Synthesis, Biological Evaluation, and Enzyme Docking Simulations of 1,5-Diarylpyrrole-3-Alkoxyethyl Ethers as Selective Cyclooxygenase-2 Inhibitors Endowed with Anti-inflammatory and Antinociceptive Activity

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A series of substituted 1,5-diarylpyrrole-3-alkoxyethyl ethers (**6**, **7**, and **8**) has been synthesized with the aim to assess if in the previously reported 1,5-diarylpyrrole derivatives (**5**) the replacement of the acetic ester moiety with an alkoxyethyl group still led to new, highly selective and potent COX-2 inhibitors. In the in vitro cell culture assay, all the compounds proved to be potent and selective COX-2 inhibitors. In the human whole blood (HWB) assay, compound **8a** had a comparable COX-2 selectivity to valdecoxib, while it was more selective than celecoxib but less selective than rofecoxib. The potential anti-inflammatory and antinociceptive activities of compounds **7a**, **8a**, and **8d** were evaluated in vivo, where they showed a very good activity against both carrageenan-induced hyperalgesia and edema in the rat paw test. In the abdominal constriction test compound **7a**, **8a**, and **8d** were able to reduce the number of writhes in a statistically significant manner. Furthermore, the affinity data of these compounds have been rationalized through enzyme docking simulations in terms of interactions with a crystallographic model of the COX-2 binding site by means of the software package Autodock 3.0.5, GRID 21, and MacroModel 8.5 using the complex between COX-2 and SC-558 (**1b**), refined at a 3 Å resolution (Brookhaven Protein Data Bank entry: 6cox)

Introduction

Cyclooxygenase [(prostaglandin (PG[a]) H synthase] catalyzes the oxygenation of arachidonic acid (AA) to prostaglandin H_2 as a first step in the synthesis of PGs, prostacyclin and thromboxane (TX)A_2.[1,2] The enzyme is expressed in mammalian cells as two distinct isozymes (named COX-1 and COX-2) that show about 60% amino acid sequence. COX-1 and COX-2 share the same catalytic activities, i.e., cyclooxygenase and peroxidase,[1] but they are differently regulated. In fact, it has been shown that COX-2 requires considerably lower levels of hydroperoxides to initiate cyclooxygenase catalysis than those required by COX-1.[2] Moreover, COX-1 seems to prefer exogenous AA, while COX-2 prefers endogenous AA.[3] Finally, COX-2 oxygenates neutral fatty acid derivatives such as endocannabinoids, which are poor substrates for COX-1.[4]

COX-1 is the major form expressed in healthy tissues and plays a role in thrombogenesis and in the homeostasis of the gastrointestinal tract.[5] COX-2 is induced in many cell types in response to bacterial endotoxin, cytokines, including IL-1, TNFα, and growth factors, and is down-regulated by dexamethasone.[5] Both enzymes are inhibited by traditional (t)nonsteroidal anti-inflammatory drugs (NSAIDs).[5–7] Inhibition of COX-2 is involved in the therapeutic effects of (t)NSAIDs, while the inhibition of COX-1 is associated with gastrointestinal toxicity.[7,8]

The identification of the selective COX-2 inhibitory prototype "sulide" compound NS-398, and the "coxib" compound DuP 697, the first diarylheterocycle, served as the rationale for the original concept that a drug having a greater selectivity for the proinflammatory inducible COX-2 isozyme, relative to the cytoprotective constitutive COX-1 isozyme, would reduce the incidence of gastrointestinal bleeding. Subsequent drug discovery programs led to the clinical development of diarylheterocycles as the major class of selective COX-2 inhibitors such as celecoxib (**1a**), rofecoxib (**2**), and valdecoxib (**3**) (Chart 1).[9]

The gastrointestinal benefit of coxibs is obliterated by the finding of increased incidence of vascular events.[10] However, the results of observational studies and randomized clinical trials have shown that the cardiovascular hazard is not restricted to selective COX-2 inhibitors but also attached to some (t)NSAIDs, such as diclofenac.[11,12] The most plausible mechanism is the suppression of COX-2-dependent prostacyclin, leaving uncon-

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[a] Abbreviations: PG, prostaglandin; AA, arachidonic acid, TX, thromboxane; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; IL-1, interleukin-1, TNFα, tumor necrosis factor-α; (t)NSAIDs, traditional nonsteroidal anti-inflammatory drugs; HWB, human whole blood.

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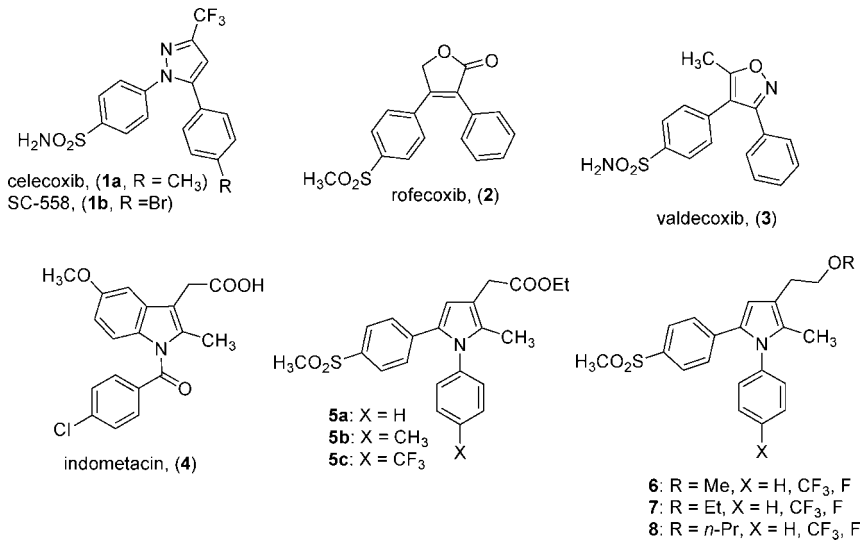
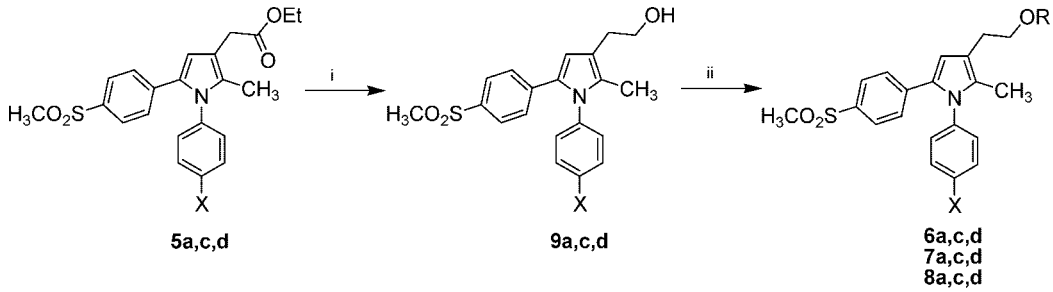
Chart 1. Structures of Compounds **1**−**8**



Scheme 1[a]



[a] Compounds: **6a**, X = H, R = Me; **7a**, X = H, R = Et; **8a**, X = H, R = *n*-Pr; **6c**, X = CF_3, R = Me; **7c**, X = CF_3, R = Et; **8c**, X = CF_3, R = *n*-Pr;**6d**, X = F, R = Me; **7d**, X = F, R = Et; **8d**, X = F, R = *n*-Pr. Reagents and conditions: (i) $LiAlH_4$, THF, room temp, 20 min; (ii) KOH, RI, DMSO, room temp, 30 min.

strained the intricate network of stimuli predisposing to thrombosis, atherogenesis, and hypertension, such as TXA_2.[10,12] The finding of a marked variability in how each person reacts to these drugs, mainly based on their genetic background,[13] encourages the development of novel compounds to increase the spectrum of therapeutic opportunities for each individual patient.[14]

Within a wide research program focused on the synthesis of new 3-substituted-1,5-diarylpyrrole derivatives **5**[15–17] as selective COX-2 inhibitors in which the pyrroleacetic and vicinal diaryl heterocyclic moieties were reminiscent of indomethacin (**4**) and of the above "coxib" family, respectively, the synthesis, the biological evaluation, and enzyme docking simulations of the hitherto unknown 1,5-diarylpyrrole-3-alkoxyethyl ethers **6**, **7**, and **8** are here reported.

Compounds **7a**, **8a**, and **8d**, with the best biological profile in terms of affinity and selectivity toward COX-2, as well as percent inhibition of the enzyme (cell culture assay), were also investigated for their in vivo anti-inflammatory and analgesic activity. Moreover, the selectivity of compound **8a** was also determined by the human whole blood assay (HWB) in vitro, which assesses the pattern of relative inhibition for human platelet COX-1 and monocyte COX-2.[17]

Results and Discussion

Scheme 1 summarizes the synthetic procedures used to obtain the 1,5-diaryl-3-(2-alkoxyethyl)pyrroles considered in this study. The target compounds were synthesized starting from appropriate ethyl acetate ester **5** prepared as previously reported.[15] By treatment with lithium aluminum hydride in dry THF at room temperature, compounds **5** were reduced to the respective alcohol derivatives **9**. These compounds were alkylated by the suitable alkyl iodide in the presence of powdered KOH in dimethylsulfoxide (DMSO), to afford the expected 1,5-diaryl-3-(2-alkoxyethyl)pyrroles **6**, **7**, and **8** in satisfactory yield.

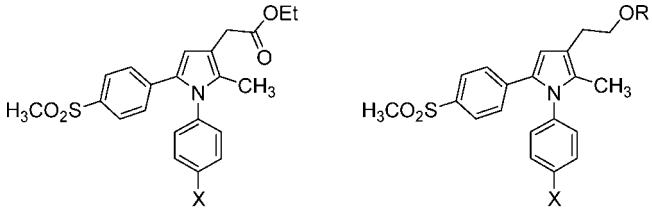
In the in vitro cell culture assay, biological evaluation of the title compounds showed that, in 1,5-diarylpyrrole derivatives **5**, the replacement of the acetic ester moiety with an alkoxyethyl group still led to new, highly selective, and potent COX-2 inhibitors, the activities of which are presented in Table 1.

The transformation of the ester moiety at position 3 of the pyrrole scaffold into an ether substituent produces a significant difference in terms of inhibitory activity. Actually, compounds **6**, **7**, and **8** are in general more potent than the corresponding previously reported esters **5**.[15]

At a first glance, these data seem to be surprising if we take into consideration the different stereoelectronic properties of the acetic ester chain with respect to the alkoxyethyl moiety and in view of their mode of interaction with one of the pockets of the COX-2 binding site.

In fact, as it has previously been reported,[15] the ester chain of compounds **5a**−**c** is clearly involved with its oxygen atoms in a network of three hydrogen bonds with the guanidino group of Arg120, in a portion of the COX-2 binding site referred to as the "classical NSAIDs carboxylate binding site" by Kurumbail and co-workers.[18] On the other hand, it is less clear how

Table 1. In Vitro COX-1 and COX-2 Inhibitory Activity of Compounds **5**, **6**, **7**, and **8**



5 **6, 7, 8**

compd	X	R	COX-1 IC_{50} (μM)[a]	COX-2 IC_{50} (μM)[a]	COX-1/COX-2 (SI)[b]
5a[c]	H		>100	0.040	>2500
5b[c]	CH_3		>100	0.480	>208
5c[c]	CF_3		>100	0.060	>1600
6a	H	Me	>100	0.048	>2083
6c	CF_3	Me	>100	0.049	>2040
6d	F	Me	>100	0.018	>5555
7a	H	Et	>100	0.015	>6666
7c	CF_3	Et	>100	0.085	>1176
7d	F	Et	>100	0.047	>2127
8a	H	*n*-Pr	>100	0.018	>5555
8c	CF_3	*n*-Pr	>100	0.110	>909
8d	F	*n*-Pr	>100	0.030	>3333

[a] Results are expressed as the mean of three experiments of the % inhibition of PGE_2 production by the test compounds with respect to control samples. [b] In vitro COX-2 selectivity index [IC_{50}(COX-1)/IC_{50}(COX-2)]. [c] See ref 15.

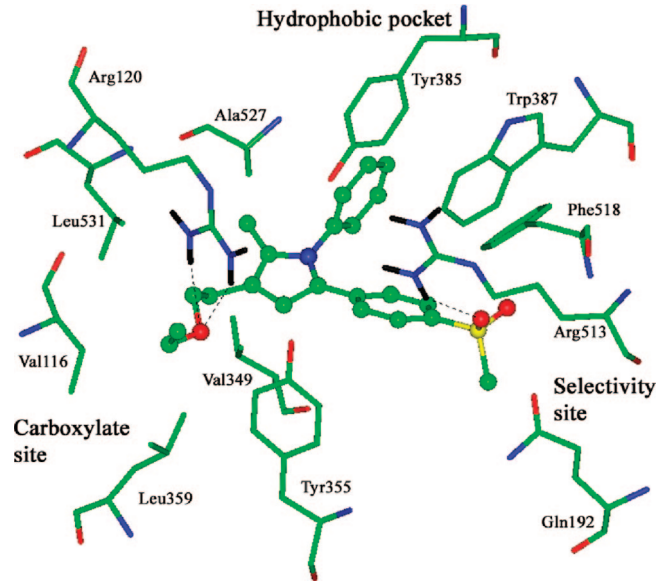


Figure 1. Graphical representation of the best pose found for compound **7a** (ball and stick notation) within the binding site of COX-2. Hydrogen bond interactions (involving the ether oxygen atom and one of the sulfone oxygens) are coded by black dashed lines. For the sake of clarity, only a few amino acids of the binding site are displayed.

the alkoxyethyl derivatives **6**, **7**, and **8,** even in the absence of the carbonyl group, are able to exploit profitable interactions, eliciting, in the in vitro using the cell culture test, a highly selective and potent COX-2 inhibitory activity. In particular, compounds **7a** and **8a** showed IC_{50} values of 0.015 and 0.018 μM, respectively, (Table 1) which are comparable to the IC_{50} of rofecoxib (0.012 μM, ref 19). This behavior may be probably due to the presence of an oxygen atom in the side chain along with the more lipophilic character of the alkoxyethyl moiety capable of tighter interactions with the hydrophobic environment of the COX-2 "carboxylate binding site". In fact, the binding mode of such compounds was characterized by the ether group located within the carboxylate site with its oxygen atom involved in hydrogen bonds with the guanidino moiety of Arg120 (Figure 1). Moreover, the alkyl portion of the ether side chain was embedded into a lipophilic region defined by Leu93, Val116, and Leu359, where the best interaction point with a C3 probe was found by means of GRID software. In fact, GRID calculations using a C3 probe, intended to analyze hydrophobic interactions between a methyl group (the probe) and the surface of COX-2,[15] found a region of very profitable interactions between the probe and the protein close to the location of the terminal methyl group of the ethyl ether chain.

Significant differences in inhibitory activity are observed when the substitution pattern is considered (e.g., the presence of alkoxy or the 4′-phenyl substituent at N1 position of the pyrrole ring or the contemporary presence of both moieties). In fact, compounds **7a** and **8a** appear to be the most active compounds in the in vitro and in vivo tests. This means that the contemporary presence of an ethoxy or a propoxy group and that of the unsubstituted 1-phenyl ring represents the best requisite in order to fit both the "carboxylate binding site" and the hydrophobic binding site.

On the contrary, in the presence of a 4′-trifluoromethyl or a 4′-fluoro substituent at 1-phenyl ring as in compounds **6c**,**d**, **7c**,**d**, and **8c**,**d**, the COX-2 inhibitory activity slightly decreases with the increase in the side chain length, with the exception of **8d**, which shows an activity (0.030 μM) higher than that of the corresponding ethyl ether analogue **7d** (0.047 μM). Interestingly, the less bulky ether in the subseries of the 4′-fluoro derivatives (compound **6d**) shows an IC_{50} of 0.018 μM. Moreover, activity of fluoro derivatives **6d**, **7d**, and **8d** was better than that of the corresponding CF_3 analogues **6a**, **7a**, and **8a**, respectively. This result appears to be in full agreement with the analysis of the Grid map generated for the fluoride probe that suggested for the 4′-fluorine substituent (as it occurs in the case of the bromine atom of SC-558, **1b**) an improving effect on the interaction with the COX-2 binding site, involving Trp387, Tyr348, and Tyr385 of the hydrophobic cavity[15] with respect to the CF_3 substituent.

Finally, the 1-aryl substituent showed the usual interaction pathway with the hydrophobic pocket of protein, as well as the aryl moiety at C5 that is in contact with the selectivity site with both hydrophobic interactions and hydrogen bonds.

On the basis of their very encouraging COX-2 inhibitory activity evidenced in the in vitro tests, compounds **7a**, **8a**, and **8d** were selected and submitted to further pharmacological tests to assess their in vivo anti-inflammatory and antinociceptive activities by carrying out the carrageenan-induced rat paw test, the paw volume test and abdominal constriction test (Tables 2 and 3).

These compounds, administered at a dose of 20 mg/kg po, showed a very good activity against carrageenan-induced hyperalgesia 30 and 60 min after administration, disappearing almost completely 2 h after treatment (Table 2). At the same time, a very good activity was demonstrated against carrageenan-induced edema in the rat paw (Table 2), with a complete remission 1 h after the administration of all compounds at the same dose of 20 mg/kg po. In the abdominal constriction test compounds **7a**, **8a**, and **8d** were able to reduce the number of writhes in a statistically significant manner at the dose of 20 mg/kg po, while at the dose of 10 mg/kg po, they were devoid of any antinociceptive efficacy (Table 3).

Among the title compounds, **8a** was selected and submitted to a further biochemical selectivity test. In particular, the HWB assay was performed to predict the actual extent of isozyme

Table 2. Effect of Compounds **7a**, **8a**, and **8d** on Hyperalgesia and Edema Induced by Carrageenan in the Rat Paw-Pressure Test[a]

		paw pressure in rats (g)				paw volume (mL)	
			after treatment				
pre-treatment		before treatment	30 min	60 min	120 min	before treatment	60 min after treatment
saline	saline	63.5 ± 4.7	60.2 ± 5.0	58.9 ± 6.3	61.4 ± 5.1	1.29 ± 0.08	1.34 ± 0.10
carrageenan	saline	62.6 ± 4.3	36.4 ± 5.2	38.4 ± 5.2	41.2 ± 4.8	1.25 ± 0.09	2.48 ± 0.07
carrageenan	**7a**	60.5 ± 4.3	59.6 ± 4.7[b]	53.8 ± 4.2[b]	49.1 ± 5.4	1.29 ± 0.10	1.37 ± 0.11[b]
carrageenan	**8a**	59.2 ± 5.2	56.3 ± 5.6[b]	59.4 ± 5.1[b]	50.6 ± 5.3	1.32 ± 0.06	1.41 ± 0.14[b]
carrageenan	**8d**	63.5 ± 4.3	52.4 ± 5.2[b]	56.8 ± 4.9[b]	52.7 ± 4.0	1.25 ± 0.09	1.45 ± 0.12[b]

[a] All compounds were administered at the dose of 20 mg kg^{-1} po. Carrageenan (100 μL, 1%) was administered ipl. 4 h before test. Test was performed 30 min after the compound injection. There were 5−6 rats per group. [b] $P < 0.01$ versus the corresponding carrageenan-treated rat.

Table 3. Effect of Compounds **7a**, **8a**, and **8d** in the Mouse Abdominal Constriction Test (Acetic Acid 0.6%)

treatment[a]	no. of mice	dose per os mg kg^{-1}	no. of writhes
CMC	20		34.7 ± 3.9
7a	10	10	26.5 ± 3.7
7a	8	20	11.4 ± 3.6[b]
8a	9	10	27.43 ± 4.1
8a	9	20	13.6 ± 4.2[b]
8d	10	10	38.1 ± 3.4
8d	10	20	16.7 ± 3.5[b]

[a] All drugs were administered per os 30 min before test. [b] $P < 0.01$ versus vehicle-treated mice.

inhibition achievable in vivo by circulating drug levels because of a number of variables are potentially able to affect drug−enzyme interaction. In particular, the use of exogenous AA in the assay for COX-1 activity in vitro, in 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 in the enzyme binding site.[19] The results showed that, in HWB assay, compound **8a** was 66-fold more potent toward COX-2 than COX-1 (Figure 2B). Interestingly, it had a comparable COX-2 selectivity to valdecoxib (Figure 2A, COX-1/COX-2 IC_{50} ratio of 52), while it was more selective than celecoxib[15] but less selective than rofecoxib (which was previously shown to be >200-fold more potent toward COX-2).[20] In fact, we have previously found that celecoxib showed a COX-1/COX-2 IC_{50} ratio of 23−29.6 in the HWB assay.[17,20] The lower selectivity showed by compound **8a** in the HWB assay versus the cell culture assay was plausible due to the use of exogenous AA and/or murine COX-isozymes.

Conclusion

A small set of novel 1,5-diarylpyrrole-3-alkoxyethyl ethers has been designed, synthesized, and tested. All of them have proved to be potent and highly selective COX-2 inhibitors in in vitro tests. In particular, compounds **7a** and **8a** appear to be equipotent with rofecoxib. In the HWB, compound **8a** was demonstrated to be as selective as valdecoxib. Enzyme docking simulations demonstrate that the binding mode of such compounds is characterized by the ether group located into the "carboxylate site" with its oxygen atom involved in a hydrogen bond with the guanidino moiety of Arg120. Moreover, the alkyl portion of the ether side chain was embedded into a lipophilic region defined by Leu93, Val116, and Leu359. The potential anti-inflammatory and antinociceptive activities of compounds **7a**, **8a**, and **8d** were evaluated in vivo, where they showed a very good activity against both carrageenan-induced hyperalgesia and carrageenan-induced edema in the rat paw, with a complete remission 1 h after the administration. In the abdominal constriction test compound **7a**, **8a**, and **8d** were able to reduce the number of writhes in a statistically significant manner at the dose of 20 mg/kg po. In conclusion, compounds **6**−**8** can be considered interesting candidates for further preclinical studies.

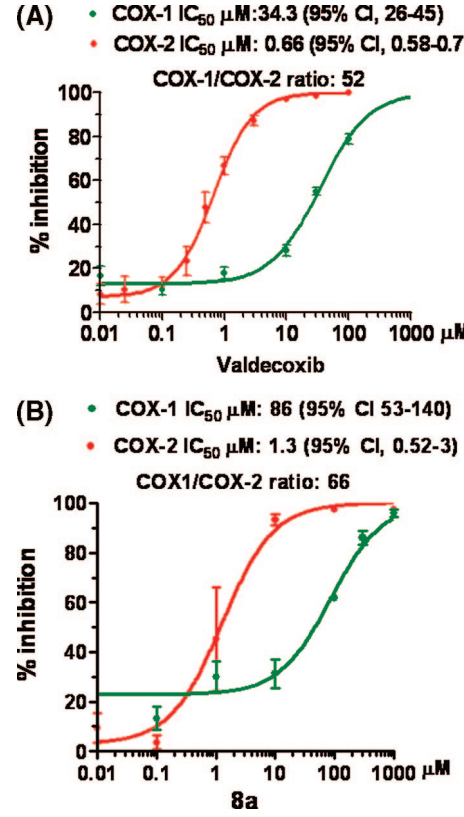


Figure 2. In vitro inhibition (whole blood assay) of COX-1 and COX-2 by valdecoxib (A) and **8a** (B). Results are expressed as average percent inhibition of three donors of prostanoid production assessed in the absence of the test compounds (control). COX-2 selectivity index [IC_{50}(COX-1)/IC_{50}(COX-2)].

Experimental Section

Chemistry. All chemicals used were of reagent grade. Yields refer to purified products and are not optimized. Melting points were determined in open capillaries on a Gallenkamp apparatus and are uncorrected. Microanalyses were carried out by means of Perkin-Elmer 240C or a Perkin-Elmer series II CHNS/O analyzer, model 2400. Merck silica gel 60 (230−400 mesh) was used for column chromatography. Merck TLC plates and silica gel 60 F_{254} were used for TLC. ^{1}H NMR spectra were recorded with a Bruker AC 200 spectrometer in the indicated solvent (TMS as internal standard). The values of the chemical shifts are expressed in ppm, and the coupling constants (*J*) are expressed in Hz. Mass spectra were recorded on a Varian Saturn 3 or a ThermoFinningan LCQ-deca spectrometer.

General Procedure for the Preparation of 1,5-Diaryl-3-(2-hydroxyethyl)pyrroles (9a,c,d). A solution of the suitable ethyl acetate ester **5a**,**c**,**d** (1.3 mmol) in dry THF (5 mL) was added

dropwise to a stirred suspension of lithium aluminum hydride (2.8 mmol) in dry THF (20 mL). After stirring under nitrogen atmosphere for 20 min, the excess of reducing agent was decomposed by careful addition of H_2O (2 mL). The inorganic material was filtered off and washed with THF. The filtrate was dried (Na_2SO_4) and evaporated under reduced pressure. The residue, purified by flash-chromatography (EtOAc/hexane 6:4 v/v), gave the expected compound.

General Procedure for the Preparation of 1,5-Diaryl-3-(2-alkoxyethyl)pyrroles (6a,c,d, 7a,c,d, 8a,c,d). A suspension of powdered KOH (0.7 mmol) in DMSO (2 mL) was stirred for 5 min. The suitable alcohol **9a**,**c**,**d** (0.2 mmol) and alkyl iodide (0.3 mmol) were added in sequence, and the mixture, after being stirred for an additional 30 min, was poured into water and extracted with dichloromethane. The organic extracts were washed with water, dried over Na_2SO_4, and evaporated to dryness in vacuo. The residue, purified by flash chromatography (EtOAc/hexane 4:6 v/v), gave the expected compound as an oil.

Biology. Compounds **6a**−**d**, **7a**−**d**, and **8a**−**d** were all evaluated for their inhibitory activity toward both COX-2 and COX-1 enzymes. Arachidonic acid was obtained from SPIBIO, Paris, France. [^{3}H-PGE_2] was from Perkin-Elmer Life Sciences (Milan, Italy). All other reagents and compounds used were obtained from Sigma-Aldrich, Milan, Italy.

Cellular Assay: Cell Culture. The murine monocyte/macrophage J774 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 25 mM Hepes, penicillin (100 u/mL), streptomycin (100 μg/mL), 10% fetal bovine serum (FBS), and 1.2% Na pyruvate (Bio Whittaker, Europe). Cells were plated in 24-well culture plates at a density of 2.5−105 cells/mL or in 10 cm diameter culture dishes (1−107 cells/10 mL per dish) and allowed to adhere at 37 °C in 5% CO_2/95% O_2 for 2 h. Immediately before the experiments, the culture medium was replaced by a fresh medium without FBS in order to avoid interference with radioimmunoassay,[21] and cells were stimulated as described.

Assessment of COX-1 Activity. The cells were pretreated with the reference standard or the test compounds (0.01−10 μM) for 15 min and incubated at 37 °C for 30 min with 15 μM arachidonic acid in order to activate the constitutive COX.[21] Stock solutions of the reference standard or test compounds were prepared in dimethyl sulfoxide, and an equivalent amount of dimethyl sulfoxide was included in control samples. At the end of the incubation, the supernatants were collected for the measurement of PGE_2 by radioimmunoassay.

Assessment of COX-2 Activity. The cells were stimulated for 24 h with *E. coli* lipopolysaccharide (LPS, 10 μg/mL) to induce COX-2 in the absence or as well as in the presence of the test compounds at the concentrations previously reported. The supernatants were collected for the measurement of PGE_2 by radioimmunoassay.

Human Whole Blood (HWB) Assay. Compound **8a** was also evaluated for COX-1 versus COX-2 selectivity by means of the HWB assay.

Subjects. Three healthy volunteers (2 females and 1 male, 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.

Effects of COX-2 Inhibitors on Whole Blood COX-2 and COX-1 Activities. Valdecoxib (0.005−50 mM) and **8a** (0.5−500 mM) were dissolved in DMSO. Aliquots of the solutions (2 μL) or vehicle were pipetted directly into test tubes to give final concentrations of 0.01−1000 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.[22] 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 LPS-induced monocyte COX-2 activity. 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 stimulated platelet COX-1 activity in response to endogenously formed thrombin.[23]

Analysis of PGE_2 and TXB_2. PGE_2 and TXB_2 concentrations were measured by previously described and validated radioimmunoassays.[22,23] 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 1:50−1:30000. [^{3}H]PGE_2 or [^{3}H]TXB_2 (4000 dpm, specific activity >100 Ci/mmol, 1:100000 dilution) and anti-TXB2 (1:120000 dilution) sera were used. The least detectable concentration was 1−2 pg/mL for both prostanoids[22,23]

Animals. Male Swiss albino mice (23−25 g) and Sprague−Dawley or Wistar rats (150−200 g) were used. Fifteen mice and four rats were housed per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed in 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. All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Paw-Pressure Test. The nociceptive threshold in the rat was determined with an analgesimeter, according to the method described by Leighton et al.[24] Threshold pressure was measured before and 30, 60, and 120 min after treatment. An arbitrary cutoff value of 250 g was adopted. To induce an inflammatory process in the rat, paw carrageenan (0.1 mL, 1%) was administered ipl 4 h before test.

Carrageenan-Induced Edema. Rat paw volumes were measured using a plethysmometer. Four hours after the injection of carrageenan (0.1 mL injection of 1.0%), the paw volume of the right hind paw was measured and compared with saline/carrageenan-treated controls. Rats received test compounds 3 h 30 min after carrageenan. The results are reported as paw volume expressed in mL.

Antinociceptive Assay. Antinociceptive activity was determined by means of a 0.6% acetic acid-induced writhing in the mouse-abdominal constriction test according to Koster.[25] The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Statistical Analysis. Triplicate wells were used for the various conditions of the treatment in the cell culture assay throughout the experiments. Results are expressed as the mean of three experiments, of the % inhibition of PGE_2 production by test compounds with respect to control samples. Data fit was obtained using the sigmoidal dose−response equation (variable slope) (GraphPad software). The IC_{50} values were calculated by the GraphPad Instat program (GraphPad software).

Results from paw-pressure and writhing tests are given as the mean ± SEM; analysis of variance (ANOVA), followed by Fisher's PLSD procedure for post hoc comparison, was used to verify the significance between two means. P values of less than 0.05 were considered significant. The data were analyzed by the StatView for the Macintosh computer program.

Computational Details. All calculations and graphical manipulations were performed on Silicon Graphics computers (Origin 300 server and Octane workstations) using the software package Autodock 3.0.5,[26] GRID 21,[27] and MacroModel 8.5[28] (equipped with an implemented version of the AMBER94 force field kindly provided by Soliva R. and co-workers[29]). The program Autodock was used to evaluate the binding mode of the new inhibitors and to explore their binding conformations within the COX-2 structure. Because Autodock does not perform any structural optimization and energy minimization of the complexes found, a molecular mechanics/energy minimization (MM/EM) approach was applied

to refine the Autodock output. Program GRID was used to map the COX-2 binding site onto a three-dimensional grid and to calculate for each grid point the interaction energy (including contributions from the van der Waals, electrostatic, and hydrogen bond interactions) between a probe and all the protein atoms.

Further details on computational protocols are in Supporting Information.

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Supporting Information Available: Physicochemical, spectroscopic, and analytical data of compounds **6**−**8** along with computational details (Autodock calculations and optimization of the COX-2-inhibitor complexes). This material is available free of charge via the Internet at http://pubs.acs.org.

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