrates) and that a direct hydrolysis to inorganic nitrate is prevalent.

2.2.6. Human whole blood (HWB) assays

We studied COX-2 selectivity of the most active nitro-oxy amides **1c** and **3a** along with the respective hydroxy derivatives **4c** and **6a**, in the HWB assays. The use of these assays allows to gain information of the potency and COX-isozyme selectivity of COX inhibitors in the presence of plasma proteins and in clinically relevant cells (i.e., monocytes, expressing COX-2 in response to LPS,⁴² a cellular target for anti-inflammatory effects; platelets expressing only COX-1,⁴³ a cellular target for gastrointestinal toxicity⁴⁴) in response to endogenously released AA. It has been suggested that HWB COX-2 inhibition in vitro can be used as a marker to predict drug efficacy in humans.⁴⁵

Compound **1c** caused a concentration-dependent inhibition of COX-1 and COX-2 activities with IC₅₀ values of 0.6 (95% CI: 0.38–0.97) and 1.7 (0.87–3.5) μM, respectively, which were not different, in a statistically significant fashion (Fig. 6A and Table 5). Its hydroxy-derivative, **4c**, inhibited HWB COX-2 and COX-1 activities with similar IC₅₀ values of 1.4 (1.1–1.8) and 1.9 (1.1–3.6) μM, respectively (Fig. 6B and Table 5). Thereby, **1c** and its hydroxy derivative **4c** proved to be potent COX-2 inhibitors even if they did not show COX-2 selectivity in the HWB assay.

As shown in Figure 7A, compound **3a** inhibited COX-2 and COX-1 activities in a concentration-dependent fashion with IC₅₀ values of 21 (14.6–30) and 140 (117–168) μM, respectively. Compound **3a** was significantly more potent towards COX-2 than COX-1 (COX-1/COX-2 IC₅₀ ratio was 6.7) (Table 5). At 100 μM, **3a** inhibited COX-2 activity by 80.2 ± 3.8% mean±SEM (*n* = 3) (considered appropriate to obtain a therapeutic effect⁴⁵), while it inhibited only marginally COX-1 activity (36.8 ± 7.8%, *n* = 3) (Fig. 7A). The corresponding hydroxyl-derivative **6a** inhibited COX-2 activity with an IC₅₀ value that was comparable to that of **3a**. In contrast, **6a** was less potent to inhibit COX-1 activity than the nitro-oxy amide **3a**. At 300 μM, COX-1 activity was reduced by 55.7 ± 6.8% and 84 ± 1.8%, by **6a** and **3a**, respectively (mean ± SEM, *n* = 3,

Table 4

Vasorelaxing properties for compounds **1a–d**, **2a–c** and **3a,b** in comparison with MAB103 and MAB124. A significant antagonism by ODQ, inhibitor of guanylate cyclase, is also indicated (+)

Compound	E _{max}	pIC ₅₀	+ODQ 1 μM
Naproxcinod	68.0 ± 3.0	6.33 ± 0.06	+
1a	49.0 ± 2.0	n.c.	+
1b	69.0 ± 8.0	5.78 ± 0.07	+
1c	68.0 ± 0.5	5.31 ± 0.05	+
2a	18.0 ± 9.0	n.c.	+
2b	25.0 ± 2.0	n.c.	+
2c	25.0 ± 1.9	n.c.	+
2d	42.0 ± 9.0	n.c.	+
3a	74.1 ± 1.0	5.38 ± 0.20	+
3b	86.0 ± 2.0	5.38 ± 0.03	+

P < 0.05) (Fig. 7A and B). Altogether these results show that compound **3a** is a more potent inhibitor of COX-2 than COX-1 up to concentrations which affect COX-2 activity by approximately 80%, considered appropriate to translate into an analgesic/anti-inflammatory effect.⁴⁵ The hydroxy derivative **6a** maintains a similar inhibitory potency towards COX-2 but it is less potent to inhibit COX-1 thus resulting in improved COX-2 selectivity (Table 5) versus the nitroxy-parent compound (**3a**). As shown in Table 5, in HWB assays celecoxib showed a COX-2 selectivity comparable to **6a**. The finding that **6a** had a potency to affect COX-2 similar to that of compound **3a** is an advisable feature because it may lead to a long-lasting therapeutic effect. The higher COX-2 selectivity of **6a** with respect to **3a** might be useful to maintain a good GI safety also when the nitrooxy compound loses its NO-releasing capacity.

2.2.7. Solubility assessment

Solubility of compounds was assessed in simulated gastric fluid (SGF-without pepsin) and phosphate buffered solution (PBS), using a multi-pulse vortexer (Glass-Col) to shake the samples. Concentration of the compounds was assessed by HPLC.

The replacement of the ester functionality with the amide moiety gave rise to more soluble molecules. Surprisingly, the introduction of a carboxylate group (**2c**) led to compounds with a lower solubility even at pH 7.4 (Table 6). This could be due to a participation of the carboxylic acid moiety to intramolecular H-bonding networks that stabilize the folding of the molecule leading to reduced solubility. On the other hand 'linear' amides showed good solubility in both SGF and PBS. The best solubility profile was displayed by glycine amide **3a** the solubility of which was higher than 200 μM in both conditions.

2.2.8. Stability insimulated gastric fluid (SGF-without pepsin), phosphate buffered solution (PBS) and rat plasma

Stability studies on MAB103 and MAB124 revealed some liability of these compounds to hydrolysis. It should be pointed out that

Table 3

Hyperalgesia and oedema reduction in the carrageenan induced inflammation for compounds **1c** and **3a** in comparison with celecoxib

Pretreat. (i.pl.)	Compd	Dose	Before treatment	Paw-pressure After treatment				Edema volume (mL)
				30 min	60 min	90 min	120 min	
Saline	CMC	—	61.2 ± 2.5	61.6 ± 3.4	58.9 ± 3.8	61.4 ± 4.1	61.4 ± 4.1	1.44 ± 0.05
Carrageenan	CMC	—	36.1 ± 3.3	32.8 ± 2.9	30.6 ± 3.2	31.8 ± 2.2	31.8 ± 2.2	2.71 ± 0.08
Carrageenan	1c	20	33.8 ± 2.9	40.7 ± 3.5** (28%)	42.8 ± 3.4** (30%)	37.8 ± 2.6 (20%)	32.7 ± 2.6 (0%)	2.63 ± 0.05 (4%)
Carrageenan	1c	40	32.9 ± 3.3	44.2 ± 4.0* (32%)	48.3 ± 4.6* (60%)	41.5 ± 3.7** (29%)	31.5 ± 2.6 (0%)	2.50 ± 0.09 (10%)
Carrageenan	3a	20	33.8 ± 2.9	55.2 ± 3.3* (80%)	52.1 ± 4.2* (70%)	47.3 ± 3.7* (50%)	39.5 ± 3.0 (26%)	1.79 ± 0.09* (76%)
Carrageenan	Celecoxib	10	31.7 ± 2.7	45.7 ± 4.2* (45%)	52.9 ± 3.1* (75%)	48.3 ± 3.4* (56%)	42.5 ± 2.9 (39%)	2.53 ± 0.05* (17%)

* *P* < 0.01.

** *P* < 0.05 in comparison with CMC treated animals.

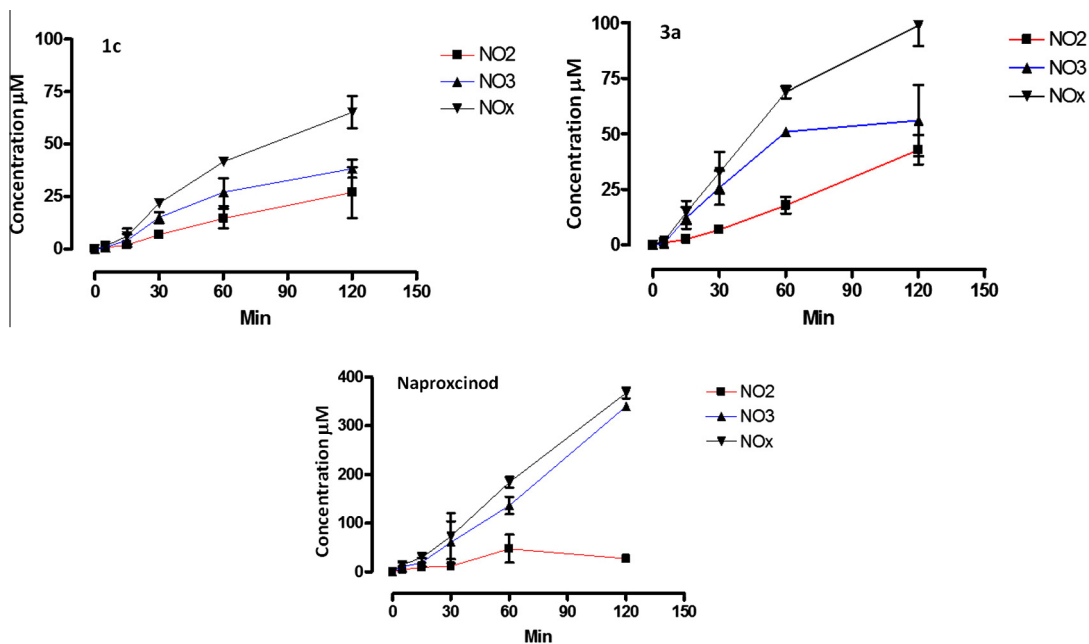


Figure 5. Time-dependent increase of the concentrations of nitrites (NO_2), nitrates (NO_3) and NO_x (nitrites + nitrates) following the incubation of 1 mM **1c**, **3a** or naproxen in rat hepatic homogenate, containing the opportune cofactors. The vertical bars indicate the standard error.

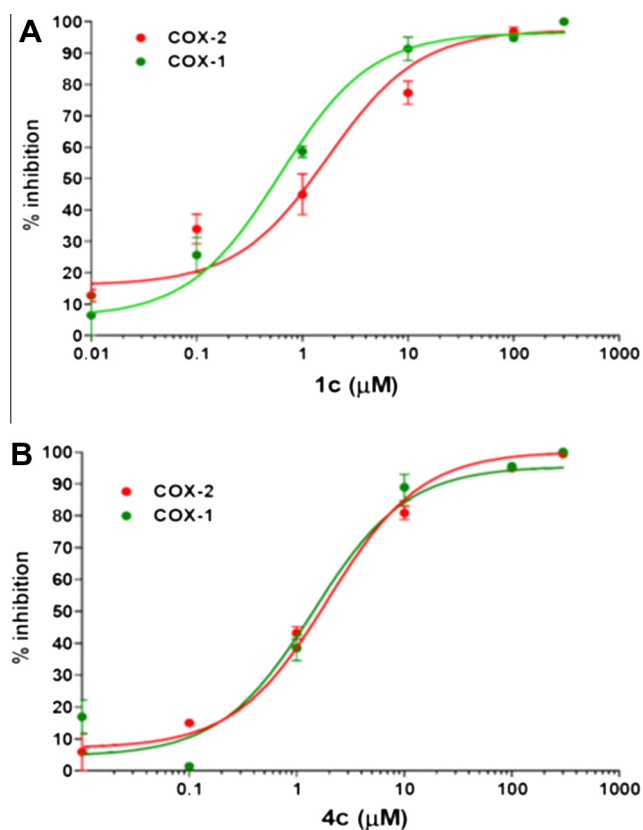


Figure 6. Effects of Compounds **1c** and **4c** on in vitro platelet COX-1 and monocyte COX-2 activity in HWB assay. Results are expressed as average percent of inhibition ($n = 3-5$, mean \pm SEM).

the presence of the amino moiety, decreased this liability when comparing the two esters,²⁶ especially in SGF (100% of parent compound detected at 120 min). Replacing the ester with the amide led to a striking stabilization of the compounds both chemically and

Table 5
IC₅₀ values and COX-2 selectivity of compounds **1c**, **4c**, **3a** and **6a**, in HWB assays

Compound	COX-1 IC ₅₀ μM^a (95% CI)	COX-2 IC ₅₀ μM^a (95% CI)	S.I. [†]
1c	0.6 (0.38–0.97)	1.7 (0.87–3.5)	0.3
4c	1.9 (1.1–3.6)	1.4 (1.1–1.8)	1.3
3a	140 (117–168)	21 (15.6–30)	6.7
6a	~300	13.7 (7.9–24)	21.9
Celecoxib	12.47 (8.6–17.9)	0.54 (0.29–0.52)	23

[†] Selectivity index (SI): selectivity of compounds towards COX-2 were determined as IC₅₀ ratios for HWB COX-1 and COX-2.

^a For IC₅₀ values, 95% confidence intervals (CI) are shown in round brackets.

enzymatically. Thus the amide bond enhanced the overall stability to hydrolysis especially for compounds belonging to the first and third subclass of amides (**1c** and **3a**) (Table 7). Compound **1c** was completely resistant to hydrolysis (in the tested conditions), whereas compound **3a** was relatively more liable in rat plasma (even though after 120 min of incubation 77% of the compound was still detected).

The serine derivative **2a** displayed the lower stability in the amide series. Again, this could be explained by means of intra-molecular interactions related to the presence of the carboxylic acid moiety; indeed, intra-molecularly, the acid group could ease any structural transformation (enzyme-mediated or not).

2.2.9. Pharmacokinetic studies

The pharmacokinetics of the most active compounds **1c** and **3a** were assessed in rats after po and iv administration of the compounds. Profiles of the plasma concentration versus time after single po and iv administration of **1c** and **3a** (10 mg/kg) are reported in Figures 8 and 9. After iv administration, **1c** was detected in plasma up to 6 h. The average clearance value was about 0.6 L/h, representing 72% of rat liver blood flow (0.83 L/h). This suggests moderate to high liver extraction, while the average final volume of distribution (V_{ss} on AUC_t) value was 367 mL which is twice the rat total body water, suggesting moderate compound distribution in tissues.

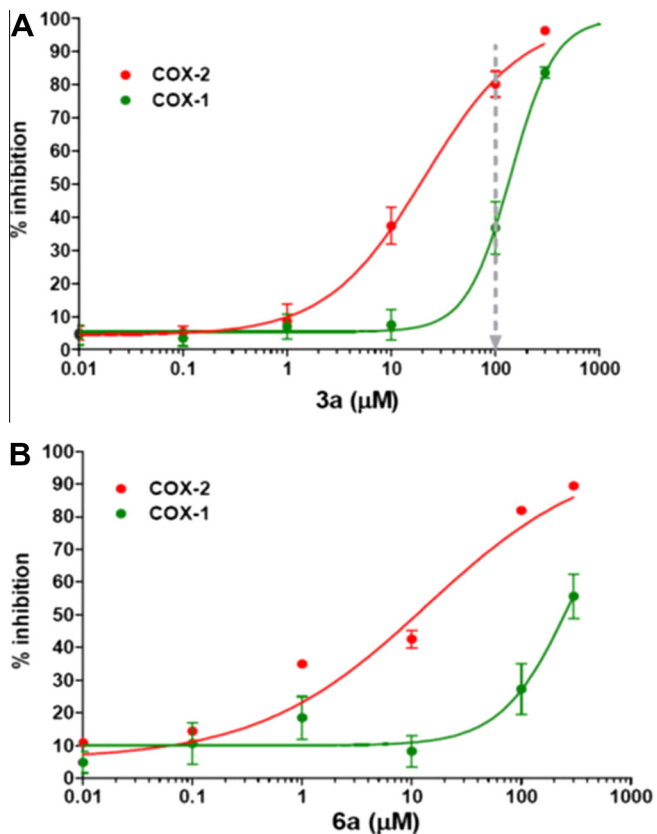


Figure 7. Effects of Compounds **3a** and **6a** on in vitro platelet COX-1 and monocyte COX-2 activity in HWB assay. Results are expressed as average percent of inhibition ($n = 3-5$, mean \pm SEM).

Table 6
Solubility assessment in SGF and PBS for compounds **1a**, **1c**, **2c**, **3a**, MAB103 and MAB124

Compound	SGF (pH 1.5) (μM)	PBS (pH 7.4) (μM)
MAB103	<1	1.6
MAB124	>200	80.0
1a	150.0	>200
1c	147.0	>200
2c	112.0	90.5
3a	>200	>200

Table 7
Stability assessment in SGF and PBS for compounds MAB103, MAB124, **1c**, **2a**, and **3a**

Compound	Time (min)	Parent molecule remained (%)		
		PBS (pH 7.4)	SGF (pH 1.5)	Rat plasma
MAB103	30	74.1	80.2	0.00
	60	53.6	65.7	0.00
	120	0.15	42.8	0.00
MAB124	30	75.0	100	0.00
	60	75.0	100	0.00
	120	75.0	100	0.00
1c	30	100	100	100
	60	100	100	100
	120	100	100	100
2a	30	75	100	71.4
	60	50	93.8	57.1
	120	37.5	87.5	28.6
3a	30	100	100	94
	60	100	100	93
	120	100	100	77

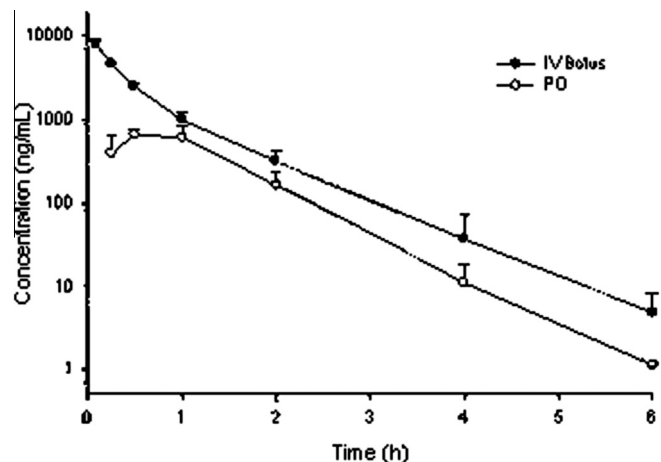


Figure 8. Profile of plasma concentration versus time after iv and po administration of **1c** (10 mg/kg).

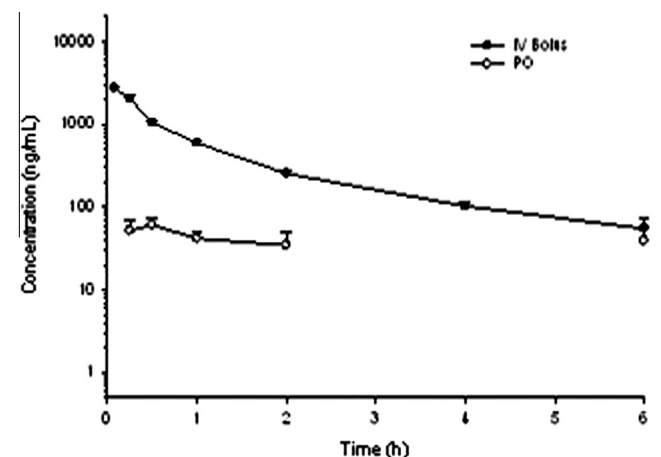


Figure 9. Profile of plasma concentration versus time after iv and po administration of **3a** (10 mg/kg).

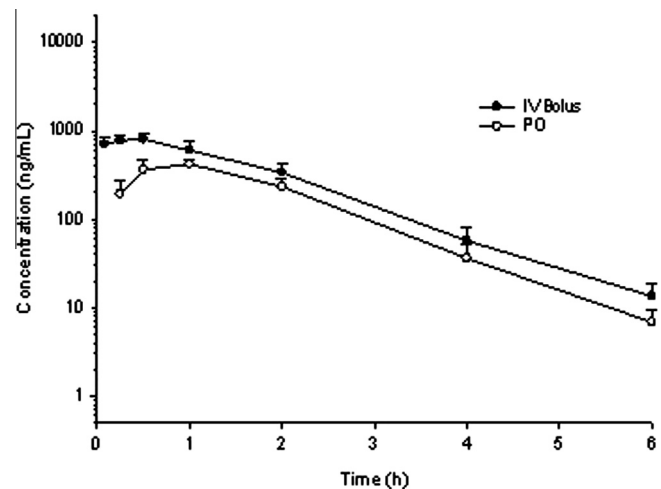


Figure 10. Mean plasma concentration versus time profiles of **4c** after iv and po administration of **1c**.

After iv administration, **3a** was detected in plasma up to 6 h. Average clearance value was about 1.12 L/h representing 136% of rat liver blood flow, this suggests high liver extraction, while final volume of distribution value (V_{ss} on AUC_t) was 1.85 L that is 11

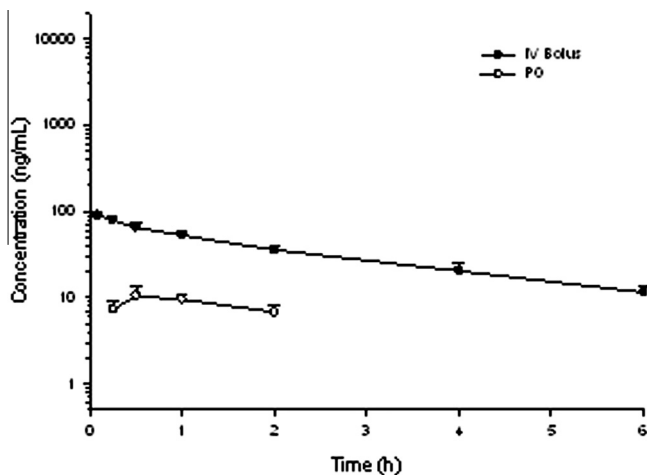


Figure 11. Mean plasma concentration versus time profiles of **6a** after iv and po administration of **3a**.

times rat total body water suggesting extensive compound distribution in tissues.

In conclusion, both **1c** and **3a** appear to be characterized by moderate to high rate of clearance and high volume of distribution. In accordance with lower liver extraction ratio, **1c** showed higher absolute bioavailability (24%) than **3a** (9%).

Considering the possibility that nitro-esters can be metabolized to the corresponding alcohols, the presence of the hydroxyl metabolites of **1c** and **3a** (**4c** and **6a**, respectively) was also measured. Profiles of the plasma concentration versus time for **4c** and **6a** after iv and po administration of **1c** and **3a** (10 mg/kg) are reported in Figures 10 and 11. Compound **4c** was detected in rat plasma up to 6 h after both iv and po administrations, while **6a** was detected in rat plasma at least up to 6 h after intravenous administrations and only up to 2 h after oral administration. For **1c**, after intravenous administration at least 39% of the parent compound is converted into metabolite **4c** (calculated on a molar base) while after oral administration 99% of the parent compound is converted into the metabolite. This suggests that after oral administration **1c** is readily metabolized to **4c**. For **3a**, after intravenous administration only 10% of the parent compound was converted to metabolite **6a** (calculated on a molar bases), while the conversion after oral administration is 12.8%, indicating that the percentage of the first pass metabolism is negligible.

3. Conclusions

The replacement of the ester moiety with an amide group gave access to a stable and soluble class of hybrid compounds. Nitroxy derivatives highlighted a very good COX-2 inhibiting profile both in vivo and in HWB. The NO-dependent vasorelaxing properties and the slow kinetic of NO release make these compounds a very promising class of pharmacodynamic hybrids. Among all derivatives, **3a** is endowed with an outstanding in vivo profile (comparable and in some extent greater than celecoxib). In HWB assays, **3a** was more potent to inhibit COX-2 than COX-1. The finding that its hydroxyl-derivative **6a** had higher COX-2 selectivity than **3a** might be a useful feature to maintain a good GI safety profile even when the nitrooxy compound loses its NO-releasing capacity.

In vivo assessment of the NO releasing properties and effects of selected compounds in suitable animal models, aimed at providing

additional evidence about the effectiveness of the approach in counteracting long-term CV toxicity triggered by COX-2 inhibitors, will be addressed in further appropriate studies.

4. Experimental section

4.1. Chemistry

All chemicals used were of reagent grade. Yields refer to purified products and are not optimized. A CEM Discovery microwave system apparatus was used for microwaved reactions. Melting points were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. Sigma–Aldrich silica gel 60 (230–400 mesh) was used for column chromatography. Merck TLC plates (silica gel 60 F 254) were used for thin-layer chromatography (TLC). Sigma–Aldrich aluminum oxide (activity II–III, according to Brockmann) was used for chromatographic purifications. Sigma–Aldrich Stratocrom aluminum oxide plates with a fluorescent indicator were used for TLC to check the purity of the compounds. ^{13}C NMR and ^1H NMR spectra were recorded with a Bruker AC 400 spectrometer in the indicated solvent (TMS as the internal standard). The values of the chemical shifts are expressed in ppm and the coupling constants (J) in hertz. Mass spectra were recorded on a API-TOF Mariner by Perspective Biosystem (Stratford, Texas, USA).

4.1.1. General procedure for the preparation of 1,5-diarylpyrrole-3-acetic acids (**7a,b**)

1 N Sodium hydroxide solution (4.83 mL) was added to a solution of ethyl 1,5-diarylpyrrole-3-acetic ester (0.67 mmol) in methanol (4.83 mL). The resulting mixture was refluxed for 1.5 h, cooled, and concentrated in vacuo. The residue was solubilized in water (5 mL) and then concentrated HCl was added dropwise until a precipitate was formed. The precipitate was filtered off to give the expected acid as a white solid. Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.²⁶

4.1.2. General procedure for the preparation of 1,5-diarylpyrrole-3-acetic nitroxyalkyl amides (**1a–d**)

Nitroxyalkylamine (**9a,b** nitrate salt) (0.3 mmol), DMAP (0.1 mmol), and EDCI (0.2 mmol) were added in sequence to a solution of the acid partner (**7a,b**) (0.1 mmol) in dichloromethane (5 mL), under nitrogen atmosphere. An excess of triethylamine (0.35 mmol) was added dropwise and the reaction was stirred at rt for 12 h. Then the mixture was quenched with water (10 mL) and extracted with chloroform (50 mL \times 3). The organic layer was washed with 1 N HCl (50 mL), NaHCO_3 saturated solution (50 mL), brine (50 mL) and dried over Na_2SO_4 . After filtration and concentration of the organic phase a crude material was obtained. The material was then purified by chromatography on silica gel/alumina (1:1) using petroleum ether/chloroform/ethyl acetate, 4:4:1 (v/v/v), as the eluent to give the desired product in good yield.

4.1.3. *N*-[2-(Nitroxy)ethyl]-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3yl]acetamide (**1a**)

Yellow powder, mp 133 °C (yield 80%). FT-IR cm^{-1} : 3300, 1640, 1595, 1520, 1278, 865. ^1H NMR (400 MHz, CDCl_3) δ (ppm): 7.71 (d, 2H, $J = 8.6$ Hz), 7.44–7.40 (m, 1H), 7.17 (d, 2H, $J = 8.6$ Hz), 7.14–7.12 (m, 1H), 6.98–6.96 (m, 1H), 6.93–6.90 (m, 1H), 6.43 (s, 1H), 6.05 (s br, 1H), 4.59 (t, 2H, $J = 7.6$ Hz), 3.63 (t, 2H, $J = 7.6$ Hz), 3.49 (s, 2H), 3.02 (s, 3H), 2.04 (s, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 169.45, 138.23, 136.12, 131.83, 131.30, 130.71, 130.66, 128.71,

127.10, 125.59, 120.71, 115.90, 113.44, 111.18, 110.42, 69.77, 44.61, 38.41, 32.21, 11.89. MS-ESI: m/z 476.12 $[M+H]^+$. HPLC: >97% pure (t_R = 7.3 min).

4.1.4. General procedure for the preparation of 1,5-diarylpyrrole-3-acetic hydroxylalkyl amides (4a–d)

Hydroxyalkylamine (**8a–b**) (1.0 mmol), HOBt (0.9 mmol), and EDCI (1.2 mmol) were added in sequence to a solution of 1,5-diarylpyrrole-3-acetic acid (**7a,b**) (0.7 mmol) in dichloromethane (20 mL), under nitrogen atmosphere. An excess of triethylamine (2.0 mmol) was added dropwise and the reaction was stirred at rt for 12 h. Then the mixture was quenched with water (10 mL) and extracted with chloroform (50 mL \times 3). The organic layer was washed with 1 N HCl (50 mL), NaHCO₃ saturated solution (50 mL), brine (50 mL) and dried over Na₂SO₄. After filtration and concentration of the organic phase a crude material was obtained. The material was then purified by chromatography on silica gel using petroleum ether/chloroform/ethyl acetate, 3:2:1 (v/v/v), as the eluent to give the desired product in good yield.

4.1.5. *N*-[2-(Hydroxy)ethyl]-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]acetamide (4a)

Yellowish powder, mp 97 °C (yield 77%). FT-IR cm^{-1} : 3623, 3315, 1645, 1520, 1280, 1100. ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.69 (d, 2H, J = 8.6 Hz), 7.43–7.37 (m, 1H), 7.19 (d, 2H, J = 8.6 Hz), 7.14–7.10 (m, 1H), 6.98–6.96 (m, 1H), 6.92–6.89 (m, 1H), 6.47 (s, 1H), 6.40 (s br, 1H), 3.70 (t, 2H, J = 7.4 Hz), 3.48 (s, 2H), 3.40 (m, 2H), 3.00 (s, 3H), 2.70 (s br, 1H), 2.04 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 169.47, 138.63, 136.32, 131.85, 131.37, 130.44, 130.56, 129.46, 127.12, 125.61, 120.31, 115.88, 113.54, 113.08, 111.29, 60.17, 44.61, 36.41, 32.21, 11.89. MS-ESI: m/z 431.14 $[M+H]^+$. HPLC: >98% pure (t_R = 4.7 min).

4.1.6. General procedure for the preparation of 1,5-diarylpyrrole-3-acetic amides (2a–d, 5a–d)

PyBOP (0.93 mmol) and triethylamine (3 mmol) were added in sequence to a solution of **7a,b** (0.78 mmol) in THF (20 mL) at rt and the mixture stirred for 1 h. Then the amino acid (**10a,b**, **11a–b**) (1.5 mmol) was added over 5 min. After 4 h the mixture was concentrated in vacuo and the oily residue was treated with 1 N KOH (10 mL). The solution was filtered through a paper filter. The liquid was collected, cooled to 0 °C and 5 N HCl was added dropwise till a white precipitate was formed. The solid obtained was filtered and dried. After re-crystallization from ethanol/petroleum ether the product was obtained in quite good yield.

4.1.7. (*R,S*)-2-[2-[1-(3-Fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]acetamide]-3-nitroxypropanoic acid (2a)

Yellow powder, mp 110 °C (yield 80%). FT-IR cm^{-1} : 3310, 1734, 1640, 1595, 1520, 1278, 865. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 11.13 (s br, 1H), 8.53 (s, br, 1H), 7.70 (d, 2H, J = 8.5 Hz), 7.49–7.47 (m, 1H), 7.22–7.20 (m, 2H), 7.18–7.16 (m, 2H), 7.03–7.01 (m, 1H), 6.50 (s, 1H), 4.79–4.76 (m, 1H), 4.62–4.60 (m, 2H), 3.51 (s, 2H), 3.10 (s, 3H), 2.09 (s, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 172.02, 169.67, 138.50, 136.13, 131.80, 131.30, 130.71, 130.66, 129.48, 127.00, 125.59, 120.56, 115.98, 113.43, 113.22, 111.25, 70.18, 55.71, 38.41, 32.21, 11.80. MS-ESI: m/z 520.11 $[M+H]^+$. HPLC: >98% pure (t_R = 4.3 min).

4.1.8. Preparation of ethyl 2-(hydroxyimino)-2-[2-methyl-5-[4-(methylsulfonyl)phenyl]-1-(4-fluorophenyl)-1*H*-pyrrol-3-yl]acetate (13)

Hydroxylamine hydrochloride (87.4 mmol) and sodium acetate (145.6 mmol) were added to a suspension of **12** (58.2 mmol) in ethanol/1,4-dioxan (100 mL, 1:1 v/v). The resulting solution was

heated at 90 °C under stirring for 40 h. The solvents were removed in vacuum and the mixture was extracted with ethyl acetate (500 mL) and washed with water (100 mL). The organic phase was dried over sodium sulfate, filtered and concentrated to afford the oxime **13** as a yellowish solid. Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.²⁷

4.1.9. General procedure for the preparation of ethyl 2-amino-2-[2-methyl-5-[4-(methylsulfonyl)phenyl]-1-(4-fluorophenyl)-1*H*-pyrrol-3-yl]acetate (14)

Formic acid (100 mL) was added to a solution of the crude oxime **13** in ethanol (100 mL) and the solution was cooled to 0 °C. Then zinc dust (252.2 mmol) was added in portions over a period of 1 h. The mixture was allowed to warm up to rt very slowly and then stirred for 19 h. Afterwards, zinc salts were filtered off and the solvents evaporated under reduced pressure. The residue obtained was dissolved in water (200 mL) and the pH adjusted to 10 with 10% NaOH solution and extracted with EtOAc (500 mL). The combined organic fractions were dried over sodium sulfate, filtered and concentrated in vacuo to afford the crude product. The material was then purified by column chromatography (silica gel) eluting with MeOH/CHCl₃ (1:20) (v/v) to afford the product **14** (50% yield). Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.²⁷

4.1.10. General procedure for the synthesis of *N*-benzyl-(ethoxycarbonyl)-[1-(4-fluorophenyl)-2-methyl-5-[4(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]methylcarbamate (15)

To a biphasic solution of amino ester **14** (2.326 mmol) in DCM (10 mL), sodium carbonate (4.6 mmol) and benzyl chloroformate (2.352 mmol) were added at 0 °C, the reaction mixture was allowed to warm up to rt and stirred for further 4 h. The mixture was then diluted with water (20 mL) and the two phases separated. The organic fraction was washed with water (50 mL \times 2), dried over sodium sulfate and evaporated to give the crude product. The residue was purified by column chromatography (silica gel) eluting with EtOAc/hexane (1:20) (v/v) to afford the desired product as white solid (65% yield). Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.²⁷

4.1.11. General procedure for the synthesis of *N*-protected (CBz) 2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]-2-amino-acetic acid (16)

A solution of NaOH (5.917 mmol) in water (3 mL) was added to a solution of amino ester **15** (2.236 mmol) in MeOH (15 mL), and the reaction mixture was stirred at rt for 17 h. The mixture was then concentrated in vacuo, diluted with water (2 mL) and the acid precipitated through the cautious addition of concentrated HCl. The solid was collected to give the product as off white solid. Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.²⁷

4.1.12. General procedure for the Preparation of 1,5-diarylpyrrole-3-acetic nitro-oxylalkyl amides (17a–b)

Nitroxyalkylamine (**22a–b**, nitrate salt) (0.3 mmol), DMAP (0.1 mmol), and EDCI (0.2 mmol) were added in sequence to a solution of the acid partner (**16**) (0.1 mmol) in dichloromethane (5 mL), under nitrogen atmosphere. An excess of triethylamine (0.35 mmol) was added dropwise and the reaction was stirred at rt for 12 h. Then the mixture was quenched with water (10 mL) and extracted with chloroform (50 mL \times 3). The organic layer was washed with 1 N HCl (50 mL), NaHCO₃ saturated solution (50 mL), brine (50 mL) and dried over Na₂SO₄. After filtration and concentra-

tion of the organic phase a crude material was obtained. The material was then purified by chromatography on silica gel/alumina (1:1) using petroleum ether/chloroform/ethyl acetate, 4:4:1 (v/v/v), as the eluent to give the desired product in good yield.

4.1.13. *N*-[2-(Nitroxy)ethyl]-(*R,S*)-[(benzyloxy)carbonyl]amino-2-(1-(3-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1*H*-pyrrol-3-yl)acetamide (17a)

White solid, 138 °C (75% yield); ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.71 (d, 2H, *J* = 8.4 Hz), 7.30–7.24 (m, 9H), 7.16 (d, 2H, *J* = 8.4 Hz), 6.88 (s br, 1H), 6.60 (s, 1H), 5.54 (d, 1H, *J* = 7.7 Hz), 5.32 (d, 1H, *J* = 7.7 Hz), 5.10 (s, 2H), 4.70 (t, 2H, *J* = 6.8 Hz), 3.49–3.46 (m, 2H), 3.03 (s, 3H), 2.14 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 169.23, 168.66, 159.78, 143.23, 139.01, 135.82, 134.65, 132.43, 131.80, 128.92, 127.98, 127.49, 127.29, 127.17, 126.89, 123.45, 123.22, 119.05, 117.41, 116.92, 115.88, 70.82, 60.09, 58.32, 44.03, 32.80, 11.04. MS-ESI: *m/z* 625.17 [M+H⁺].

4.1.14. General procedure for the preparation of 1,5-diarylpyrrol-glycine hydroxyalkyl amides (6a,b)

Ammonium formate (1.3 mmol) and Pd/C (0.07 g) were added to a solution of **17a,b** (0.32 mmol) in isopropanol (2 mL). The solution was microwave irradiated at 80 °C for 5 min (power 150 W, pressure 170 psi). The reaction mixture was passed through Celite® and then poured into water (20 mL). The pH was adjusted to 10–12 (using NaOH 10% solution) and the mixture extracted with chloroform (100 mL × 3). The organic layer was then dried over Na₂SO₄. Filtration and concentration of the organic phase gave a material, which was identified to be the product without the need of any further purification.

4.1.15. *N*-[3-(Hydroxy)propyl]-2-amino-2-[1-(3-fluorophenyl)-2-methyl-5-[4-methylsulfonyl]phenyl]-1*H*-pyrrol-3-yl]acetamide (6a)

Yellowish powder, mp 115 °C (>95% yield). FT-IR cm⁻¹: 3400, 1750, 1620, 1518, 1350, 990. ¹H NMR (400 MHz, DMSO-*d*₆) δ (ppm): 8.50 (s, br, 1H), 7.66 (d, 2H, *J* = 8.4 Hz), 7.26 (d, 2H, *J* = 8.4 Hz), 7.12–7.08 (m, 3H, *J* = 8.4 Hz), 7.04–7.02 (m, 1H), 6.51 (s, 1H), 4.65 (s br, 1H), 4.26 (s, 1H), 4.15 (t, 2H, *J* = 7.4 Hz), 3.48–3.45 (m, 2H), 3.09 (s, 3H), 2.17 (s, 3H), 1.92–1.89 (m, 2H), 1.80 (s, br, 2H). ¹³C NMR (100 MHz, DMSO-*d*₆) δ (ppm): 169.40, 159.12, 145.31, 144.71, 141.49, 138.06, 131.44, 129.23, 128.41, 127.00, 126.29, 122.42, 121.47, 116.10, 111.29, 62.10, 58.11, 44.07, 33.23, 30.12, 11.06. MS-ESI: *m/z* 460.16 [M+H⁺]. HPLC: >96% pure (*t*_R = 4.31 min).

4.1.16. General procedure for the synthesis of *tert*-butyl (ethoxycarbonyl)-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]methyl carbamate (18)

Boc anhydride (4.65 mmol) was added to a solution of amino ester **14** (2.23 mmol) in MeOH (15 mL) and the reaction mixture stirred at rt for 18 h. The mixture was then diluted with water (20 mL), adjusted the pH to 2–3 with 1 N HCl and extracted with EtOAc (100 mL × 3). The combined organic fractions were washed with water (100 mL × 2). Dried over sodium sulfate and evaporated to give the crude product. The residue was purified by column chromatography (silica gel) eluting with EtOAc/hexane (1:20) (v/v) to afford the desired product as white solid (63% yield). Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.²⁷

4.1.17. General procedure for the synthesis of *n-tert*-butyl (ethoxycarbonyl)-[2-[1-(4-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]-2-amino-acetic acid (19)

A solution of NaOH (5.917 mmol) in water (3 mL) was added to a solution of amino ester **18** (2.23 mmol) in MeOH (15 mL), and the

reaction mixture stirred at rt for 17 h. The mixture was then concentrated in vacuo, diluted with water (2 mL) and the acid precipitated through the cautious addition of concentrated HCl. The solid was collected to give the product as off white solid. Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.²⁷

4.1.18. General procedure for the preparation of 1,5-diarylpyrrole-3-acetic nitro-oxyalkyl amides (20a,b)

Nitroxyalkylamine (**9a,b**, nitrate salt) (0.3 mmol), DMAP (0.1 mmol), and EDCI (0.2 mmol) were added in sequence to a solution of acid partner **19** (0.1 mmol) in dichloromethane (5 mL), under nitrogen atmosphere. An excess of triethylamine (0.35 mmol) was added dropwise and the reaction was stirred at rt for 12 h. Then the mixture was quenched with water (10 mL) and extracted with chloroform (50 mL × 3). The organic layer was washed with 1 N HCl (50 mL), NaHCO₃ saturated solution (50 mL), brine (50 mL) and dried over Na₂SO₄. After filtration and concentration of the organic phase a crude material was obtained. The material was then purified by chromatography on silica gel/alumina (1:1) using petroleum ether/chloroform/ethyl acetate, 4:4:1 (v/v/v), as the eluent to give the desired product in good yield.

4.1.19. *N*-[2-(Nitroxy)ethyl]-(*R,S*)-2-[(*tert*-butyl)oxycarbonyl]amino-[2-(1-(3-fluorophenyl)-2-methyl-5-(4-(methylsulfonyl)phenyl)-1*H*-pyrrol-3-yl)]acetamide (20a)

White solid, mp 102 °C (70% yield). FT-IR cm⁻¹: 3330, 2895, 1688, 1645, 1579, 1228, 1155, 1101, 867. ¹H NMR (400 MHz, CDCl₃): δ (ppm) 7.68 (d, 2H, *J* = 8.4 Hz), 7.43–7.40 (m, 1H), 7.30–7.28 (m, 1H), 7.22–7.19 (m, 2H), 7.13–7.09 (m, 2H), 6.63 (s, 1H), 6.52 (s br, 1H), 5.59–5.56 (m, 1H), 5.40 (s br, 1H), 4.69 (t, 2H, *J* = 7.3 Hz), 4.19 (t, 2H, *J* = 7.3 Hz), 3.05 (s, 3H), 2.15 (s, 3H), 1.46 (s, 9H). ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 169.24, 168.70, 159.20, 138.70, 135.63, 134.18, 132.43, 129.18, 128.42, 127.11, 126.66, 123.00, 123.13, 120.18, 117.33, 81.91, 60.05, 58.34, 44.03, 32.21, 30.50, 27.82, 11.24. MS-ESI: *m/z* 591.18 [M+H⁺].

4.1.20. General procedure for the preparation of 1,5-diarylpyrrol-glycine nitro-oxyalkyl amides (3a,b)

An excess of trifluoroacetic acid (4 mL) was added dropwise at 0 °C to a solution of the Boc-protected **20a,b** (0.40 mmol) in dioxane (2 mL). The solution was microwave irradiated at 60 °C for 40 min (power 150 W, pressure 170 psi). The reaction mixture was poured into ice/water (20 mL) and the pH adjusted to 12–13 (using ammonia solution) and the mixture extracted with chloroform. The organic layer was then dried over sodium sulfate. The filtration and concentration of the organic phase gave a crude material, which was purified through column chromatography (alumina) using MeOH/DCM 1:20 (v/v) to obtain the product as a yellowish powder.

4.1.21. *N*-[2-(Nitroxy)ethyl]-2-amino-2-[1-(3-fluorophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]acetamide (3a)

Yellowish powder, mp 144 °C (81% yield). FT-IR (neat, cm⁻¹) v: 3330, 2890, 1654, 1590, 1300, 1147, 1102, 899. ¹H NMR (400 MHz, CDCl₃) δ (ppm): 7.67 (d, 2H, *J* = 8.6 Hz), 7.45–7.40 (m, 1H), 7.23 (d, 2H, *J* = 8.6 Hz), 7.09–7.07 (m, 1H), 6.96–6.94 (m, 1H), 6.84–6.81 (m, 1H), 6.40 (s br, 1H), 6.51 (s, 1H), 4.67 (t, 2H, *J* = 7.4 Hz), 4.23 (s, 1H), 3.47–3.44 (m, 2H), 3.00 (s, 3H), 2.20 (s, 3H), 1.80 (s, br, 1H). ¹³C NMR (100 MHz, CDCl₃) δ (ppm): 169.33, 159.91, 145.23, 144.51, 141.22, 138.00, 131.32, 129.55, 128.42, 127.91, 126.19, 122.45, 121.20, 117.26, 116.09, 68.34, 58.18, 43.07, 33.15, 11.06. MS-ESI: *m/z* 491.13 [M+H⁺]. HPLC: >98% pure (*t*_R = 4.89 min).

4.1.22. General procedure for the preparation of nitro-oxyalkyl amines (9a,b) and amino acids (11a,b)

Fuming nitric acid (3 mL) was added to dichloromethane in a 100 mL round-bottomed flask. The solution was allowed to reach 0 °C and then hydroxyalkylamine (**8a–b**) (or amino acid **10a–b**) (16 mmol) was added dropwise. After 50 min of stirring, acetic anhydride (2 mL) was added dropwise over 2 min. After 45 min a precipitate was formed and then filtered. The solid was then crystallized with hot chloroform/ethanol to give the product as nitrate salt.

4.1.23. 2-(Nitro-oxy)ethyl amine nitrate salt (9a)

Off-white crystals, mp 89 °C (yield 90%). FT-IR cm^{-1} : 1636, 889. ^1H NMR (400 MHz, $\text{MeOH-}d_4$) δ (ppm): 4.80 (t, 2H), 3.40 (t, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm): 69.17, 38.49. MS-ESI: m/z 107.05 $[\text{M}+\text{H}^+]$.

4.1.24. HPLC methods

The purity of the title compounds was assessed by means of a Waters Alliance 2695 instrument equipped with an UV–vis Waters PDA 996 as the detector. Millennium Empower with Windows XP was used. For compounds **1a–d**, **3a,b,4a–d**, and **6a,b** a Gemini-NX C18 (100 × 2 mm, 3 μm) column at 35 °C was used at a flow rate of 0.3 mL/min. The eluent was (A) H_2O plus 0.1% formic acid or (B) CH_3CN plus 0.1% formic acid and a gradient from 90% A to 90% B was run in 13 min for **1a–d** and **4a–d**. Using the analytical conditions reported above, the retention times of the compounds ranged between 4.3 and 8.6 min. The eluent was (A) ammonium bicarbonate 10 mM (pH 9) or (B) CH_3CN and a gradient from 50% A to 90% B was run in 13 min for **3a,b** and **6a,b**. Using the analytical conditions reported above, the retention times of the compounds ranged between 2.7 and 4.2 min.

For compounds **2a–d** and **5a,b** a Acquity BEH C18 (100 × 2.1 mm, 1.7 μm) column at 30 °C was used at a flow rate of 0.3 mL/min. The eluent was (A) 5 mM ammonium acetate or (B) CH_3CN and a gradient from 90% A to 90% B was run in 9 min. Using the analytical conditions reported above, the retention times of the compounds ranged between 4.7 and 8.5 min. The eluent was (A) ammonium bicarbonate 10 mM (pH 9) or (B) CH_3CN and a gradient from 50% A to 90% B was run in 13 min for **3a,b** and **6a,b**. Using the analytical conditions reported-above, the retention times of the compounds ranged between 4.23 and 8.57 min. All the synthesized compounds were generally more than 95% pure.

4.2. Biology and pharmacology

4.2.1. In vitro anti-inflammatory studies

The in vitro profiles of compounds **1a–d**, **2a–d**, **3a,b**, **4a–d**, **5a–d**, and **6a,b** related to their inhibitory activity towards both COX-1 and COX-2 isoenzymes, were evaluated through cell-based assay employing murine monocyte/macrophage J774 cell lines. The cell line was grown in DMEM supplemented with 2 mM glutamine, 25 mM HEPES, 100 units/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 10% fetal bovine serum (FBS), and 1.2% sodium pyruvate. Cells were plated in 24-well culture plates at a density of 2.5×10^5 cells/mL or in 60 mm diameter culture dishes (3×10^6 cells per 3 mL per dish) and allowed to adhere at 37 °C in 5% CO_2 for 2 h. Immediately before the experiments, culture medium was replaced with fresh medium and cells were stimulated as described previously.^{26–28,33–40} The evaluation of COX-1 inhibitory activity was achieved pre-treating cells with test compounds (10 μM) for 15 min and then incubating them at 37 °C for 30 min with 15 μM arachidonic acid to activate the constitutive COX. For the compounds with COX-1% inhibition higher than 50% (at 10 μM), the

cells were treated also with lower concentrations (0.01–1 μM). At the end of the incubation, the supernatants were collected for the measurement of prostaglandin E_2 (PGE_2) levels by a radioimmunoassay (RIA). To evaluate COX-2 activity, cells were stimulated for 24 h with *Escherichia coli* lipopolysaccharide (LPS, 10 $\mu\text{g}/\text{mL}$) to induce COX-2, in the absence or presence of test compounds (0.01–10 μM). Celecoxib was utilized as a reference compound for the selectivity index. The supernatants were collected for the measurement of PGE_2 by means of RIA. Throughout the time the experiments lasted, triplicate wells were used for the various conditions of treatment. Results are expressed as the mean, for three experiments, of the percent inhibition of PGE_2 production by test compounds with respect to control samples. The IC_{50} values were calculated with GraphPadInstat, and the data fit was obtained using the sigmoidal dose-response equation (variable slope) (GraphPad).

4.2.2. Ex vivo vasorelaxing activity

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609. The effects of the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g). After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding. The aortae were immediately excised, freed of extraneous tissues and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five mm wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; CaCl_2 1.80; MgSO_4 1.05; NaH_2PO_4 0.41; NaHCO_3 11.9; glucose 5.5), thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with a computerised system (Biopac). After an equilibration period of 60 min, the endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (30 mM)-precontracted vascular rings. A relaxation <10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation $\geq 10\%$ (i.e. significant presence of the endothelium), were discarded. From 30 to 40 min after the confirmation of the endothelium removal, the aortic preparations were contracted by a single concentration of KCl (30 mM) and when the contraction reached a stable plateau, 3-fold increasing concentrations of the tested compounds (1 nM–10 μM) were added. Preliminary experiments showed that the KCl (30 mM)-induced contractions remained in a stable tonic state for at least 40 min. The same experiments were carried out also in the presence of a well-known GC inhibitor: ODQ 1 μM which was incubated in aortic preparations after the endothelium removal confirmation. The vasorelaxing efficacy was evaluated as maximal vasorelaxing response (E_{max}), expressed as a percentage (%) of the contractile tone induced by KCl 30 mM. When the limit concentration 10 μM (the highest concentration, which could be administered) of the tested compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM, evoked by this limit concentration. The parameter of potency was expressed as pIC_{50} , calculated as negative logarithm of the molar concentration of the tested compounds evoking a half reduction of the contractile tone induced by KCl 30 mM. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as mean \pm standard error, for 6–10 experiments. Two-way ANOVA

was selected as statistical analysis, $P < 0.05$ was considered representative of significant statistical differences. Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism 4.0).

4.2.3. Determination of the formation of nitrites and nitrates, in rat liver homogenate

The experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609. The liver homogenates were obtained from male normotensive Wistar rats (250–350 g). After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding. The portal vein was immediately cannulised and the liver was perfused with 4 °C-cold homogenation buffer (composition: K_2HPO_4 100 mM; EDTA 1 mM; KCl 15 mM; sucrose 0.25 M and ethanol 0.1%, pH 7.4). After about 5 min of slow perfusion, the liver was minced with scissors and washed with cold homogenation buffer. Then, it was dried with filter paper, weighted and resuspended (1:5 w/v) in cold homogenation buffer. The sample was finally homogenated in Turrax. In a vial, 400 μ L of the above homogenate were mixed with 500 μ L of assay buffer (composition: K_2HPO_4 100 mM; EDTA 1 mM; pH 7.4), containing glutathione, NADH and NADPH (all at the final concentration of 1 mM). The vials were then thermostated at 37 °C, and 100 μ L of a DMSO solution of the tested NO-donor were added (final concentration of the tested compound = 1 mM), allowing the release of NO, which in turn is rapidly converted to the stable inorganic metabolites, i.e. nitrites and nitrates. Aliquots (100 μ L) of the above medium were collected at selected intervals (5, 15, 30, 60 and 120 min) and added into a beaker containing 1.9 ml of an aqueous solution of H_2SO_4 (0.1 M) and KI (0.1 M). This solution allow nitrite ions to be exhaustively and instantaneously reduced to NO, which is amperometrically titrated by a NO-selective electrode connected to an Apollo 4000 free radical analyzer (World Precision Instrument), allowing us to determine the concentration of the nitrites previously formed in the biological sample. In parallel experiments, a Nitrate Reductor (World Precision Instrument) was constantly kept immersed into the vial. This tool allowed nitrates to be constantly converted to nitrites. The concentration of nitrites in these sample is defined as NO_x, which reflects the sum of all the nitrites and nitrates previously derived from NO. Opportune calibration curves were previously obtained with standard solutions of sodium nitrite.

4.2.4. Ex vivo human whole blood (HWB) assay

Compounds **1c**, **4c**, **3a**, and **6a** were evaluated for COX-1 versus COX-2 selectivity in HWB assay. Three to five healthy volunteers (aged 29 ± 3 years) were enrolled to participate in the study after its approval by the Ethical Committee of the University of Chieti. Informed consent was obtained from each subject. Compounds (0.005–150 mM) were dissolved in DMSO. Aliquots of the solutions (2 μ L) or vehicle were pipetted directly into test tubes to give final concentrations of 0.01–300 μ M in whole blood samples. To evaluate COX-2 activity, 1 mL aliquots of peripheral venous blood samples containing 10 IU of sodium heparin were incubated in the presence of LPS (10 μ g/mL) or saline for 24 h at 37 °C, as previously described.⁴² The contribution of platelet COX-1 was suppressed by pretreating the subjects with aspirin (300 mg, 48 h) before sampling. Plasma was separated by centrifugation (10 min at 2000 rpm) and kept at –80 °C until assayed for PGE₂ as an index of monocyte COX-2 activity. Moreover, peripheral venous blood samples were drawn from the same donors when they had not taken any NSAID during the 2 weeks preceding the study. Aliquots (1 mL) of whole blood were immediately transferred into glass tubes and allowed to clot at 37 °C for 1 h. Serum was separated by centrifugation (10 min at 3000 rpm) and kept at –80 °C until as-

sayed for TXB₂. Whole blood TXB₂ production was measured as a reflection of maximally platelet COX-1 activity in response to endogenously formed thrombin.⁴³

4.2.5. Analysis of PGE₂ and TXB₂

PGE₂ and TXB₂ concentrations were measured by previously described and validated radioimmunoassays.^{42,43}

4.2.6. Statistical analysis

For the HWB assays, results are expressed as the mean \pm SEM of three to five experiments. The % inhibition of TXB₂ (for COX-1 HWB assay) and PGE₂ (for COX-2 HWB assay) production by test compounds was calculated respect to control samples. Data fit was obtained using the sigmoidal dose–response equation (GraphPad software). The IC₅₀ values were calculated with GraphPad InStat, and the data fit was obtained using the sigmoidal dose–response equation (GraphPad). P values of less than 0.05 were considered significant.

4.2.7. In vivo analgesic and anti-inflammatory study

In vivo anti-inflammatory activity of the new compounds was also assessed. Male Swiss albino mice (23–25 g) and Sprague-Dawley or Wistar rats (150–200 g) were used. The animals were fed with a standard laboratory diet and tap water ad libitum and kept at 23 (1 °C with a 12 h light/dark cycle, light on at 7 a.m.). The paw pressure test was performed by inducing an inflammatory process by the intraplantar (ipl) carrageenan administration 4 h before the test. In the administered intraplantar (ipl) carrageenan 4 h before the test. The carrageenan-induced paw oedema test was also performed, evaluating the paw volume of the right hind paw 4 h after the injection of carrageenan and comparing it with saline/carrageenan-treated controls. The analgesic activity of compounds was also assessed by performing the abdominal constriction test, using mice into which a 0.6% solution of acetic acid (10 mL/kg) had been injected intra-peritoneal (ip). The number of stretching movements was counted for 10 min, starting 5 min after administration.

4.2.8. Solubility assessment in phosphate buffered saline and simulated gastric fluid

Stock solutions of test compounds (20 mM) were prepared in DMSO. A standard phosphate buffered saline (PBS) 0.01 M (pH 7.4) was used to determine the solubility in neutral pH conditions; a simulated gastric fluid (SGF) 0.05 M solution without pepsin (pH was adjusted to 1.5 using concentrated HCl) was instead used to check the solubility of compounds in acid medium. Seven point calibration standards (1, 5, 10, 25, 50, 100 and 200 μ M) were prepared from each 20 mM solution stock by serial dilution. Two microlitres of each test compound (stock solution 20 mM in DMSO) were added to 198 μ L of PBS and SGF in two duplicate wells in multiscreen solubility filter plate, covered and shaken for 90 min at 300 rpm using a Multi-pulse Vortexer (Glas-Col). The sample was filtered using a Millipore manifold filter assembly and collected in an acceptor plate and then analysed on HPLC together with the standards solutions (HPLC-Waters Separations Module 2695 with photodiode array detector).

4.2.9. Stability assessment in phosphate buffered saline, simulated gastric fluid and rat plasma

Stock solutions of test compounds (1 mM) were prepared in DMSO. A standard phosphate buffered saline 0.01 M (pH 7.4) was used to determine the stability in neutral pH conditions; a simulated gastric fluid 0.05 M solution without pepsin (pH was adjusted to 1.5 using concentrated HCl) was instead used to check the

stability of compounds in acid medium. Rat plasma (Sprague-Dawley rat) was used to underline the sensitivity of test compounds to plasma enzymes; in this case, Na₂EDTA dehydrate was used as anticoagulant for the experiment. An aliquot of each medium of 588 μL is taken and stored in a 2 mL Eppendorf tube. Afterwards, 12 μL of 1 mM solution of each test compound is added to each medium. The samples are incubated in an hybridisation oven. At each time point (0, 30, 60, and 120 min), a 100 μL aliquot is taken and quenched immediately with 300 μL of 100% ice cold acetonitrile (containing 60 ng/mL of internal standard, i.e. phenacetine) and stored at –80 °C. All the sample then are centrifuged at 14,000 rpm for 5 min. The supernatant is transferred into an HPLC vial and analysed on LC–MS/MS (Shimadzu-API4000).

4.2.10. Pharmacokinetic study

Male Han Wistar rats, three animals per dosage route, were treated with each compound solution (5% DMSO, 5% CHREMO-PHOR EL, 0.1% Tween 80 in phosphate buffer pH 7 (150 mM) at the dose of 10 mg/kg/5 mL intravenously and orally. Approximately 0.3 mL of blood per time point was taken from left jugular vein using a syringe. The samples were then centrifuged at 12,000 rpm for 5 min at 4 °C. Plasma was separated, placed in pre-labeled Eppendorf tubes, and stored at –80 °C pending the assay. LC–MS/MS analysis was conducted on Applied Biosystem API 4000 Qtrap mass spectrometer operating in ESI positive ion mode with the LLOQ of 1 ng/mL for **1c** and **4c** and LLOQ of 15 ng/mL for **3a** and 5 ng/mL for **6a**. Data were elaborated using Analyst 1.5.1 software. Agilent 1200 series HPLC system was used for chromatographic separation through Phenomenex Luna 3u Phenyl Hexyl (2.1 × 50 mm, 3.5 μm) 3.5 μm and Zorbax Sb C18, 2.1 × 50 mm columns, heated at 45 °C. Compounds **1c** and **4c** and **3a** and **6a** separation was achieved in gradient conditions at a flow rate of 0.4 mL. Mobile phase composition in both cases was 0.1% HCOOH in H₂O/CH₃CN, 10/90 (v/v). Compounds quantification was done at following MRM transitions: **1c**: *m/z* 476 > 342, **4c**: *m/z* 431 > 342 and **3a**: *m/z* 491 > 369, **6a**: *m/z* 446 > 368.

Extraction method consisted in plasma protein precipitation of rat plasma samples with CH₃OH into 96-deep well plates 1 mL round (DW Greiner). Samples were mixed and centrifuged for 15 min at 3700 rpm at 6 °C and 5 μL of the supernatant was injected into the LC/MS/MS system.

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Supplementary data

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