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Novel Analgesic/Anti-Inflammatory Agents: Diarylpyrrole Acetic Esters Endowed with Nitric Oxide Releasing Properties

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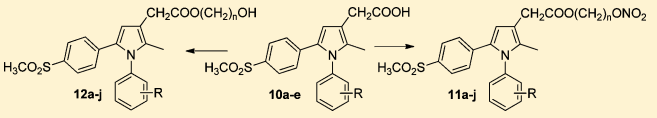
Supporting Information

ABSTRACT: The design of compounds that are able to inhibit cyclooxygenase (COX) and to release nitric oxide (NO) should give rise to drugs endowed with an overall safer profile for the gastrointestinal and cardiovascular systems. Herein we report a new class of pyrrole-derived nitrooxy esters (**11a−j**), cyclooxygenase-2 (COX-2) selective inhibitors endowed with NO releasing properties, with the goal of generating new molecules able to both strongly inhibit this isoform and reduce the related adverse side effects. Taking into account the metabolic conversion of nitrooxy esters into corresponding alcohols, we also studied derivatives **12a−j**. All compounds proved to be very potent and selective COX-2 inhibitors; nitrooxy derivatives displayed interesting ex vivo NO-dependent vasorelaxing properties. Compounds **11c**, **11d**, **12c**, and **12d** were selected for further in vivo studies that highlited good anti-inflammatory and antinociceptive activities. Finally, two selected compounds (**11c** and **12c**) tested in human whole blood (HWB) assay proved to be preferential inhibitors of COX-2.



INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) selective for cyclooxygenase-2 (COX-2), named coxibs, were developed to overcome the gastrointestinal (GI) side effects associated with the use of traditional NSAIDs (*t*-NSAIDs).[1,2] Large randomized clinical trials highlighted how almost all highly selective COX-2 inhibitors were endowed with safer GI profiles compared to *t*-NSAIDs.[1−3] The withdrawal of rofecoxib from the marketplace has been related to a small but significant increase of thrombotic events associated with the chronic use of rofecoxib in patients with a history of colorectal adenoma.[4] Numerous studies of clinical pharmacology and pharmacoepidemiology as well as studies performed in animal models showed quite convincingly that selective inhibition of COX-2 by coxibs translates into a cardiovascular (CV) hazard. The most plausible hypothesis is that they cause the inhibition of the vasorelaxing and antithrombotic prostacyclin which is generated mainly by the activity of constitutively expressed COX-2, in endothelial cells, in vivo.[5,6] This CV hazard is shared with *t*-NSAIDs through the same mechanism, i.e., profound inhibition of endothelial COX-2-dependent prostacyclin associated with incomplete inhibition of antithrombotic factors deriving from platelet COX-1, resulting in an impaired platelet function and increased risk of thromboembolic accidents.[7] The COX-inhibiting nitric oxide (NO) donors (CINODs) represent a new class of agents that may offer an improved safety profile having a dual mechanism of action involving COX inhibition and NO donation.

NO is a pleyotropic radicalic molecule involved in vascular smooth muscle tone modulation, angiogenesis, thrombosis, endothelium permeability, and neuronal transmission. This endogenous autacoid exerts its physiological roles mainly through the activation of guanylate cyclase (GC), which gives rise to increasing levels of c-GMP and, as a consequence, to smooth muscle tone relaxation, platelet aggregation inhibition, and chemokine and cytokine down-regulation (which accounts for the inhibitory activity on leukocyte adhesion), finally

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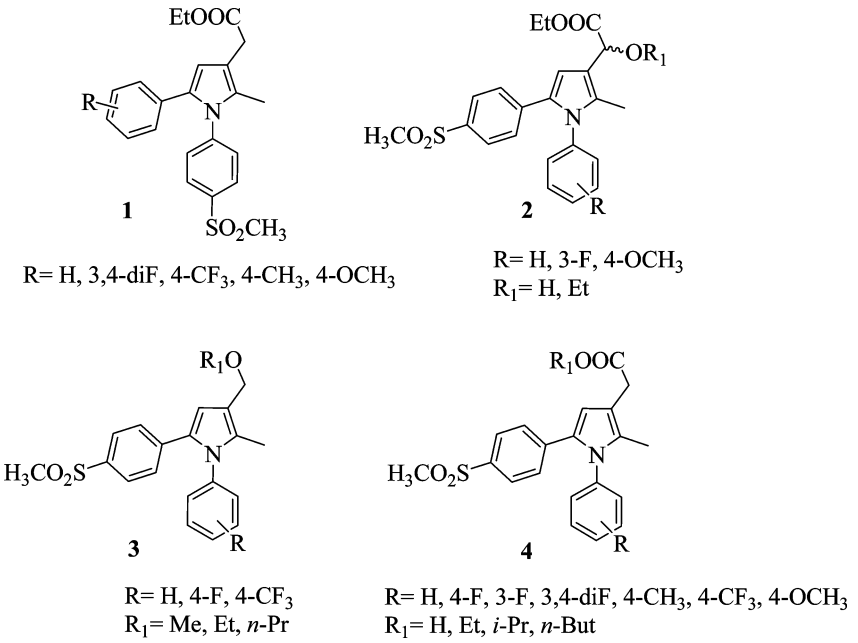

Figure 1. New class of pyrrole-containing antiflogistic/analgesic agents (**1**−**4**) as selective COX-2 inhibitors.

leading to an antithrombotic mode of action.[8] It is also worth noting that NO is endowed with a cardioprotective function, and gene knock-out studies demonstrated that NO depletion leads to an increased incidence of myocardial infarction. It is well-known and understood that organic nitrates as well as some other compounds (nitrous thiols, furoxan *N*-oxides)[9] are endowed with releasing property as exogenous "NO donors", mimicking its actions in such a way to be employed as therapeutic agents.[9,10] On these grounds, the synthesis of hybrid molecules, which conjugate a COX-2 inhibiting feature with a NO releasing moiety, could bring about new anti-inflammatory and analgesic agents, with an improved safety profile for the CV system versus NSAIDs (both *t*-NSAIDs and coxibs) which are associated with renal and occlusive vascular events.[7,11] Some CINODs, such as nitronaproxen,[12−14] are known in literature and underwent clinical development studies. Nitronaproxen has been reported have reduced renal and gastrointestinal toxicity compared to naproxen.[11,15,16] Though the approach of combining an NO-donor moiety to *t*-NSAIDs was widely applied, only few examples can be found in the design of COX-2 inhibiting nitric oxide donors.[17] An interesting prodrug of rofecoxib, able to release NO, was reported as well as nitroxy-substituted 1,5-diarylimidazoles and nitroxy substituted analogues of celecoxib.[18−24]

In the past, we reported several investigations concerning the development of a new class of pyrrole-containing antiflogistic/analgesic agents (compounds **1**−**4**, Figure 1) as selective COX-2 inhibitors.[25−31] Structure−activity relationships of these compounds were examined in depth. In addition to the impact by the substitution pattern on the phenyl ring at N1 (activity decreasing in the order H > 3-F = 4-F > 3,4-diF > 4-OMe > 4-CF_3 > 4-CH_3) and, of course, to the presence of the *p*-methylsulfonyl substituent in the C5 pyrrole ring, the side chain at C3 appeared to be crucial for the COX-2 inhibition potency. Only aliphatic side chains were previously explored, and no information was available about the effect exerted by the introduction into the side chain of other substituents (Figure 1). Thus, starting on these grounds, we decided to investigate the effects exerted on COX-2 inhibitory activity by the introduction of a NO donor moiety on this scaffold. Manipulation of the 3-position of the scaffold allowed us to retain COX-2 inhibitory properties in terms of both potency and selectivity and to introduce a NO-releasing moiety with appropriate characteristics. Conversely, manipulation of the scaffold in other positions led to the loss of COX-2 inhibition. We report herein the syntheses and the biological activities of new derivatives **11a**−**j** (Scheme 1), obtained by the combination of selective COX-2 inhibitors and nitrooxyalkyl chains in order to obtain effective analgesics and anti-inflammatory agents CV-sparing. These compounds have been designed by combining some 1,5-diarylpyrroles that were selected on the basis of their COX-2 inhibitory activity[31] and NO-donor alkyl chains with different length. Furthermore, considering that the nitrooxyalkyl groups are subjected to in vivo metabolism, we also synthesized the corresponding hydroxyl derivatives (compounds **12a**−**j**) of compounds **11a**−**j**. All the target compounds were evaluated in vitro for their ability to inhibit COX-1 and COX-2 in a J774 murine macrophage cell line. In order to evaluate their NO-releasing properties, the vasorelaxing activities of compounds **11a**−**j** were evaluated ex vivo on isolated rat aortic rings. The in vivo anti-inflammatory and analgesic activities for selected compounds were also evaluated in several animal models. Among all compounds, derivatives **11c** and **12c**, a nitro ester and its hydroxy-related derivative, were selected to evaluate their selectivity ex vivo, performing a human whole blood (HWB) assay.

CHEMISTRY

The synthesis of the target compounds is described in Scheme 1. Briefly, the reaction of methyl vinyl ketone with 4-(methylthio)-benzaldehyde in a microwave apparatus gave intermediate **5**,[30] which was transformed into the corresponding 4-methylsulfonyl derivative **6** by means of Oxone oxidation.[32] Intermediate **6** cyclized to yield the expected 1,5-diarylpyrroles **7a**−**e** in the presence of the appropriate arylamine (45 min by employing a microwave apparatus).[30] Regioselective acylation of **7a**−**e** with

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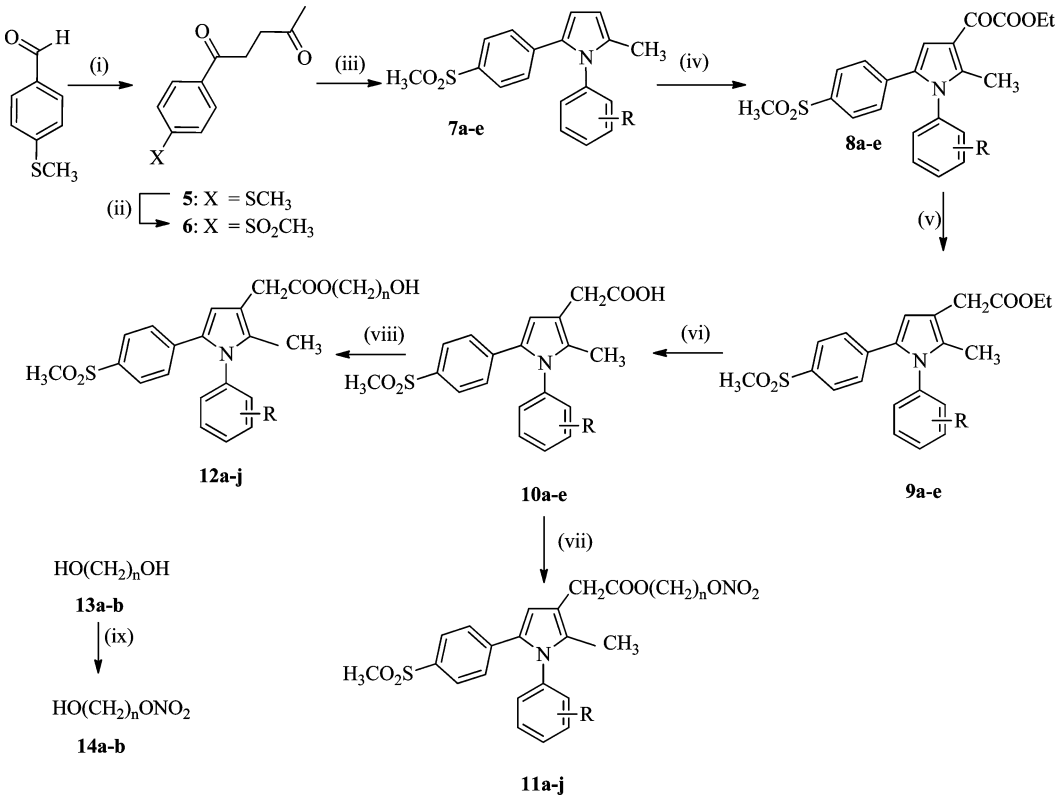
Scheme 1. Synthesis of Compounds 11a–j and 12a–j[a]



[a]Compounds: **7a**, R = H; **7b**, R = 3-F; **7c**, R = 4-F; **7d**, R = 4-SCH_3; **7e**, R = 4-OCH_3; **8a**, R = H; **8b**, R =3-F; **8c**, R = 4-F; **8d**, R = 4-SCH_3; **8e**, R = 4-OCH_3; **9a**, R = H; **9b**, R = 3-F; **9c**, R = 4-F; **9d**, R = 4-SCH_3; **9e**, R = 4-OCH_3; **10a**, R = H; **10b**, R = 3-F; **10c**, R = 4-F; **10d**, R = 4- SCH_3; **10e**, R = 4-OCH_3; **11a**, R = H, n = 2; **11b**, R = H, n = 3; **11c**, R = 3-F, n = 2; **11d**, R = 3-F, n = 3; **11e**, R = 4-F, n = 2; **11f**, R = 4-F, n = 3; **11g**, R = 4-SCH_3, n = 2; **11h**, R = 4-SCH_3, n = 3; **11i**, R = 4-OCH_3, n = 2; **11j**, R = 4-OCH_3, n = 3; **12a**, R = H, n = 2; **12b**, R = H, n = 3; **12c**, R = 3-F, n = 2; **12d**, R = 3-F, n = 3; **12e**, R = 4-F, n = 2; **12f**, R = 4-F, n = 3; **12g**, R = 4-SCH_3, n = 2; **12h**, R = 4-SCH_3, n = 3; **12i**, R = 4-OCH_3, n = 2; **12j**, R = 4-OCH_3, n = 3; **13a**, n = 2; **13b**, n = 3; **14a**, n = 2; **14b**, n = 3. Reagents and conditions: (i) CH_2=CHCOMe, TEA, 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide, microwave, 15 min; (ii) Oxone, MeOH/H2O, room temp, 2 h; (iii) $RPhNH_2$, p-toluensulfonic acid, EtOH, microwave, 45 min; (iv) EtOCOCOCl, $TiCl_4$, CH_2Cl_2, room temp, 4 h; (v) Et_3SiH, TFA, room temp, 2 h; (vi) 1 N NaOH, MeOH, 1 h; (vii) **14a,b**, EDCI, DMAP, CH_2Cl_2, room temp, 3h; (viii) **13a,b**, EDCI, DMAP, CH_2Cl_2, room temp, 3 h; (ix) HNO_3, CH_3COOH, $(CH_3CO)_2O$, EtOAc, room temp, 20 h.

ethoxalyl chloride in the presence of titanium tetrachloride gave the α-keto ester derivatives **8a-e**, which were in turn reduced by means of triethylsilane in trifluoroacetic acid to give the pyrrole ethyl esters **9a–e**.[30] Treatment of **9a–e** with 1 N NaOH in MeOH for 1 h gave the corresponding acetic acids **10a–e**. Target compounds **12a–j** and **11a–j** were finally obtained by reacting acetic acids **10a–e** with the appropriate diols **13a,b** or hydroxyalkyl nitrates **14a,b** (previously prepared by reacting **13a,b** with nitric acid, acetic acid, and acetic anhydride overnight)[33] in the presence of EDCI and DMAP for 3 h.

RESULTS AND DISCUSSION

All compounds were tested in the J774 murine macrophage assay to evaluate their inhibitory potency and selectivity on both COX isoforms (Table 1). All compounds inhibited COX-2 activity at concentrations ranging from micromolar to nanomolar values. In particular, nitrated compounds (**11a–j**) displayed much better biological profiles than those of the corresponding alcohol compounds (**12a–j**). Similar trends have been previously observed by other authors.[19,20] This result could be due to an electronic interaction of the nitrate group or to other interactions of this moiety at the inner hydrophobic channel of the enzyme. Nonetheless, it is noteworthy that especially in the meta-fluorine substituted (**11c**, **11d**) and para-methoxy substituted (**11i**, **11j**) subclasses, the activity seems to be related to the side chain length; an inverse relationship is found between side chain length and inhibitory concentration. Compound **11c**, which is characterized by a two-carbon side chain, showed a 19 nM activity on COX-2 isoenzyme, while compound **11d** (three-carbon side chain) displayed a 3-fold higher activity with a 7.3 nM inhibitory concentration. This trend is also found in increased form comparing compounds **11i** (0.17 μM) and **11j** (2.4 nM). On the other hand, nitrated compounds bearing a para-fluorine, a para-methylsulfonyl, or an unsubstituted phenyl ring at N1 of the central core displayed activities that are not influenced by length of the side chain (Table 1). The results obtained for the hydroxylated derivatives (**12a–j**) showed a similar trend concerning the inhibition of COX-2 isozyme; the only exception is found in compound **12b** which is devoid of any activity. No correlation, however, between the length of the hydroxyl compound side chain and the activity can be postulated. All compounds, either in the form of nitroester or corresponding hydroxyl or acid derivatives, showed outstanding selectivity toward COX-2. Compounds **11a–j** were further evaluated to assess their

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Table 1. In Vitro COX-1 and COX-2 Inhibitory Activity of 11a–j, 12a–j, and Celecoxib

compd	R	*n*	concn (μM)	COX-1 inhibition (%)	COX-1 IC_{50} (μM)[a]	COX-2 inhibition (%)	COX-2 IC_{50} (μM)[a]	selectivity index[b]
10a[c]	H		0.01		>10	13	1	>10
			0.1			17		
			1			43		
			10	0		100		
10b[d]			0.01		>10	40	0.028	>357.1
			0.1			60		
			1			89		
			10	4		96		
10c			0.1		>10	25	0.5	>20
			1			60		
			10			100		
			0.1		>10	25	0.5	>20
10d			0.01		>10	47	0.013	>769.2
			0.1			73		
			1			94		
			10	45		98		
10e[d]			0.1		>10	47	0.17	>58.8
			1			62		
			10	0		90		
11a	H	2	0.01		>10	22	0.0430	>232.6
			0.10			68		
			1.00			91		
			10.00	19		100		
11b	H	3	0.01		>10	13	0.0420	>238.1
			0.10			76		
			1.00			91		
			10.00	27		90		
11c	3-F	2	0.01		>10	43	0.0190	>526.3
			0.10			67		
			1.00			92		
			10.00	36		96		
11d	3-F	3	0.01		1.1	55	0.0073	150.7
			0.10	20		83		
			1.00	50		97		
			10.00	75		97		
11e	4-F	2	0.01		>10	31	0.0290	>344.8
			0.10			72		
			1.00			100		
			10.00	0		100		
11f	4-F	3	0.01		>10	25	0.0372	>268.8
			0.10			71		
			1.00			90		
			10.00	28		91		
11g	4-SCH_3	2	0.01		>10	38	0.0170	>588.2
			0.10			84		
			1.00			100		
			10.00	0		100		
11h	4-SCH_3	3	0.01		>10	40	0.0270	>370.4
			0.10			61		
			1.00			90		
			10.00	0		99		
11i	4-OCH_3	2	0.100	12	6.4	34	0.1700	37.6
			1.00	30		89		
			10.00	55		97		
11j	4-OCH_3	3	0.001		>10	39	0.0024	>4166.7
			0.01			67		
			0.1			90		
			1	0		90		
12a	H	2	0.10		>10	51	0.0960	>104.2
			1.00			92		
			10.00	22		100		

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Table 1. continued

compd	R	*n*	concn (μM)	COX-1 inhibition (%)	COX-1 IC_{50} (μM)[a]	COX-2 inhibition (%)	COX-2 IC_{50} (μM)[a]	selectivity index[b]
12b	H	3	0.100			0		
			1.00			0		
			10.00			100		
12c	3-F	2	0.01		>10	30	0.0850	>117.6
			0.100			50		
			1.00			78		
			10.00	33		80		
12d	3-F	3	0.01		>10	40	0.0230	>434.8
			0.10			66		
			1.00			80		
			10.00	0		85		
12e	4-F	2	0.01		>10	25	0.0390	>256.4
			0.10			68		
			1.00			92		
			10.00	21		100		
12f	4-F	3	0.01		>10	22	0.0560	>178.6
			0.10			60		
			1.00			90		
			10.00	0		199		
12g	4-SCH_3	2	0.01		3.18	24	0.0850	37.4
			0.10	11		50		
			1.00	40		85		
			10.00	62		87		
12h	4-SCH_3	3	0.10		>10	34	0.2600	>38.5
			1.00			74		
			10.00	36		85		
12i	4-OCH_3	2	0.10		>10	2	1.1000	>9.1
			1.00			52		
			10.00	5		84		
12j	4-OCH_3	3	0.10		>10	3	0.7000	>14.3
			1.00			64		
			10.00	27		93		
celecoxib			0.01	0	3.84	32	0.0610	>63.0
			0.10	4		52		
			1.00	31		80		
			10.00	64.7		100		

[a]Results are expressed as the mean (n = 3) of the % inhibition of PGE_2 production by the test compounds with respect to control samples. [b]IC_{50}(COX-1)/IC_{50}(COX-2). [c]See ref 25. [d]See ref 30.

efficacy and potency in determining NO-dependent vasorelaxing responses in a suitable experimental model of vascular smooth muscle and in particular in endothelium-denuded rat aortic rings (Table 2). Again, the side chain length seems to be the crucial feature for this activity: the two-carbon chain-bearing compounds (**11a**, **11c**, **11e**, **11g**, **11i**) are always endowed with higher vasorelaxing effects than the related three-carbon chain-bearing derivatives (**11b**, **11d**, **11f**, **11h**, **11j**). In particular, all two-carbon chain-bearing derivatives exhibited vasorelaxing efficacy (E_{max}) higher than 50% and potency values (pIC_{50}) ranging between 5.47 (**11e**) and 6.75 (**11i**), while the efficacy parameters of all three-carbon chain-bearing derivatives were always lower than 50%, thus rendering the calculation of the parameters of pIC_{50} impossible. The experiments carried out in the presence of guanylate cyclase (GC) inhibitor 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) confirmed that the vasorelaxing effects were due to the release of NO, since such a pharmacological inhibition of GC significantly antagonized the vasodilator responses evoked by the nitrooxy derivatives **11a**–**j** (Figure 2). These experiments also showed that the corresponding alcohol derivatives **12a**–**j** were devoid of significant vasorelaxing effects (data not shown), further emphasizing the fact that the pharmacodynamic feature is only due to the nitrooxy group and its biotransformation to NO. GTN, selected as reference drug endowed with rapid NO-releasing properties,

Table 2. Evaluation of Efficacy and Potency in Determining NO-Dependent Vasorelaxing Responses of 11a–j and GTN

compd	R	*n*	R′	E_{max}[a]	pIC_{50}[a]
11a	H	2	NO_2	65 ± 2	5.76 ± 0.08
11b	H	3	NO_2	44 ± 8	<5
11c	3-F	2	NO_2	69 ± 4	6.48 ± 0.06
11d	3-F	3	NO_2	39 ± 1	<5
11e	4-F	2	NO_2	58 ± 5	5.47 ± 0.07
11f	4-F	3	NO_2	41 ± 2	<5
11g	4-SCH_3	2	NO_2	65 ± 1	6.34 ± 0.06
11h	4-SCH_3	3	NO_2	41 ± 8	<5
11i	4-OCH_3	2	NO_2	77 ± 2	6.75 ± 0.05
11j	4-OCH_3	3	NO_2	41 ± 11	<5
GTN				73 ± 2	6.90 ± 0.07

[a]E_{max} represents the vasorelaxing efficacy, while the parameter of potency is expressed as pIC_{50}.

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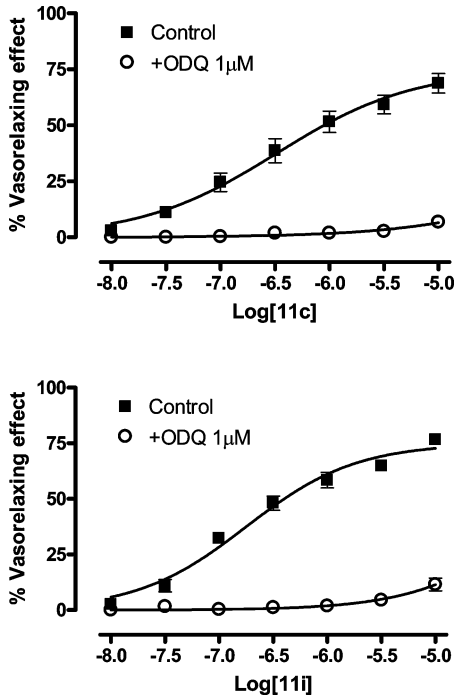

Figure 2. Representative concentration–response curves relative to the vasorelaxing effects evoked by compounds **11c** and **11i**, recorded on endothelium-denuded rat aortic rings in the absence (control, black squares) or in the presence (white circles) of 1 μM ODQ (inhibitor of guanylate cyclase). The vasorelaxing responses are expressed as a percentage of the contractile effect induced by 30 mM KCl. The vertical bars indicate the standard error.

showed strong vasorelaxing effects and higher levels of vasorelaxing potency (Table 2). Experimental evidence suggests that the compounds reported herein ensure a NO release that is slower than that for GTN, thus acting as "modulated" NO donors, which are preferred for hybrid drugs. The meta-fluorine subclass of compounds (**11c,d** and **12c,d**) was selected for further in vivo studies. The results related to the writhing test emphasized the strong dependence of the activity on the side chain length (Table 3). Even though compounds bearing three-carbon side chains (Table 1) proved to be more active than the corresponding two-carbon side chain bearing ones in vitro, compounds with two-carbon side chains, such as nitrated and nitro-free derivatives **11c** and **12c**, showed better analgesic profiles than those found for three-carbon side chain derivatives. The 70% writhes reduction was associated with the activities of both **11c** and **12c** at their maximum dosage (40 and 30 mg kg^{-1}, respectively), while compounds **11d** and **12d** showed a lower activity. Findings reported for carrageenan induced hyperalgesia and edema show that all compounds are endowed with good pharmacological profiles (Table 4). As discussed for the writhing test, compounds bearing two-carbon side chains were able to cut down carrageenan induced pain. Compound **12c** exerted a unique profile: 80% of hyperalgesia reduction 30 min after the treatment, when administered at a dose of 30 mg kg^{-1}. A comparison of **12c** with celecoxib clearly emphasizes that their profiles nearly resemble each other until 60 min after treatment, with the only difference being that **12c** can be associated with a faster onset of the effect. As shown for **12c**, the nitro-related compound **11c** is characterized by the same fast effect but its efficacy is abated after 60 min. The shorter duration of the analgesic effect of **11c** allows us to

Table 3. Effect of 11c, 11d, 12c, 12d, Celecoxib, and Vehicle (CMC) in the Mouse Abdominal Constriction Test (Acetic Acid 0.6%)

compd[a]	no. mice	dose po, mg/kg (μmol/kg)[b]	no. writhes[c]
CMC	43		32.6 ± 2.1
11c	12	20 (42)	17.1 ± 2.3*
11c	12	40 (84)	9.4 ± 2.5*
11d	18	20 (41)	25.7 ± 2.8^
11d	10	40 (82)	24.9 ± 2.2*
12c	8	3 (7)	26.9 ± 3.0*
12c	8	10 (23)	17.4 ± 2.9*
12c	8	20 (46)	16.2 ± 2.8*
12c	8	30 (70)	8.9 ± 2.1*
12d	10	20 (45)	25.3 ± 2.8
12d	10	40 (90)	15.6 ± 2.2*
celecoxib	10	10 (26)	13.4 ± 2.6*

[a]All compounds were administered per os 30 min before test. [b]The doses are expressed in mg/kg. The equivalent doses in μmol/kg are indicated in parentheses. [c]*, $P < 0.01$ versus vehicle-treated mice. ^, $P < 0.05$.

hypothesize that its related hydroxyl derivative **12c** is not the main metabolite of **11c**. Indeed, the complex metabolic pathway that ensures the biotransformation of the nitrooxy derivatives, leading to the production of NO and the related hydroxyl derivatives, is widely considered as the most usual metabolic destiny of organic nitrates.[34] Nevertheless, recent experimental evidence suggests that this is not a unique pathway. For example, the pharmacokinetic feature of some nitrooxy derivatives of aspirin indicates that alternative pathways involving the formation of *O*-glutathionyl metabolites can play a relevant role in the metabolism of organic nitrates.[35] A more detailed characterization of the pharmacokinetic profile of **11c** is currently being investigated. Compounds **11d** and **12d** performed well but significantly less well than **12c**. The antiedemigenic activity of compounds is remarkable albeit not comparable to celecoxib. The extent of selective inhibition gained through the cell-based assay is, however, not to be considered the most reliable one; indeed, the use of exogenous AA in the assay for COX-1 activity in vitro, in the murine monocyte/macrophage J774 cell line, might have caused a loss in COX-1 affinity for an AA-dependent allosteric activation of COX-1, which induces a conformation change within the enzyme binding site.[36] With the aim of assessing COX-2 selectivity, the HWB assay was carried out on selected compounds **11c** and **12c** (nitro ester and nitro-free one, respectively). In particular, the assay was performed to predict the actual extent of isozyme inhibition achievable in vivo by circulating drug levels, since a number of variables are potentially able to affect drug–enzyme interactions. As shown in Figure 3 and in Table 5, compounds **11c** and **12c** inhibited platelet COX-1 activity in clotting blood incubated for 60 min at 37 °C in a concentration-dependent fashion, with different IC_{50} values of 9.6 μM (95% CI of 6.3–14.6 μM) and 12.2 μM (95% CI of 7 –21 μM), respectively, the effect of the compounds on inducible COX-2 activity in LPS-stimulated HWB in vitro. **11c** and **12c** showed as well similar inhibitions of monocyte COX-2 activity with IC_{50} values of 3.2 μM (95% CI of 2–5.5 μM) and 1.7 μM (95% CI of 0.94–3 μM), respectively. Hence, **11c** was 3-fold and **12c** was 7.3-fold more potent toward COX-2 than toward COX-1

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Table 4. Effect of 11c,d, 12c,d, Celecoxib, and Vehicle (CMC) on Hyperalgesia and Edema Induced by Carrageenan in the Rat Paw Pressure Test

pretreatment, ipl	treatment, po	paw pressure (g) dose mg/kg (μmol/kg)[a]	before treatment	after treatment[b] 30 min	60 min	120 min	volume (mL)[b] 60 min
saline	CMC		62.6 ± 2.1	62.5 ± 1.8	63.4 ± 2.2	62.7 ± 2.5	1.33 ± 0.07
carrageenan	CMC		61.9 ± 2.0	33.7 ± 2.6	35.1 ± 2.3	34.8 ± 2.3	2.44 ± 0.08
carrageenan	**11c**	20 (42)	32.6 ± 2.7	40.1 ± 3.4^	35.3 ± 2.8	ND	2.52 ± 0.06
carrageenan	**11c**	40 (84)	29.8 ± 3.1	54.1 ± 3.7*	38.7 ± 3.1	ND	2.17 ± 0.08*
carrageenan	**11c**	100 (210)	31.9 ± 3.0	57.2 ± 3.5*	36.5 ± 3.8	ND	1.88 ± 0.08*
carrageenan	**11d**	20 (41)	62.5 ± 3.1	43.2 ± 3.2	45.1 ± 3.7^	40.7 ± 2.6	2.05 ± 0.08^
carrageenan	**11d**	40 (82)	63.2 ± 3.3	45.5 ± 3.7*	44.9 ± 4.2^	36.8 ± 3.8	1.96 ± 0.09*
carrageenan	**12c**	30 (70)	31.8 ± 2.5	55.7 ± 3.1*	52.3 ± 2.8*	39.3 ± 3.5	2.51 ± 0.05
carrageenan	**12d**	20 (45)	62.6 ± 3.1	41.2 ± 3.0	45.8 ± 3.6^	36.4 ± 3.1	ND
carrageenan	**12d**	40 (90)	61.3 ± 3.0	43.1 ± 3.6	48.8 ± 3.6*	44.4 ± 3.3^	ND
carrageenan	celecoxib	10 (26)	61.5 ± 3.4	54.3 ± 3.9*	57.1 ± 4.0*	54.9 ± 3.6*	1.36 ± 0.07*

[a]The doses are expressed in mg/kg. The equivalent doses in μmol/kg are indicated in parentheses. [b]*, $P < 0.01$ versus vehicle-treated mice. ND, not determined. ∧, $P < 0.05$.

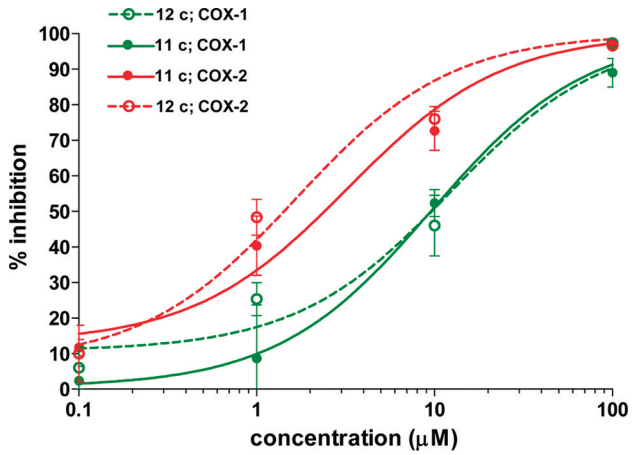

Figure 3. Effects of **11c** and **12c** on COX-1 and COX-2 activity in vitro in human whole blood. Results are expressed as average percent of inhibition ($N = 3$, mean ± SD) from three separate experiments.

(Figure 3 and Table 5); however, a dramatic change in COX-2 selectivity in HWB assay versus J774 models was observed. The drop of selectivity in the HWB assay in relation to that by the cell-based assay can be potentially explained by different sensitivities of mice and human COXs enzymes, as adequately described in the literature for other COX-2 inhibitors.[26,37]

MOLECULAR MODELING SIMULATIONS

The COX-2 inhibition data for selected compounds **11c,d** and **12c,d** were rationalized through docking simulations using a crystallographic model of the COX-2 active site [complex between COX-2 and SC-558, refined at 3 Å resolution (Brookhaven Protein Data Bank entry: 6cox)][25] by means of AutoDock 4.0 (for details see the Supporting Information).

Those docking simulations suggest that the binding modes observed for hydroxylalkyl esters **12c,d** and nitro diesters **11c,d** are very similar to the ones previously reported for 1,5-diarylpyrrole-3-alkoxyethyl ether **3**[27] and 1,5-diarylpyrrole-3-acetic ester **4**.[25] In particular, the hydroxyethyl ester moiety of compound **12c** establishes multiple interactions with the active site: a simple hydrogen bond between Tyr355 and the alcoholic oxygen atom as well as a simultaneous double hydrogen bond interaction of Arg120 with alcoholic and carboxylic oxygen atoms. Moreover, in the docked conformation of **12c**, an intramolecular hydrogen bond was observed between the OH group and the carboxylic oxygen that induces the side chain to adopt a pseudocyclic conformation (Figure 4a). This distinctive feature is not conserved in the interaction of hydroxypropyl chain-bearing derivative; that is, **12d** (Figure 4b) is able to inhibit COX-2 activity with higher potency (IC_{50} = 23 nM) compared to compound **12c** (IC_{50} = 85 nM). In fact, the hydroxypropyl chain of **12d** allows for an additional hydrogen bond with Val116, while the hydrogen bond between Arg120 and the carboxylic oxygen atom is retained. In this way, the lipophilic pocket defined by Val116, Leu93, and Leu359 allows the hydroxypropyl ester side chain of **12d** to be accommodated in a more profitable manner compared to the case of hydroxyethyl chain of **12c**. For the nitrooxyalkyl derivatives **11c,d** docking simulations demonstrated that the side chains of these compounds interact with Arg120, adopting a conformation similar to the hydroxypropyl chain of compound **12d**. Different from the hydroxypropyl ester side chain of **12d**, the nitrooxyethyl and nitrooxypropyl acetate moieties of the highly active derivative **11d** (IC_{50} = 7.3 nM) and **11c** (IC_{50} = 19 nM) interact with the COX-2 active site by means of a hydrogen bond between Ser119 and one of the oxygen atom of the NO_2 group (Figure 5), confirming what Wey et al. previously suggested for polar-end tethers.[20] Thus, independently from the length of the spacer between the acetate moiety and the nitroester, both residues Val116 and Ser119 seem to elicit the same effect on compounds **11c,d** and **12d**, stretching the side chains toward the solvent accessible surface.

Furthermore, the substituted phenyl ring in position 1 of the pyrrole core is located in the "hydrophobic pocket" defined by hydrophobic (Leu384) and aromatic (Tyr385, Trp387, and Phe518) residues; this finding corresponds to those observed for compounds **3** and **4**. Finally, the *p*-methylsulfonylphenyl moiety at position 5 of the diarylpyrrole scaffold of compounds **11c,d** and **12c,d** occupies the same space as for compounds **3** and **4**, being accommodated within the so-called "selectivity site". In fact, the entire *p*-methylsulfonylphenyl group of the inhibitor undregoes profitable interactions with COX-2

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Table 5. In vitro Inhibition (HWB) of COX-1 and COX-2 by 11c, 12c, and Celecoxib[a]

compd	R	*n*	R′	COX-1 IC_{50} (μM)	COX-2 IC_{50} (μM)	SI
11c	3-F	2	NO_2	9.6 (6.3−14.6)	3.2 (2−5.5)	3
12c	3-F	2	OH	12.2 (7−21)	1.7 (0.94−3)	7.3
celecoxib				12.47 (8.6−17.9)	0.54 (0.29−0.52)	23

[a] IC_{50} values of COX-1 and COX-2 are reported as the mean of three different experiments performed in duplicate. In parentheses, the 95% confidence intervals (CI) are shown.

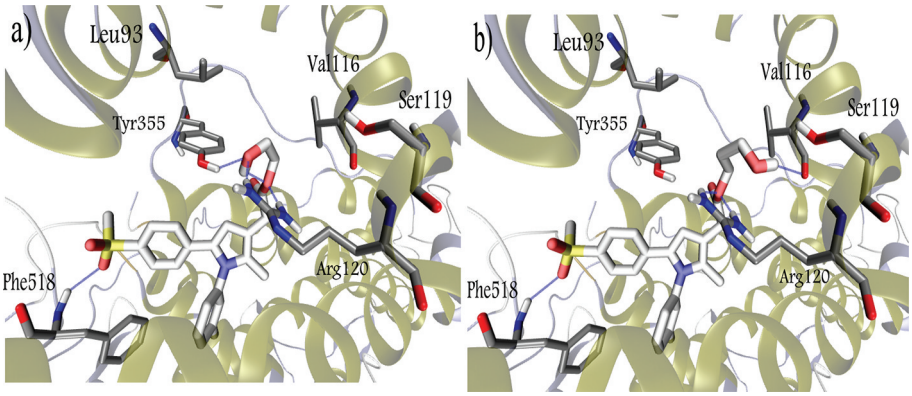

Figure 4. (a) Hydroxyethyl ester **12c** and (b) hydroxypropyl ester **12d** docked into the COX-2 cyclooxygenase site. The main residues and the inhibitors are represented as licorice. Hydrogen bonds are represented as blue dotted lines. For the sake of clarity, only a few amino acids of the binding site are displayed.

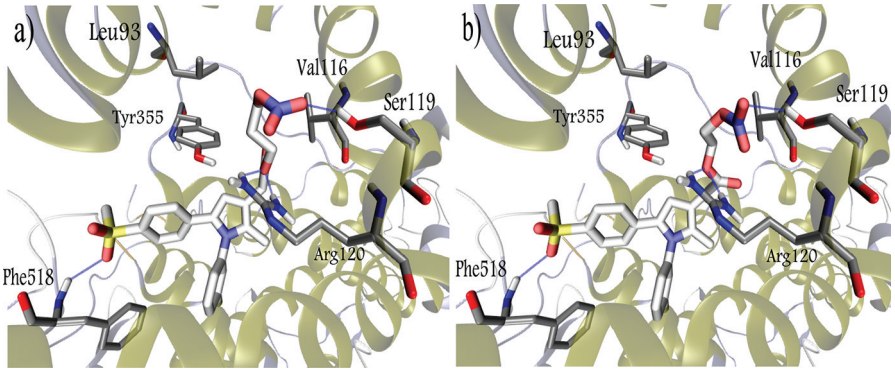

Figure 5. (a) Nitrooxypropyl ester **11d** and (b) nitrooxyethyl ester **11c** docked into the COX-2 cyclooxygenase site. The main residues and the inhibitors are represented as licorice. Hydrogen bonds are represented as blue dotted lines. For the sake of clarity, only a few amino acids of the binding site are displayed.

residues. The oxygen atoms of the sulfone groups show hydrogen bond contacts with the terminal NH_2 group of Gln192 and with the backbone NH group of Phe518, while the aromatic ring interacts with the side chain of hydrophobic amino acids, such as Leu352 and Val523.

Not surprisingly, the docking calculations reported herein were not able to explain the different COX-2 inhibitory activity of compounds **11c** and **11d**. More detailed and time-consuming metadynamics simulations could potentially reveal a possible alternative binding mode of these inhibitors within COX-2, as it was very recently reported by Limongelli et al.[38] who by means of a metadynamic-based approach illuminated the highly dynamical character of selective 1,5-diarylpyrrole derivatives/COX-2 recognition process.

In addition, as for compound **11c**, the energy difference (Δ_E = −0.4059 hartree) between the docked conformation (see Figure 5b) and the conformation resulting from single crystal X-ray diffraction (see Supporting Information.) provides further support to the binding mode herein proposed.

CONCLUSIONS

The preclinical evaluation of a new class of pyrrole-derived compounds displayed satisfactory profiles. Nitrooxy derivatives, acting as hybrid molecules, conjugate a COX-2 inhibiting feature with a NO releasing moiety. In vitro tests highlighted a profound inhibition of COX-2 isozyme both in rats and in humans. Their vasorelaxing properties are dependent on NO release. Moreover, it was shown that compounds **11c**, **11d**, **12c**, and **12d** are endowed with good anti-inflammatory and antinociceptive activities. Nevertheless, it still remains to be addressed whether the NO-releasing property of this new class of compounds would translate into inhibition of platelet function, thus counteracting the possible CV hazard related to COX-2-dependent prostacyclin inhibition.

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EXPERIMENTAL SECTION

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 the Stetter and Paal–Knorr reactions. Melting points were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. Microanalyses were performed on a Perkin-Elmer 240C or a Perkin-Elmer series II CHNS/O analyzer 2400 instrument. 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 ^{1}H 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.

HPLC Analysis. 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 and working at 333 nm. Millennium Empower with Windows XP was used. A Phenomenex LUNA C8, 5 μm (150 mm × 4.6 mm) column (code 00F-4249-E0) at 40 °C was used as the chromatographic column at a flow rate of 1.0 mL/min. The eluent was (A) a 65/15/20 (v/v/v) 10 mM (pH 2.5) KH_2PO_4/MeOH/acetonitrile mixture or (B) a 15/75/10 (v/v/v) 10 mM (pH 2.5) KH_2PO_4/MeOH/acetonitrile mixture. A gradient from 100% A to 100% B was run in 25 min, and then isocratic conditions at 100% B were maintained for an additional 10 min. Using the analytical conditions reported above, the retention times of the compounds range between 7.44 and 17.25 min. All the synthesized compounds were generally more than 95% pure.

1-[4-(Methylthio)phenyl]pentane-1,4-dione (5). 4-Methylthiobenzaldehyde (11.97 mL, 90 mmol), triethylamine (19.5 mL, 600 mmol), methyl vinyl ketone (5.8 mL, 90 mmol), and 3-ethyl-5-(2-hydroxyethyl)-4-methylthiazolium bromide (14 mmol) were put into a round-bottomed flask equipped with a stir bar. The flask was inserted into the cavity of the microwave system apparatus, and the reaction was run for 15 min (150 W, 70 °C, 170 psi).[27] The residue obtained was treated with 2 N HCl (10 mL) until pH 2 was reached. After the extraction with ethyl acetate, the organic layer was washed with aqueous sodium bicarbonate and water. The organic fractions were dried over Na_2SO_4, filtered, and concentrated to give a crude orange liquid. After crystallization from cyclohexane, intermediate **5** was isolated as white needles (78% yield). Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.[25−31]

1-[4-(Methylsulfonyl)phenyl]pentane-1,4-dione (6). To a solution of **5** (35 mmol) in methanol (250 mL), a solution of Oxone (61.4 mmol) in water (150 mL) was added over a period 15 min. The reaction mixture was stirred for 2 h at room temperature, then diluted with water (400 mL) and extracted with dichloromethane. The organic layer was washed with brine (200 mL), water (200 mL) and dried over Na_2SO_4. After filtration and concentration under reduced pressure, compound **6** was obtained as a white solid (>92% yield). Physicochemical, spectroscopic, and analytical data were consistent with those reported in the literature.[25−31]

General Procedure for the Preparation of 1,5-Diarylpyrroles (7a–e). Following the Paal–Knorr condensation conditions, compound **6** (2.28 mmol) was dissolved in ethanol (2 mL) in a round-bottomed flask equipped with a stir bar. Corresponding aniline (2.28 mmol) and *p*-toluenesulfonic acid (0.17 mmol) were added. The flask was inserted into the cavity of the microwave system apparatus and heated (150 W for 45 min, temperature 160 °C, pressure 150 psi).[27] The reaction mixture was cooled to room temperature and subsequently concentrated. The crude material was purified by chromatography on aluminum oxide using cyclohexane/ethyl acetate, 3:1 (v/v), as the eluent to give the expected 1,5-diarylpyrroles **7a–e** as solids in satisfactory yields.

1-[(4-Methylthio)phenyl]-2-methyl-5-[4-(methylsulfonyl)-phenyl]-1*H*-pyrrole (7d). Yellowish needles, mp 105–110 °C (yield 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.60 (d, 2H, J = 8.6 Hz), 7.26 (d, 2H, J = 8.5 Hz), 7.17 (d, 2H, J = 8.6 Hz), 6.98 (d, 2H, J = 8.5 Hz), 6.39 (m, 1H), 6.11 (m, 1H), 3.01 (s, 3H), 2.51 (s, 3H), 2.06 (s, 3H). MS-ESI: m/z 380 (M + Na^+).

General Procedure for the Preparation of 1,5-Diarylpyrrol-3-glyoxylic Esters (8a–e). To a solution of the appropriate pyrrole [**7a–e** (4.82 mmol)] in dry dichloromethane (5 mL) at 0 °C under nitrogen atmosphere, ethoxalyl chloride (4.82 mmol) and $TiCl_4$ (4.82 mmol) were added in sequence. The solution was stirred for 4 h at room temperature and then quenched with water. The mixture was extracted with chloroform, and the organic layer was washed with brine, dried, and evaporated in vacuo. Purification of the residue by chromatography on silica gel employing petroleum ether/chloroform/ethyl acetate, 4:3:1 (v/v/v), as the eluent gave a solid which, after recrystallization from hexane, afforded the expected product.

Ethyl 2-[1-[(4-Methylthio)phenyl]-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]glyoxylate (8d). Yellowish needles, mp 105–110 °C (yield 77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.60 (d, 2H, J = 8.6 Hz), 7.25 (d, 2H, J = 8.5 Hz), 7.17 (d, 2H, J = 8.6 Hz), 7.03–6.99 (m, 3H,), 4.10 (q, 2H, J = 7.0 Hz), 3.00 (s, 3H), 2.54 (s, 3H), 2.09 (s, 3H), 1.55 (t, 3H, J = 7.0 Hz). MS-ESI: m/z 480 (M + Na^+).

General Procedure for the Preparation of Ethyl 1,5-Diarylpyrrole-3-acetic Esters (9a–e). To a solution of the suitable 1,5-diarylpyrrol-3-glyoxylic ester (**8a–e**) (2.3 mmol) in TFA (9 mL, 0.12 mol), stirred at 0 °C under nitrogen atmosphere, triethylsilane (0.75 mL, 4.7 mmol) was slowly added, and the mixture was reacted for 2 h at room temperature. Then the mixture was made alkaline (pH 12) with 40% aqueous ammonia and extracted with chloroform. The organic solution was dried over Na_2SO_4, filtered, and evaporated in vacuo. The resulting residue was purified by chromatography on silica gel using petroleum ether/chloroform/ethyl acetate, 4:3:1 (v/v/v), as the eluent to give a solid which, after recrystallization from ethanol, afforded the desired product.

Ethyl 2-[1-[(4-Methylthio)phenyl]-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]acetate (9d). Yellowish needles, mp 123–127 °C (yield 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.60 (d, 2H, J = 8.6 Hz), 7.25 (d, 2H, J = 8.5 Hz), 7.17 (d, 2H, J = 8.6 Hz), 6.99 (d, 2H, J = 8.5 Hz), 6.46 (s, 1H), 4.10 (q, 2H, J = 7.0 Hz), 3.51 (s, 2H), 3.00 (s, 3H), 2.54 (s, 3H), 2.09 (s, 3H), 1.55 (t, 3H, J = 7.0 Hz). MS-ESI: m/z 466 (M + Na^+).

General Procedure for the Preparation of 1,5-Diarylpyrrole-3-acetic Acids (10a–e). To a solution of the appropriate ethyl 1,5-diarylpyrrole-3-acetic ester (**9a–e**) (0.67 mmol) in methanol (4.83 mL) was added 4.83 mL of 1 N NaOH. The resulting mixture was refluxed for 1.5 h, cooled, and concentrated in vacuo. The residue was solubilized in water and then acidified with concentrated HCl. The precipitate was filtered off to give the expected acid as a white solid.

2-[1-[(4-Methylthio)phenyl]-2-methyl-5-[4-(methylsulfonyl)-phenyl]-1*H*-pyrrol-3yl]acetic Acid (10d). White needles, mp 147–152 °C (yield >95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 11.00 (br s, 1H), 7.60 (d, 2H, J = 8.6 Hz), 7.25 (d, 2H, J = 8.5 Hz), 7.17 (d, 2H, J = 8.6 Hz), 7.00 (d, 2H, J = 8.5 Hz), 6.49 (s, 1H), 3.02 (s, 3H), 2.55 (s, 3H), 2.10 (s, 3H). MS-ESI: m/z 416 (M + H^+).

General Procedure for the Preparation of 1,5-Diarylpyrrole-3-acetic Nitroxyalkyl and Hydroxylalkyl Esters (11a–j, 12a–j). To a solution of the opportune 1,5-diarylpyrrole-3-acetic acid **10a–e** (0.1 mmol) in dichloromethane (5 mL), under nitrogen atmosphere, an excess of alcohol [(**13a–c**, **14a–c**) 0.3 mmol], DMAP (0.1 mmol), and EDCI (0.2 mmol) were added in sequence. The reaction was quenched with water after 3 h and extracted with chloroform. The organic layer was washed with 1 N HCl, $NaHCO_3$ saturated solution, brine and dried over Na_2SO_4. The filtration and concentration of the organic phase gave a crude material, which was purified by chromatography on silica gel using petroleum ether/chloroform/ethyl acetate, 4:4:1 (v/v/v), as the eluent to give the desired product in good yield.

2-(Nitroxy)ethyl 2-[1-[(4-Methylthio)phenyl]-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3yl]acetate (11g). Yellow

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powder, mp 118−123 °C (yield 70%). FT-IR: 1738 cm^{-1} (νC−O), 1280 cm^{-1} (ν_{as}C−O−C), 1144 cm^{-1} (ν_{s}C−O−C), 1625 cm^{-1} (ν_{as}O-NO_2), 843 cm^{-1} (ν_{s}O-NO_2). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.80 (d, 2H, J = 8.6 Hz), 7.24 (d, 2H, J = 8.5 Hz), 7.17 (d, 2H, J = 8.6 Hz), 6.99 (d, 2H, J = 8.5 Hz), 6.51 (s, 1H), 4.73 (t, 2H, J = 6.3 Hz), 4.27 (m, 2H, J = 6.3 Hz), 3.50 (s, 2H), 3.00 (s, 3H), 2.55 (s, 2H), 2.10 (s, 3H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 10.89 (CH_3 pyrrolic), 29.79 (CH_3−S), 32.21 (CH_2), 44.60 (CH_3 sulfonyl), 60.41 (OCH_2), 69.90 (CH_2ONO_2), 112.96, 113.50, 115.89, 120.67, 125.39, 127.00, 130.59, 130.70, 131.30, 131.80, 136.10, 138.00, 177.03 (COO). MS-ESI: m/z 505 (M + H^+). HPLC: >98% pure (t_R = 14.19 min).

2-Hydroxyethyl 2-[1-(4-Methylthiophenyl)-2-methyl-5-[4-(methylsulfonyl)phenyl]-1*H*-pyrrol-3-yl]acetate (12g). Yellow powder, mp 110−112 °C (yield 55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.80 (d, 2H, J = 8.6 Hz), 7.24 (d, 2H, J = 8.5 Hz), 7.17 (d, 2H, J = 8.6 Hz), 7.00 (d, 2H, J = 8.5 Hz), 6.48 (s, 1H), 4.25 (t, 2H, J = 6.8 Hz), 3.89 (t, 2H, J = 6.8 Hz), 3.52 (s, 2H), 2.55 (s, 3H), 2.07 (s, 3H), 2.01 (br s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 10.89 (CH_3 pyrrolic), 29.79 (CH_3−S), 32.20 (CH_2), 44.54 (CH_3 sulfonyl), 60.40 (OCH_2), 66.29 (CH_2OH), 112.96, 113.50, 115.90, 120.67, 125.39, 127.01, 130.63, 130.71, 131.27, 131.80, 136.10, 138.00, 177.00 (COO). MS-ESI: m/z 460 (M + H^+). HPLC: >97% pure (t_R = 9.32 min.)

General Procedure for the Preparation of Hydroxylalkyl Nitrates (14a,b). To a solution of diol (**13a,b**, 20 mmol) in ethyl acetate (100 mL) were added nitric acid (65% w/v, 16 mmol), glacial acetic acid (97 mmol), and acetic anhydride (97% w/v, 59 mmol). The mixture was allowed to react overnight. The reaction was quenched with 20% KOH solution. The pH was adjusted to 12−13, and the acqueous phase was extracted with ethyl acetate (3 × 100 mL), washed with NaCl saturated solution, and dried over Na_2SO_4. After filtration, the organic layers were concentrated in vacuo, and the crude product was purified by chromatography on silica gel, using petroleum ether/chloroform/ethyl acetate, 3:1:1 (v/v/v), as eluent, to obtain the expected nitroester in good yield.

2-Nitroxyethanol (14a). Yellowish oil (yield 70%). FT-IR: 1623 cm^{-1} (ν_{as}O-NO_2), 850 cm^{-1} (ν_{s}O-NO_2).^{1}H NMR: 4.67 (t, 2H, J = 6.9 Hz), 4.20 (t, 2H, J = 6.9 Hz), 2.10 (br s, 1H). MS-ESI: m/z 108 (M + H^+).

Pharmacology. *In Vitro Anti-Inflammatory Study.* The in vitro profiles of compounds **11a−j** and **12a−j**, related to their inhibitory activity toward 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 μg/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, the culture medium was replaced with fresh medium and cells were stimulated as described previously.[39] The evaluation of COX-1 inhibitory activity was achieved by pretreating 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 also treated 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 μg/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 GraphPad Instat, and the data fit was performed using the sigmoidal dose−response equation (variable slope) (GraphPad).

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 anesthesia, 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. The 5 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 to a computerized 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 of <10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation of ≥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). When the contraction reached a stable plateau, 3-fold increasing concentrations of compounds (1nM to 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: 1 μM ODQ 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 30 mM KCl. When the limit concentration of 10 μM (the highest concentration that could be administered) of the tested compounds did not produce the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage of the contractile tone induced by 30 mM KCl, evoked by this limit concentration. The parameter of potency was expressed as pIC_{50}, calculated as the negative logarithm of the molar concentration of the tested compounds evoking a half reduction of the contractile tone induced by 30 mM KCl. 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 the mean ± standard error for 6−10 experiments. Two-way ANOVA was used for statistical analysis, and P < 0.05 was considered representative of significant statistical differences. Experimental data were analyzed by a computer fitting procedure (software: GraphPad Prism, version 4.0).

In Vitro Human Whole Blood (HWB) Assay. Compounds **11c** and **12c** were also evaluated for COX-1 versus COX-2 selectivity in HWB assay. Three healthy volunteers (two females and one male, aged 29 ± 3 years) were enrolled to participate in the study after its approval by the Ethical Committee of the University of Chieti, Italy. Informed consent was obtained from each subject. Compounds **11c** (0.05−50 mM) and **12c** (0.05−50 mM) were dissolved in DMSO. Aliquots of the solutions (2 μL) or vehicle were pipetted directly into test tubes to give final concentrations of 0.1−100 μM 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 μg/mL) or saline for 24 h at 37 °C, as previously described.[40] 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 PGE_2 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 assayed for TXB_2. Whole blood TXB_2 production was measured as a reflection of maximally platelet COX-1 activity in response to endogenously formed thrombin.[41]

Analysis of PGE_2 and TXB_2. PGE_2 and TXB_2 concentrations were measured by previously described and validated radioimmunoassays.[40,41] Unextracted plasma and serum samples were diluted in the standard diluent of the assay (0.02 M phosphate buffer, pH 7.4) and assayed in a volume of 1.5 mL at a final dilution of 1:50 to 1:30000. [^{3}H]PGE_2 or [^{3}H]TBX_2 (3000 cpm, specific activity of >100 Ci/mmol, 1:100000 dilution) and anti-TBX_2 (1:120000 dilution) sera were used. The least detectable concentration was 1−2 pg/mL for both prostanoids.[40,41]

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. The carrageenan-induced paw edema 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 intraperitoneal (ip). The number of stretching movements was counted for 10 min, starting 5 min after administration.

Computational Details. *Inhibitor Setup.* The structures of the inhibitors used in the docking simulations were generated by means of Extensible Computational Chemistry Environment (ECCE) software[42] and then geometry optimized by means of NWChem (100 steps of RHF, 6-31G*). Partial atomic charges RESP were calculated by means of the NWChem and then used in the following docking simulations.[43] All the relevant torsion angles were treated as rotatable during the docking process, thus allowing a search of the conformational space.

Enzyme Setup. The COX-2 protein was set up for docking as follows: polar hydrogens were added by means of ECCE software, and Kollman united-atom partial charges were assigned.[44] The ADDSOL utility of AutoDock was used to add solvation parameters to the protein structures, and the grid maps representing the proteins in the docking process were calculated using AutoGrid. The grids, one for each atom type in the inhibitor plus one for the electrostatic interactions, were chosen to be large enough to include not only the cyclooxygenase sites but also a significant part of the protein around it. As a consequence, the dimensions of grid maps were 59 × 45 × 59 points with a grid point spacing of 0.375 Å for COX-2 for all docking calculations.

Docking Calculations. Compounds **11c,d** and **12c,d** were docked into the enzymes using AutoDock, version 4.0. Docking simulations of the compounds were carried out using the Lamarckian genetic algorithm and through a protocol with an initial population of 300 randomly placed individuals, a maximum number of 25 million energy evaluations, a mutation rate of 0.02, a crossover rate of 0.80, and an elitism value of 1. The pseudo Solis and Wets algorithm with a maximum of 300 interactions was applied for the local search. Two-hundred independent docking runs were carried out for each inhibitor, and the resulting conformations that differed by less than 2.0 Å in positional root-mean-square deviation (rmsd) were clustered together. Cluster analysis was performed by selecting the most populated cluster, which in all cases coincided with the one endowed with the best energy.

■ ASSOCIATED CONTENT

Supporting Information

Physicochemical, spectroscopic, and analytical data of compounds along with single crystal X-ray diffraction results of compound **11c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

t-NSAID, traditional nonsteroidal anti-inflammatory drug; COXIB, cyclooxygenase-2 inhibitor; COX, cyclooxygenase; GI, gastrointestinal; NO, nitric oxide; AA, arachidonic acid; GC, guanylate cyclase; c-GMP, cyclic guanosine monophosphate; CV, cardiovascular; CINOD, cyclooxygenase-inhibiting nitric oxide donor; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMAP, dimethylaminopyridine; Ach, acetylcholine; DMEM, Dulbecco's modified Eagle's medium; PGE_2, prostaglandin E_2; FBS, fetal bovine serum; LPS, lipopolysaccharide; RIA, radioimmunoassay; TX, thromboxane; CMC, carboxymethylcellulose; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; ipl, intraplantar:; po, per os; ip, intraperitoneal; GTN, glyceryl trinitrate; E_{max}, maximal vasorelaxing response

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