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Enlarging the NSAIDs Family: Ether, Ester and Acid Derivatives of the 1,5-Diarylpyrrole Scaffold as Novel Anti-Inflammatory and Analgesic Agents

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Abstract: The development of the coxib family has represented a stimulating approach in the treatment of inflammatory disorders, such as arthritis, and for the management of acute pains, in relation to the well-known traditional Non-Steroidal Anti-inflammatory Drugs (t-NSAIDs). Prompted by the pursuit for new cyclooxygenase-2 (COX-2) inhibitors, endowed with fine tuned selectivity and high potency, in the past years we have identified novel classes of ether, ester and acid molecules characterized by the 1,5-diarylpyrrole scaffold as potentially powerful anti-inflammatory molecules (**12-66**). All compounds proved to exert an *in vitro* inhibition profile as good as that shown by reference compounds. Compounds bearing a *p*-methylsulfonylphenyl substituent at C5 displayed the best issues. In particular, ester derivatives proved to perform the best *in vitro* profile in terms of selectivity and activity toward COX-2. The cell-based assay data showed that an increase of hindrance at the C3 side chain of compounds could translate to activity enhancement. The human whole blood (HWB) test let to highlight that submitted compounds displayed 5-10 fold higher selectivity for COX-2 vs COX-1 which should translate clinically to an acceptable gastrointestinal safety and mitigate the cardiovascular effects highlighted by highly selective COX-2 inhibitors. Finally, to assess *in vivo* anti-inflammatory and analgesic activity three different tests (rat paw pressure, rat paw oedema and abdominal constriction) were performed. Results showed good *in vivo* anti-inflammatory and analgesic activities. The issues gained with these classes of compounds represent, nowadays, a potent stimulus for a further enlargement of the NSAIDs family. In this review we describe the results obtained by our research group on this topic.

Keywords: COX-2 inhibitors, COX-1 inhibitors, Anti-inflammatory agents, Analgesic agents, Celecoxib, Pyrrole derivatives, Coxibs, *t*-NSAIDs.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are efficacious drugs for the management of musculoskeletal symptoms. They are a chemically heterogenous group of compounds which shares the same mechanism of action, i.e. the inhibition of prostanoids [1]. Prostanoids [prostaglandin(PG)E_2, PGF_{2a}, thromboxane(TX)A_2 and prostacyclin(PGI_2)] are biologically active derivatives of arachidonic acid(AA) generated through the activity of prostaglandin H synthases (popularly known as COX-1 and COX-2) [2]. Although COX-1 and COX-2 share the same catalytic activities, cyclooxygenase and peroxidase [3], they are differently regulated both catalytically and transcriptionally. 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 [4]. Moreover, the COX-2 activity occurs at lower levels of free AA than COX-1 activity [5]. Another important difference between the two pathways of prostanoid biosynthesis is related to the regulation of the expression of COX-1 and COX-2 genes. In fact, while COX-1 has the structural features of an "housekeeping" gene, COX-2 is an immediate early gene [6, 7a-c]. Thus, COX-1 is constitutively expressed in almost all tissues and it is responsible for those physiological functions such as gastrointestinal cytoprotection, platelet aggregation and vascular smooth muscle tone modulation [8, 9]. On the other hand, COX-2, the second isoform, identified as an inducible enzyme, is highly expressed in response to interleukin-1β (IL-1β) and other inflammatory stimuli [9]. However, this strict separation of the COX-1 and COX-2 function, as constitutive and inducible, respectively, is now considered naïve. In fact, in-depth knowledge of COX-isoform biology has shown that COX-1 can be up-regulated in some circumstances (such as cell differentiation) and COX-2 can be constitutively expressed in physiologic conditions, such as in central nervous system, vasculature and kidney [10a-e]. Due to the fact that COX-1 is constitutively expressed in the GI system and that prostanoids generated by this pathway, play several protective actions for this system, it was proposed "the COX-2 hypothesis" which claimed that at comparable COX-2 inhibiting doses, selective COX-2 inhibitors (coxibs) would be as effective as traditional (t) NSAIDs and cause less gastrointestinal adverse effects putatively due to COX-1 inhibition [6, 11]. Thus, several coxibs, structurally unrelated to tNSAIDs (Chart **1**), were developed [ie celecoxib (**6**), SC588 (**7**) rofecoxib (**8**), valdecoxib (**9**), parecoxib (**10**) and later etoricoxib (**11**) and lumiracoxib (**12**)] (Chart **2**) [12a, b].

The development of whole blood assay to assess selectivity towards COX-isozymes by tNSAIDs and coxibs was an essential tool to cluster NSAIDs based on their pharmacodynamics, i.e. their inhibition towards the activity of platelet COX-1 versus that of COX-2 in monocytes described by COX-1/COX-2 IC_{50} ratios (ratio of the concentration which inhibits by 50% the activity of COX-1 and COX-2 *in vitro*) [12a, 13a-b]. The assessment of COX-1/COX-2 IC_{50} *in vitro* values describes an experimental COX-isoenzyme selectivity which mirrors the chemical features of the different NSAIDs. However, it has been shown that COX-2 selectivity of NSAIDs is a continuous variable and there is not a cut-off value of COX-1/COX-2 IC_{50} ratio which separates tNSAIDs from coxibs [11, 12a]. Thus, the tNSAID diclofenac shows a COX-2 *in vitro* selectivity comparable to that of the coxib celecoxib [12a]. The

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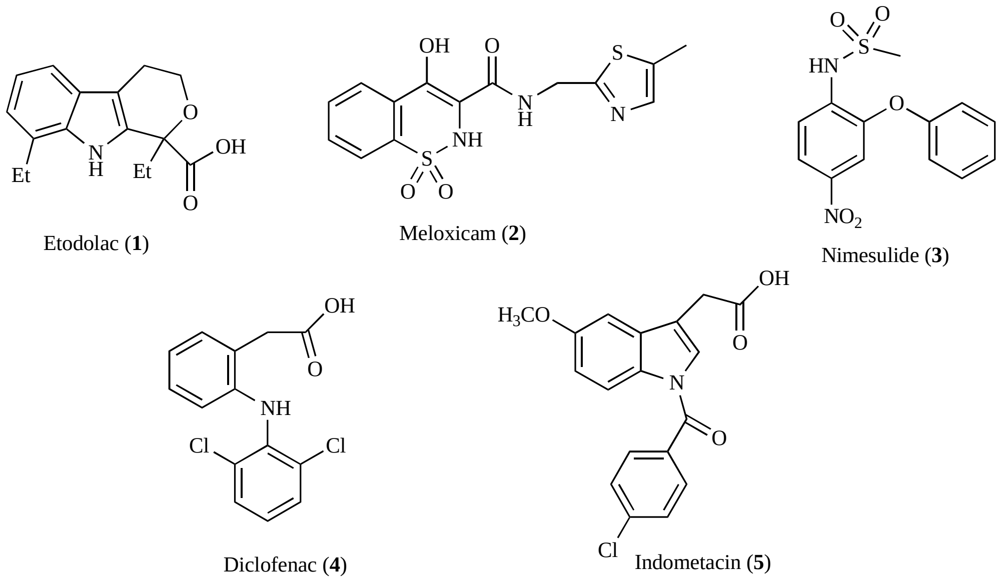


Etodolac (**1**)

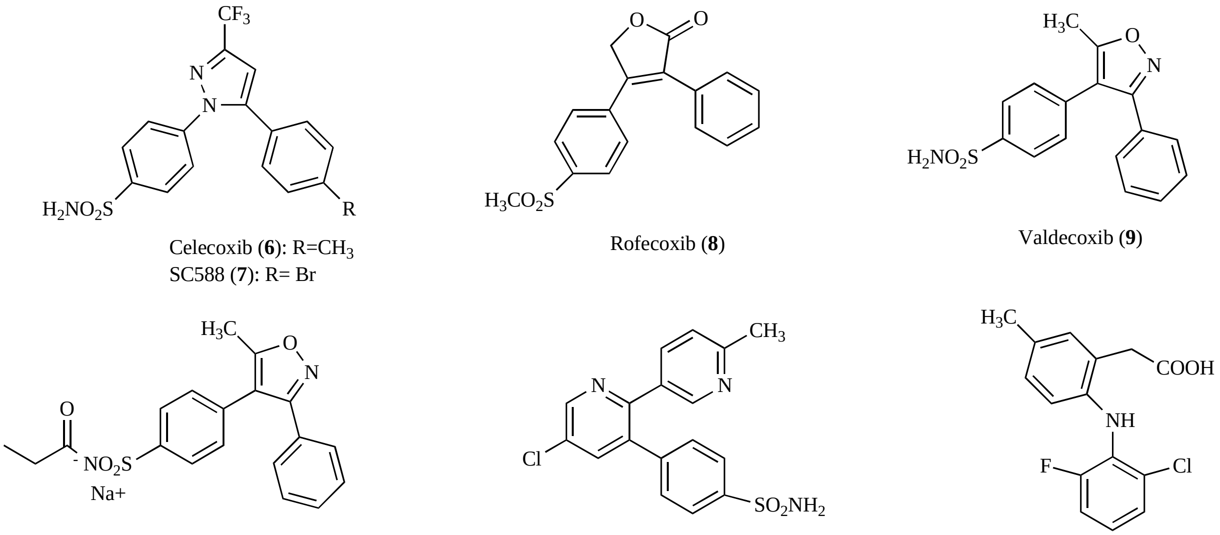
Meloxicam (**2**)

Nimesulide (**3**)

Diclofenac (**4**)

Indometacin (**5**)

Chart 1. Traditional Nonsteroidal Anti-inflammatory Drugs.



Celecoxib (**6**): R=CH_3
SC588 (**7**): R= Br

Rofecoxib (**8**)

Valdecoxib (**9**)

Parecoxib sodium (**10**)

Etoricoxib (**11**)

Lumiracoxib (**12**)

Chart 2. The Coxib Family.

whole blood assay also permit to estimate "achieved" COX-isoenzyme selectivity in humans when the drugs are administered at therapeutic doses (this is called COX-2 selectivity *ex vivo*). The "achieved " COX selectivity is obtained by assessing the degree of inhibition of COX-1 and COX-2 by circulating drug concentrations. Achieved selectivity of NSAIDs varies as a consequence of the administered dose [12a]. The assessment of COX-2 selectivity *ex vivo* allows to predict the clinical outcomes (both therapeutic and toxic effects) when these drugs are administered to humans [12a].

NSAIDs are characterized by different COX-1/COX-2 selectivity, and different pharmacokinetic and toxicological profiles [12a,b].

Reduced incidence of serious gastrointestinal (GI) adverse effects compared to tNSAIDs has been demonstrated for 2 highly selective COX-2 inhibitors [e.g. rofecoxib (**8**) and lumiracoxib (**12**)] in large randomized clinical trials (RCTs) [14 a,b]. This was a proof of concept that sparing COX-1 in the GI tract and possibly in platelets translates to a safer GI profile [11]. In fact, COX-1 is constitutively expressed in the stomach and platelets [15], whereas COX-2 does not appear to be expressed or is expressed at very low levels. However, both COX-isozymes are a source of cytoprotective prostanoids [16a]. COX-1-derived prostanoids (mainly PGE_2) are involved in the constitutive defense mechanism operating in physiological conditions [11]. In contrast, endogenous PGE_2 derived from COX-2 plays an important part in the spontaneous healing of gastric ulcers [16a-d]. This is supported by clinical data showing that coxibs are still associated with a small risk of upper gastrointestinal bleed (UGIB), though smaller than that caused by tNSAIDs [14 a,b]. Interestingly, agents with a coincident inhibition of both COX-1 and COX-2 were the ones associated with the highest relative risk of UGIB [17]. The use of tNSAIDs at the lowest effective dose may be a strategy to reduce GI toxicity. In fact, it was found that low-dose ibuprofen (a nonselective NSAID) has comparable UGIB risk as rofecoxib (**8**), and celecoxib (**6**)[17].

Despite dose reduction is recommended to reduce GI toxicity, it has to be claimed that this strategy may reduce their clinical efficacy. The assessment of a biomarker which may predict the anti-inflammatory and analgesic effects of NSAIDs, such as the evaluation of whole blood COX-2 inhibition, showed that circulating drug concentrations which inhibit COX-2 by 80% are appropriate to cause clinical efficacy (analgesia) [12a, 18].

An important clinical issue associated with the use of NSAIDs, is the possible increased risk of cardiovascular side-effects, such as thrombotic events, hypertension and heart failure. It has been recently evidenced that the use of NSAIDs (both coxibs and tNSAIDs) in the general population is associated with a 35% increased risk of non fatal myocardial infarction (MI) [relative risk 1.35, (95% CI), 1.23 to 1.48] [19]. The excess risk increased with increasing treatment duration and daily dose. Using a translational medicine approach, i.e. from proof of concept in cells and experimental animals to studies in humans, it was possible to show that inhibition of COX-2-dependent prostacyclin is the most plausible mechanism involved in cardiovascular hazard by NSAIDs. COX-2 is among the endothelial genes upregulated by uniform laminar shear stress (LSS), characteristically associated with atherosclerotic lesion-protected areas [10c]. We have recently enlightened a central role played by LSS-induced COX-2–dependent prostacyclin in restraining endothelial inflammation [20]. Thus, inhibition of COX-2-dependent prostacyclin might contribute to acceleration of atherogenesis in patients taking tNSAIDs and NSAIDs selective for COX-2 [20].

In mice, it has been demonstrated that endothelial COX-2 dependent prostacyclin modulates thrombosis induced *in vivo* by photochemical injury to the vasculature [21] using different approaches, i.e. the administration of the COX-2 selective inhibitor 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU), deletion of PGI_2 receptor (IP) and specific deletion of COX-2 in endothelial cells.

Single-nucleotide polymorphisms (SNPs) within the IP have been identified and, interestingly, the R212C variant is associated with defective prostacyclin signalling, i.e. reduced generation of the second messenger c-AMP after activation of the receptor [22]. Importantly, Arehart *et al.* [22] have shown that patients who were carriers of the SNP R212C were associated with increased cardiovascular disease and events, analogously to COX-2 inhibition.

The increased incidence of thrombotic events associated with profound inhibition of COX-2-dependent prostacyclin by coxibs and tNSAIDs can be mitigated, even if not obliterated, by a complete suppression of platelet COX-1 activity [23 a,b].

However, most tNSAIDs and coxibs are not able to inhibit almost completely and persistently platelet COX-1 activity to determine an *in vivo* inhibition of platelet function [23a,b]. This explains why they shared comparable cardiovascular toxicity [19]. Among all NSAIDs, a CV safer drug is naproxen and this is due to its pharmacodynamics and pharamcokinetics, i.e. the drug inhibits profoundly platelet COX-1 activity and this effect persists throughout dosing interval due to its long half-life (approximately 17 hours) [19]. However, naproxen is one of the more toxic drug for the GI system due to its profound inhibitory effect for the GI prostanoids.

We found that the extent of inhibition of COX-2–dependent prostacyclin may represent an independent key determinant of the increased risk of MI among NSAIDs , which does not affect platelet function, a property shared by coxibs and most tNSAIDs, and that the assessment of whole blood COX-2 might represent a surrogate end-point to predict the cardiovascular risk of these drugs [19]. However, appropriate clinical studies should be performed to verify this preliminary finding.

Reduction of the dose is recommended and presumably will limit the number of patients exposed to a cardiovascular hazard by NSAIDs. It will not, however, eliminate the risk on an individual level because there is a marked variability in how different people react to these drugs, based, at least in part, on their genetic background [24].

The planning for novel NSAIDs with a different chemical structure should be important on the light of marked variability in the response to these drugs in humans both for the therapeutic effects and for the toxic effects.

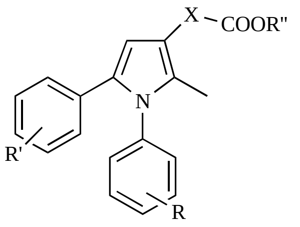
Thus, we started a wide research programme, aimed at discovering new pyrrole-containing anti-inflammatory agents and focused on the synthesis of 1,5-diarylpyrrole-3-acetic ketoesters, acids, esters, and ethers, as new COX-2 selective inhibitors in which the pyrroleacetic and vicinal diaryl heterocyclic moieties were reminiscent of indometacin (**5**) and of the coxib family, respectively [25a-h]. The results obtained by us on this topic are reported below.

ETHER, ESTER AND ACID DERIVATIVES OF THE 1,5-DIARYLPYRROLE SCAFFOLD

Having a look at the chemical structures reported in Chart **2**, with the exclusion of etoricoxib (**11**) and lumiracoxib (**12**), it could be recognized how diaryl-substituted penta-atomic heterocylcles could be particularly well shaped for originating potent and selective COX-2 inhibitors. Indeed, the diaryl substitution decoration was found to be an essential feature for generating potent selective inhibitors able to fulfil all the required interactions [26].

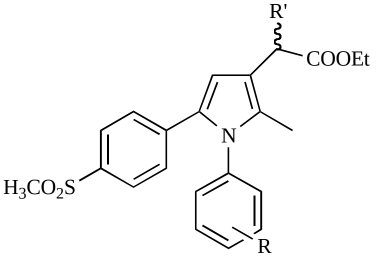
The diaryl substitution pattern can be also recognized in a number of penta-atomic heterocylcles, particularly imidazoles and pyrazoles, for example, in the class of Mitogen-activated protein (MAP) kinase inhibitors [27a,b]. Liverton and co-workers reported interesting issues on diarylpyrroles as p38 kinase inhibitors. In particular, the issues showed that the presence of two aryl moieties on adjacent positions was a crucial parameter in determining activity [28]. As reported by McIntyre, the presence of a central π-electron excessive aromatic heterocycle bearing two aryls on adjacent positions is the main feature for identifying kinases inhibitors [29]. The diaryl-heterocyclic motif can also be found when dealing with cannabinoid receptor modulators. Rimonabant, a diaryl pyrazole, is the first prototype of this class of CB1 receptor modulators but there are a lot of closely related molecules which have been submitted to clinical testing [30]. In order to decrease the possibility of unwanted potentially unsafe interaction with other targets (i.e. kinases) and to optimize absorption, distribution, metabolism, and excretion (ADME) characteristics, we chose the pyrrole nucleus as the core heterocycle. The pyrrole nucleus and properly substituted pyrroles are moieties present in several natural and safe substances [31]. This heterocycle is quite stable from a metabolic standpoint, it does not give rise to reactive intermediates by metabolic activation and it is endowed with a suitable hydrophilicity. As drugs, pyrroles have been described for various uses including treatment of inflammation, pain and cardiovascular diseases. Khanna and co-workers described a series of diaryl-pyrroles as COX-2 selective inhibitors. Both *in vitro* and *in vivo* issues suggested that the decorated central pyrrole was crucial for activity and the enzymatic properties of such a class of derivatives was closely dependent on the substitution pattern [32]. On these grounds, at the very beginning of our research programme aimed at discovering new pyrrole-containing antiinflammatory agents, we focused our attention on the synthesis of 1,5-diarylpyrrole-3-acetic acids and esters (**12-43**) (Chart **3**) [25a,b,f]. These compounds were characterized by: *i)* a *p*-methylsulfonylphenyl substituent, responsible for the COX-2 selective inhibition [present in rofecoxib (**8**) and etoricoxib (**11**)] at C5 (**12-32**) or N1(**33-43**) of the pyrrole nucleus; *ii)* a second phenyl ring decorated with different groups such as H, 3-F, 4-F, 3,4-F_2, 4-CF_3, 4-OCH_3 and 4-CH_3; *iii)* a ketoacid, ketoester, acetic acid and acetic ethyl ester chain at C3.

A structure-activity relationship (SAR) analysis of compounds **12-43**, supported by molecular docking simulations of these inhibi-



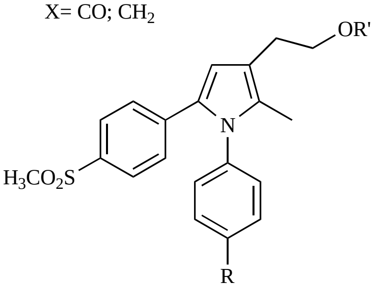
12-43

R=R'= H; 4-CH_3; 4-OCH_3; 4-CF_3; 4-F; 3-F; 3,4-F_2; 4-SO_2CH_3
R"= H; Et
X= CO; CH_2



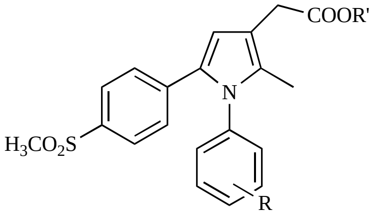
44a-c-49a-c

R= H; 4-OCH_3; 3-F; 3,4-F_2
R'= OH; OEt



50-58

R= H; F; CF_3
R'= CH_3; C_2H_5; *n*-C_3H_7



59-66

R= H; 4-OCH_3; 3-F; 3,4-F_2
R'= *i*-C_3H_7; *n*-C_4H_9

Chart 3. Structures of Compounds **12-66**.

tors within the COX-2 binding site, allowed us to rule out several considerations: *i)* the position of the *p*-methylsulfonylphenyl substituent was very important for activity (in general compounds **12-32** being more active than **33-43**); *ii)* surprisingly, the acetic ethyl ester chain at C3 of the pyrrole ring led to compounds more active than the corresponding acids and ketoesters; *iii)* substituents and substitution pattern on the phenyl ring at N1 influenced activity in the following order: 3-F = 4-F > 3,4-F_2 > 4-O CH_3 > H > 4-CF_3 > 4-CH_3 [25a,b.f].

On the basis of these results, carrying on the project we planned the synthesis of new derivatives (**44-49**) [25c,f]. Thus, keeping the most convenient fluorine substitution (namely the 3-F substituent at the N1 phenyl ring), as well as the 4-methoxyphenyl and the 3,4-difluorine-phenyl groups and an unsubstituted phenyl ring at position 1 of the pyrrole, we introduced a hydroxyl or an ethoxyl chain at the methylene of the C3 alkyl chain (leading to the corresponding chiral alcohol and ether derivatives **44a-c-49a-c**, respectively, Chart **3**) to evaluate the presence of any kind of stereoselective interaction at the enzyme's active site and to check the possible variation of activity induced by different enantiomers [25c,f]. Enantiomerically pure samples were obtained, at mg-scale, by high performance liquid chromatography (HPLC) on the amylose-based Chiralpak IA chiral stationary phase. In particular, the absolute configuration of **(-)-44c** was determined by single-crystal X-ray diffraction analysis [25d]**.** The findings of X-ray structure analysis allowed us to assign unambiguously the (R)-configuration to the **(-)-44c** enantiomer. Then, the stereochemistry of remaining pyrrole derivatives was empirically assigned by circular dichroism spectroscopy. All the enantiomers showed a very good activity, even though, in general they proved to be less active than corresponding previously synthesized derivatives lacking a stereogenic center at the α-position of the side chain [25c,f].

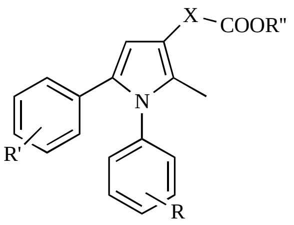
In order to expand the knowledge about the SAR of this class of compounds, the search for further functionalizations on the pyrrole nucleus spurred us to transform 1,5-diarylpyrrole-3-acetic esters into the hitherto unknown 3-alkoxyethyl ethers **50-58** (Chart **3**) [25e,f]. Biological evaluation of these compounds showed that in the class of 1,5-diarylpyrrole-3-acetic alkylesters, the replacement of the acetic ester moiety with an alkoxyethyl group still led to new, highly selective and potent COX-2 inhibitors [25e,f].

Finally, we pursued the synthesis and the biological activity of derivatives **59-66**, in which longer side chains were introduced at C3 on the basis of the hypothesis that such chains could better interact with hydrophobic residues of the receptor's carboxylate pocket. In particular, compounds **59-66**, bearing an *i*-propyl or a *n*-butyl ester function, a N1 phenyl ring substituted with various moieties previously found to be important for activity (namely, 3-F, 3,4-diF, 4-OCH_3) and a C5 *p*-methylsulfonyl phenyl moiety, showed outstanding activities.

BIOLOGICAL AND PHARMACOLOGICAL EVALUATION

All compounds were subjected to biological evaluation through *in vitro* testing (J774 Murine Macrophage Assay) and the results are reported in Tables **1**, **2**, **3** and **4**. The *in vitro* estimation of the title compounds was performed to assess their inhibitory activity on both COX-1 and COX-2, hence their selectivity toward the latter. *In vitro* activity is defined as the concentration required to inhibit the activity of the isoenzymes, while selectivity toward COX-2, referred to as Selectivity Index (SI), is described as the ratio of the concentrations required to inhibit the activity of both isoenzymes by 50% (IC_{50}) (i.e. IC_{50}(COX-1)/ IC_{50}(COX-2)]. The *in vitro* issues showed that all compounds were very selective and that, in general, the activity was a function of the substitution pattern present on the pyrrole ring. More specifically the compounds bearing the *p*-methylsulfonylphenyl group at position 5 of the pyrrole nucleus (compounds **12-32** and **44-66**), could be considered the most selective, probably because of a more efficacious interaction with the enzyme active site with respect to the corresponding N1 *p*-methylsulfonylphenyl derivatives (compounds **33-43**). Compounds showing an interesting biological profile were chosen and further investigated through pharmacological experimentations. Thus,

Table 1. Chemical Structures and *In Vitro* COX-1 and COX-2 Inhibitory Activity of Compounds 12-43 by Using Cell-Based Assay

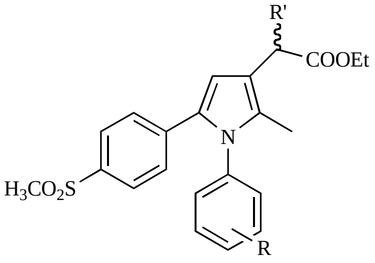


Compd	R	R’	R”	X	COX-1 IC_{50} (μM)^{a}	COX-2 IC_{50} (μM)^{a}	% of COX-2 Inhibition (10μM)	% of COX-2 Inhibition (1μM)	COX-1/ COX-2 (SI)
12	H	4-SO_2CH_3	C_2H_5	CO	>100	4.3	54	43	>23
13	4-CH_3	4-SO_2CH_3	C_2H_5	CO	>100	1.9	77	33	>92
14	4-CF_3	4-SO_2CH_3	C_2H_5	CO	>100	9.9	51	33	>10
15	4-F	4-SO_2CH_3	C_2H_5	CO	>100	11.0	51	19	>9
16	3-F	4-SO_2CH_3	C_2H_5	CO	>100	0.90	75	55	>100
17	3,4-F_2	4-SO_2CH_3	C_2H_5	CO	>100	8.4	55	8	>12
18	4-OCH_3	4-SO_2CH_3	C_2H_5	CO	>100	0.94	70	59	>100
19	H	4-SO_2CH_3	H	CH_2	>100	1.0	100	43	>100
20	4-CH_3	4-SO_2CH_3	H	CH_2	>100	0.43	100	74	>200
21	4-CF_3	4-SO_2CH_3	H	CH_2	>100	0.11	100	69	>900
22	4-F	4-SO_2CH_3	H	CH_2	>100	ND^{b}	ND^{b}	ND^{b}	ND^{b}
23	3-F	4-SO_2CH_3	H	CH_2	>100	0.028	96	89	>3500
24	3,4-F_2	4-SO_2CH_3	H	CH_2	47	0.26	98	70	38
25	4-OCH_3	4-SO_2CH_3	H	CH_2	>100	0.17	90	62	>580
26	H	4-SO_2CH_3	C_2H_5	CH_2	>100	0.04	84	79	>2500
27	4-CH_3	4-SO_2CH_3	C_2H_5	CH_2	>100	0.48	81	67	>208
28	4-CF_3	4-SO_2CH_3	C_2H_5	CH_2	>100	0.06	87	80	>1600
29	4-F	4-SO_2CH_3	C_2H_5	CH_2	>100	0.010	100	100	>10000
30	3-F	4-SO_2CH_3	C_2H_5	CH_2	>100	0.010	100	91	>10000
31	3,4-F_2	4-SO_2CH_3	C_2H_5	CH_2	>100	0.020	100	87	>5000
32	4-OCH_3	4-SO_2CH_3	C_2H_5	CH_2	>100	0.026	90	87	>4000
33	4-SO_2CH_3	H	C_2H_5	CO	>100	ND^{b}	30	0	ND^{b}
34	4-SO_2CH_3	4-CH_3	C_2H_5	CO	>100	ND^{b}	25	0	ND^{b}
35	4-SO_2CH_3	4-CF_3	C_2H_5	CO	>100	ND^{b}	34	30	ND^{b}
36	4-SO_2CH_3	4-F	C_2H_5	CO	>100	ND^{b}	12	0	ND^{b}
37	4-SO_2CH_3	3,4-F_2	C_2H_5	CO	>100	ND^{b}	29	0	ND^{b}
38	4-SO_2CH_3	4-OCH_3	C_2H_5	CO	>100	ND^{b}	49	21	ND^{b}
39	4-SO_2CH_3	H	C_2H_5	CH_2	>100	ND^{b}	27	0	ND^{b}
40	4-SO_2CH_3	4-CH_3	C_2H_5	CH_2	>100	1.96	79	33	>50
41	4-SO_2CH_3	4-CF_3	C_2H_5	CH_2	>100	ND	ND	ND	ND
42	4-SO_2CH_3	4-F	C_2H_5	CH_2	>100	0.40	75	50	>250
43	4-SO_2CH_3	4-OCH_3	C_2H_5	CH	>100	0.55	78	59	>200
celecoxib					5.1	0.079	95	80	65

[a]Results are expressed as the mean (n=3) of the % inhibition of PGE_2 production by test compounds with respect to control samples. [b]Not Determined.

selectivity of compounds was determined by the human whole blood assay [13a,b], which permit to estimate the “achieved” COX inhibition *ex vivo* by circulating drug levels. Results indicated a dramatic change in terms of COX selectivity in these experimental conditions. Finally, to assess *in vivo* anti-inflammatory and analgesic activity, three different tests (rat paw pressure, rat paw oedema and abdominal constriction, Tables **5**, **6**, and **7**, respectively) were performed. The submitted compounds, administered at a dose ranging from 5 to 20 mg/kg p.o., showed very good activities against carrageenan-induced hyperalgesia 30 min after administration. The analgesic activities at this time were very similar to that obtained with celecoxib (10 mg/kg p.o.) (Table **5**). Good activity profiles were displayed against carrageenan-induced rat paw oedema, with almost complete remission 1h after the administration (20 mg/kg

Table 2. Chemical Structures and *In Vitro* COX-1 and COX-2 Inhibitory Activity of Compounds 44-49 by Using Cell-Based Assay

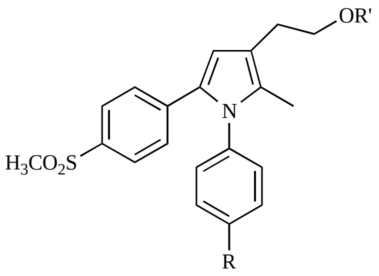


Compd	R	X	COX-1 IC$_{50}$ (μM)[a]	COX-2 IC$_{50}$ (μM)[a]	% of COX-2 Inhibition (10μM)	% of COX-2 Inhibition (1μM)	COX-1/ COX-2 (SI)
(+/-)44a	H	OH	>100	0.30	100	78	>600
(+)-(S)44b	H	OH	>100	0.18	92	79	>550
(-)-(R)44c	H	OH	>100	0.15	92	86	>650
(+/-)45a	3-F	OH	>100	0.12	100	70	>800
(+)-(S)45b	3-F	OH	>100	1.10	84	56	>90
(-)-(R)45c	3-F	OH	>100	0.075	92	86	>1300
(+/-)46a	4-OCH_3	OH	>100	0.12	100	82	>800
(+)-(S)46b	4-OCH_3	OH	>100	0.079	100	88	>1200
(-)-(R)46c	4-OCH_3	OH	>100	0.15	93	86	>650
(+/-)47a	H	OC_2H_5	>100	0.18	100	90	>800
(+)-(S)47b	H	OC_2H_5	>100	0.10	100	81	>1000
(-)-(R)47c	H	OC_2H_5	>100	0.14	86	77	>700
(+/-)48a	3-F	OC_2H_5	>100	0.10	100	74	>1000
(+)-(S)48b	3-F	OC_2H_5	>100	0.25	100	86	>400
(-)-(R)48c	3-F	OC_2H_5	>100	0.81	100	73	>100
(+/-)49a	4-OCH_3	OC_2H_5	>100	0,13	ND[b]	ND[b]	>800
(+)-(S)49b	4-OCH_3	OC_2H_5	>100	0.14	100	100	>700
(-)-(R)49c	4-OCH_3	OC_2H_5	>100	0.09	100	87	>1100
celecoxib			5.1	0.079	95	80	65

[a] Results are expressed as the mean (n=3) of the % inhibition of PGE_2 production by test compounds with respect to control samples.
[b] Not Determined.

p.o) (Table **6**). Moreover, a dose-dependent anti-nociceptive activity was observed in the abdominal constriction test (Table **7**). The *in vitro* test highlighted the acetic esters derivatives **30** and **59** as the most active compounds [25b,g]. These molecules displayed excellent IC$_{50}$ values (in the range of nanomolar concentrations) and selectivity indexes higher than those shown by reference compounds (up to 10000). Moving the fluorine substituent of compound **30** from the *meta* to the *para*-position of the N1 phenyl ring, as for compound **29**, the activity was maintained nearly by, while the simultaneous presence of two fluorine atoms at the *meta* and *para* positions (compound **31**) led to a slight reduction of both activity and selectivity (Table **1**) [25b]. SAR data indicated that the *p*-methylsulfonylphenyl group at the C5 phenyl ring and a fluorine substituent at the N1 phenyl ring are cooperative determinants for both potent and selective COX-2 inhibition. The analysis of bulky compounds revealed that the ester function, present in compound **59**, could be responsible for the potency and selectivity performed by this class of hindered compounds: this led to the suggestion that an increase of hindrance at C3 could translate to activity enhancement [25g]. The exception is performed by the fluorinated series of compounds, whose ethyl ester derivatives seem to be more selective than the bulkier analogues (*i*-propyl and *n*-butyl derivatives). Since the hydrolysis of the ester can occur at least to some extent *in vivo*, ester compounds **26-32** and **59-66** were evaluated in comparison with the corresponding acid (**19-25**) [25g]. In general the ester derivatives proved to be more effective and selective toward COX-2 with respect to acid derivatives and only in the case of 3-F phenyl substituted derivatives the acid compound **23** and the esters compounds **30**, **61** and **62** displayed closer potencies. The biological evaluation also affords to outline that the activity characterizing the acetic compounds **26-32** rapidly decreases when a carbonyl group is introduced at C3, making the side chain to be sterically frozen, to obtain the glyoxylic compounds **12-18** [25a,b]. Compounds **44-49**, characterized by the insertion of a stereogenic carbon at position 3 of the pyrrole core, were synthesized in order to evaluate the presence of stereoselective interactions at the enzyme's active site. *In vitro* cell-based assay showed IC$_{50}$ values toward COX-2 in the submicromolar concentration [with the only exception of (+)-(S)-**45b**], while compounds were all inactive toward COX-1 at the maximum tested dose (100 μM). All the enantiomers showed a very good activity, even though, in general they proved to be less active than corresponding derivatives lacking a stereogenic center at the C3 side chain α-position. Biological evaluation of 3-alkoxyethyl ether compounds **50-58** showed that the replacement of the acetic ester moiety with an alkoxyethyl group produced a significant difference in terms of inhibitory activity. Actually, compound **53** is more potent than the corresponding ester compounds **26** but in general 3-alkoxyethyl ethers are less potent than the above-cited esters

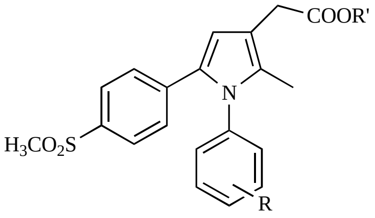
Table 3. Chemical Structures and *In Vitro* COX-1 and COX-2 Inhibitory Activity of Compounds 50-58 by Using Cell-Based Assay



Compd	R	R'	COX-1 IC_{50} (μM)[a]	COX-2 IC_{50} (μM)[a]	% of COX-2 Inhibition (10μM)	% of COX-2 Inhibition (1μM)	COX-1/ COX-2 (SI)
50	H	CH_3	>100	0.048	ND[b]	ND[b]	>2083
51	F	CH_3	>100	0.018	ND[b]	ND[b]	>5555
52	CF_3	CH_3	>100	0.049	ND[b]	ND[b]	>2040
53	H	C_2H_5	>100	0.015	ND[b]	ND[b]	>6666
54	F	C_2H_5	>100	0.047	ND[b]	ND[b]	>2127
55	CF_3	C_2H_5	>100	0.085	ND[b]	ND[b]	>1176
56	H	n-C_3H_7	>100	0.018	ND[b]	ND[b]	>5555
57	F	n-C_3H_7	>100	0.030	ND[b]	ND[b]	>3333
58	CF_3	n-C_3H_7	>100	0.110	ND[b]	ND[b]	>909
celecoxib			5.1	0.079	95	80	65

[a]Results are expressed as the mean (n=3) of the % inhibition of PGE_2 production by test compounds with respect to control samples. [b]Not Determined.

Table 4. Chemical Structures, and *In Vitro* COX-1 and COX-2 Inhibitory Activity of Compounds 59-66 by Using Cell-Based Assay



Compd	R	R'	COX-1 IC_{50} (μM)[a]	COX-2 IC_{50} (μM)[a]	% of COX-2 Inhibition (10μM)	% of COX-2 Inhibition (1μM)	COX-1/ COX-2 (SI)
59	H	i-C_3H_7	>100	0.0073	100	100	> 13600
60	H	n-C_4H_9	>100	0.014	88	85	> 7100
61	3-F	i-C_3H_7	>100	0.043	91	85	> 2300
62	3-F	n-C_4H_9	>100	0.024	100	87	> 4100
63	3,4-F_2	i-C_3H_7	>100	0.021	90	85	> 4700
64	3,4-F_2	n-C_4H_9	>100	0.03	90	86	> 3300
65	4-OCH_3	i-C_3H_7	>100	0.022	100	88	> 4500
66	4-OCH_3	n-C_4H_9	>100	0.038	92	89	> 2600
celecoxib			5.1	0.079	95	80	65

[a]Results are expressed as the mean (n=3) of the % inhibition of PGE_2 production by test compounds with respect to control samples.

[25e]. These data can be easily explained if we take into consideration the different stereoelectronic properties of the acetic ester chain with respect to the alkoxyethyl moiety. On the other hand, it is less clear how the alkoxyethyl derivatives**,** even in the absence of the carbonyl group, are able to exert highly selective and potent COX-2 inhibitory activities. The human whole blood assay indicated that the issues gained through the cell-based assay are in some extent reversed. In fact, celecoxib showed a COX-1/COX-2 IC_{50} ratio of 23 in the human whole blood assay (Table **8**), whereas a COX-1/COX-2 IC_{50} ratio 64.5 was found in the cell-culture test. The reversion is much more meaningful when considering compound **30**: in the human whole blood its COX-1/COX-2 IC_{50} ratio was about 9.7 while it showed a >10000 ratio *in vitro* (Tables **1** and **8**). The discrepancy between the results obtained by using the human whole blood assay and those found in the cell-based assay could be due to a different inhibitor sensitivity showed by the mouse and human COX isoenzymes, as already reported for other anti-inflammatory compounds. Thus, a dramatic loss in COX inhibition

Table 5. Effect of Selected Active Compounds on Hyperalgesia by Carrageenan in the Rat Paw-Pressure Test[a]

Pre-treatment	Treatment	Paw pressure (g)			
		Before treatment	30 min	60 min	120 min
saline	Saline	62.0±4.3	62.6±5.2	63.5±4.6	64.2±5.2
carrageenan	Saline	61.9±5.1	38.7±5.3	35.8±4.5	40.1±5.0
carrageenan	**19**	63.1±3.9	48.6±4.3	51.8±3.6	41.6±3.7
carrageenan	**23**	62.7±4.2	54.8±3.7	55.2±3.8	43.1±3.9
carrageenan	**24**	59.2±3.4	58.3±3.4	54.1±4.4	48.6±3.4
carrageenan	**25**	61.2±3.6	52.3±3.7	39.8±4.1	36.2±3.2
carrageenan	**30**	61.7±3.9	55.2±3.6	49.8±4.1	43.4±4.9
carrageenan	**(+/-)-45a**	59.6±2.8	54.8±3.8	45.2±4.60	47.5±4.6
carrageenan	**53**	60.5 ± 4.3	59.6 ± 4.7	53.8 ± 4.2	49.1 ± 5.4
carrageenan	**56**	59.2 ± 5.2	56.3 ± 5.6	59.4 ± 5.1	50.6 ± 5.3
carrageenan	**57**	63.5 ± 4.3	52.4 ± 5.2	56.8 ± 4.9	52.7 ± 4.0
carrageenan	**59**	60.8 ± 4.3	56.2 ± 4.8	51.8 ± 5.3	48.8 ± 3.7
carrageenan	**60**	57.5 ± 3.7	52.5 ± 4.9	46.3 ± 5.3	42.6 ± 3.8
carrageenan	**61**	63.2 ± 3.8	59.5 ± 5.4	57.9 ± 4.1	45.2 ± 4.3
carrageenan	**62**	62.4 ± 3.2	49.7 ± 4.1	46.9 ± 5.5	45.2 ± 4.8
carrageenan	**63**	64.8 ± 3.8	59.5 ± 2.9	52.3 ± 4.9	58.3 ± 3.4
carrageenan	**64**	58.3 ± 3.6	52.7 ± 5.0	59.2 ± 6.3	56.6 ± 4.5
carrageenan	**65**	58.6 ± 4.1	51.0 ± 5.5	56.7 ± 4.4	41.7 ± 4.1
carrageenan	**66**	60.5 ± 3.0	53.4 ± 4.8	51.4 ± 5.0	50.8 ± 5.2
carrageenan	**celecoxib**	62.7±3.9	56.5±3.8	58.2±4.4	55.2±5.1

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

Table 6. Effect of Selected Active Compounds on Carrageenan-Induced Oedema in the Rat Paw-Pressure Test[a]

Pre-treatment	treatment	Before treatment	60 min after
saline	saline	1.25 ± 0.09	1.29 ± 0.10
carrageenan	saline	1.22 ± 0.08	2.33 ± 0.08
carrageenan	**19**	1.23 ± 0.10	1.86 ± 0.07
carrageenan	**23**	1.28 ± 0.05	1.35 ± 0.09
carrageenan	**24**	1.32 ± 0.10	1.42 ± 0.09
carrageenan	**25**	1.27± 0.08	1.81± 0.12
carrageenan	**30**	1.24±0.11	1.29±0.14
carrageenan	**(+/-)-45a**	1.37±0.12	1.40±0.13
carrageenan	**53**	1.29 ± 0.10	1.37±0.11
carrageenan	**56**	1.32 ± 0.06	1.41 ± 0.14
carrageenan	**57**	1.25 ± 0.09	1.45 ± 0.12
carrageenan	**59**	1.29 ± 0.09	1.26 ± 0.12
carrageenan	**60**	1.31 ± 0.10	1.68 ± 0.15
carrageenan	**61**	1.17 ± 0.11	1.31 ± 0.09
carrageenan	**62**	1.35 ± 0.09	1.71 ± 0.11
carrageenan	**63**	1.29 ± 0.10	1.39 ± 0.12
carrageenan	**64**	1.23 ± 0.08	1.52 ± 0.09
carrageenan	**65**	1.16 ± 0.07	1.41 ± 0.08
carrageenan	**66**	1.33 ± 0.08	1.45 ± 0.14
carrageenan	**celecoxib**	1.28±0.14	1.33±0.10

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

Table 7. Effect of Selected Active Compounds in the Mouse Abdominal Constriction Test (Acetic Acid 0.6%)

Treatment[a]	**number of mice**		
		dose (p.o. mg kg^{-1})	**number of writhes**
CMC	20		31.4 ± 4.3
19	11	10	27.3 ± 3.2
19	10	20	22.2 ± 2.9
23	9	10	31.6 ± 3.2
23	8	20	17.5 ± 3.1
24	12	10	29.3 ± 3.0
24	10	20	15.7 ± 2.6
25	10	10	30.9± 3.3
25	10	20	21.8± 2.1
30	10	10	28.9 ± 23.9
30	7	20	15.7 ± 3.6
(+/-)-45a	8	10	26.3 ± 3.6
(+/-)-45a	8	20	16.1 ± 2.7
53	10	10	26.5 ± 3.7
53	8	20	11.4 ± 3.6
56	9	10	27.43 ± 4.1
56	9	20	13.6 ± 4.2
57	10	10	38.1 ± 3.4
57	10	20	16.7 ± 3.5
59	8	1	33.6 ± 3.0
59	8	5	28.5 ± 3.3
59	8	15	25.5 ± 2.8
60	8	20	23.9 ± 3.4
61	12	5	39 .5 ± 3.8
61	9	20	17.7 ± 2.9
62	8	20	24.4 ± 4.1
63	10	5	35 .1 ± 4.6
63	12	20	21.5 ± 3.9
63	10	40	19.8 ± 3.2
64	12	5	34.4 ± 3.5
64	30	20	13.7 ± 4.0
65	11	5	42.2 ± 4.0
65	12	20	18.0 ± 3.5
66	8	10	21.6 ± 2.9
66	10	20	15.9 ± 3.3
celecoxib	12	10	14.2 ± 2.3

[a] All drugs were administered *per os* 30 min before test.

potency and drop in selectivity when using the human whole blood assays are shared with other selective COX-2 inhibitors such as valdecoxib [33,34], etoricoxib [33,34], lumiracoxib [35] and rofecoxib [36,37] as well as (t)NSAID such as nimesulide [33,38] and meloxicam [39,40].

In vivo tests highlighted an increase of activity along with the increase of hindrance of C3 side chain. In the rat paw-pressure test, the activities of esters compounds **59-66** proved to be better than those of corresponding acid compounds **19** and **23-25**. All these bulkier esters proved to be very effective and long lasting, being, in such a test, a relevant analgesia still present 2 hours after administration. Conversely, the corresponding acid compounds **19** and **23-25** highlighted a remarkable lower activity especially after 2 hours, with the exception of compound **24**. Ethyl esters (compounds **30** and **(+/-)-45a**) displayed an activity which tended to fade at longer time (1h), disappearing almost completely 2 h after administration. 3-Alkoxyethyl-ether (compounds **53**, **56** and **57**) showed activities comparable to those reported for bulky esters (compounds **59-66**). In parallel, a very good activity was demonstrated by all subjected compounds against carrageenan-induced rat paw oedema (Table **3**), with a complete remission 1 h after the administration (20 mg/kg p.o.). In the abdominal constriction test all compounds were able to reduce the number of wrights in a statistically significant manner at a dose of 20 mg/kg p.o. The issues regarding the *in vivo* profiles characterizing the ester derivatives can be better understood when taking into account their metabolic features. Preliminary studies

Table 8. Assessment of IC_{50} Values (i.e.the Concentration of the Drug Required to Inhibit COX Activity by 50%) for Platelet COX-1 and Monocyte COX-2 Activities for Celecoxib, Valdecoxib, and a Selection of the Most Active Compounds by Using the Human whole Blood Assays [13a, b] *In Vitro*. Selectivity Index is COX-1/COX-2 IC_{50} Ratio

Compd	IC_{50} (μM)		Selectivity Index (SI)
	COX-1	COX-2	
19	132	7	19
23	125	17	7
30	20.4	2.1	9.7
(+/-)-45a	21.3	3.8	5.6
56	86	1.3	66
59	20	2.5	8
61	24	2	12
Celecoxib	12.5	0.54	23
Valdecoxib	34.3	0.66	52

were made on derivative **59** and its related acid **19** to underline their pharmacokinetics. The results of this study showed that compound **59** was characterized by a 42% of relative oral bioavailability and by a high clearance value. After both oral and endovenous administration of **59**, the presence of the acid **19** was detected thus underlining that hydrolysis of the ester moiety did occur. It was also observed that the size of metabolism was independent by the route of administration [25g]. Hence, the instability of the ester moiety could in some extent confirm what found by the *in vivo* studies: indeed, the long-lasting pharmacological activity characterizing bulkier esters could be representative for a prodrug-like system, in which the pharmacological aspects of the ester are dependent on both the activity related to the ester itself and the acid which is continuously released *in vivo*, as displayed by pharmacokinetics. This could also account for the longer effect characterizing bulky esters (Table **5**) in relation to ethyl ones. In fact, the former are characterized by a relative slow metabolism (hence a slower generation of the related acid) due to the hindered esterified moiety that leads to an enhanced pharmacological effect. Nevertheless, the composite metabolic pathway that characterize esters represents a great challenge in terms of drug development. Indeed, both pharmacokinetics and *in vivo* tests did not disclose the actual agent responsible for activity: we are aware about the fact that both the acid and the ester are available for promoting the pharmacological effect, but we do not know the real extent of each. Furthermore, we could hypothesize that after *in vivo* administration the ester would be directly involved in the interaction with the biophase but we are not able to demonstrate it with the available data. To enlarge our knowledge about the importance of a C3 side chain on the pyrrole core of our compounds we will follow a bioisosteric replacement of the ester moiety which proved to be quite sensitive to hydrolysis (both chemical and enzyme-dependent). This kind of replacement will allow us to correlate the obtained results with the 3-alkoxyethyl-ether compounds, which share the same scaffold with the ester derivatives but are not sensitive to the same metabolic route (hydrolysis), in order to understand if the integrity of the C3 side chain is a crucial aspect for activity or if different substitution patterns can be brought about to obtain the pharmacological effect.

COMPUTATIONAL STUDIES

Cyclooxygenase inhibitors belonging to the coxib family (such as celecoxib and SC-558) show a similar binding mode with the two isoforms, in agreement with that recently reported in the literature [41]. The differences involve in particular, Arg513 of COX-2, that interacts by a hydrogen bond with one of the sulfonamide oxygen atoms of SC-558 (as found in the complex coded 6cox in the protein data bank, Fig. **1**, upper panel) [42], and the corresponding His513 of COX-1, that was unable to contact celecoxib (entry 3kk6, Fig. **1**, lower panel). Moreover, reciprocal orientation between Val434 and Phe518 of COX-2 is significantly different from that of Ile434 and Phe518 of COX-1 [41].

Two computational approaches were applied to pyrrole compounds, based on molecular docking/structural optimization and on the generation of Grid maps for different probes. Results from calculations led to the identification of a binding mode for the new inhibitors, to the rationalization of their major structure-activity relationships, and, finally, to the suggestion of structural modifications to obtain new derivatives with higher affinity for COX-2.

To study the binding mode of the new compounds toward COX-2, pyrrole derivatives were submitted to molecular docking simulations and structural optimization of the resulting ligand-COX-2 complexes. However, the software Autodock, chosen among docking programs available, was checked for its ability to find reliable poses (orientations) and conformations of ligands into the COX-2 binding site. For this purpose, the crystal structure of the enzyme co-crystallized with SC558 (**7**), an inhibitor of the coxib family, was used to set the optimal parameters of Autodock runs and to verify that such parameters could be applied to handle flexible diaryl five-membered inhibitors. In particular, the COX-2 atomic coordinates used in the modeling studies were taken from the Protein Data Bank entry 6cox. Residues belonging to chain B and three *N*-acetyl-D-glucosamine residues present in chain A of the crystal structure were removed and excluded from all the calculations. The structure of the inhibitor was extracted from the complex with COX-2 and then re-docked into the enzyme binding site by means of Autodock. As a result, a very good agreement between the calculated (from docking simulations) and experimental (from the crystallographic complex) coordinates of SC558 (**7**) was found, the root mean square deviation (calculated on the heavy atoms) between the best ranking docked and minimized conformation of SC558 (**7**) and the corresponding crystallographic pose being of 0.27 Å. Considering that the computational protocol was able to identify conformers of the inhibitor very similar to that found in the X-ray structure, this approach could be considered as a useful modeling procedure to be applied for finding the orientations and interactions of ligands inside the binding site of COX-2. The same set of Autodock parameters used during the preliminary calculations on SC558 (**7**) was also applied to the new pyrrole derivatives. However, considering that Autodock does not perform any structural optimization of the complexes obtained, a molecular mechanics/energy minimization approach was also applied to refine docking output structures. All the amino acid residues at a 5 Å distance from ligand atoms were subjected to minimization, while the remaining amino acids were kept fixed.

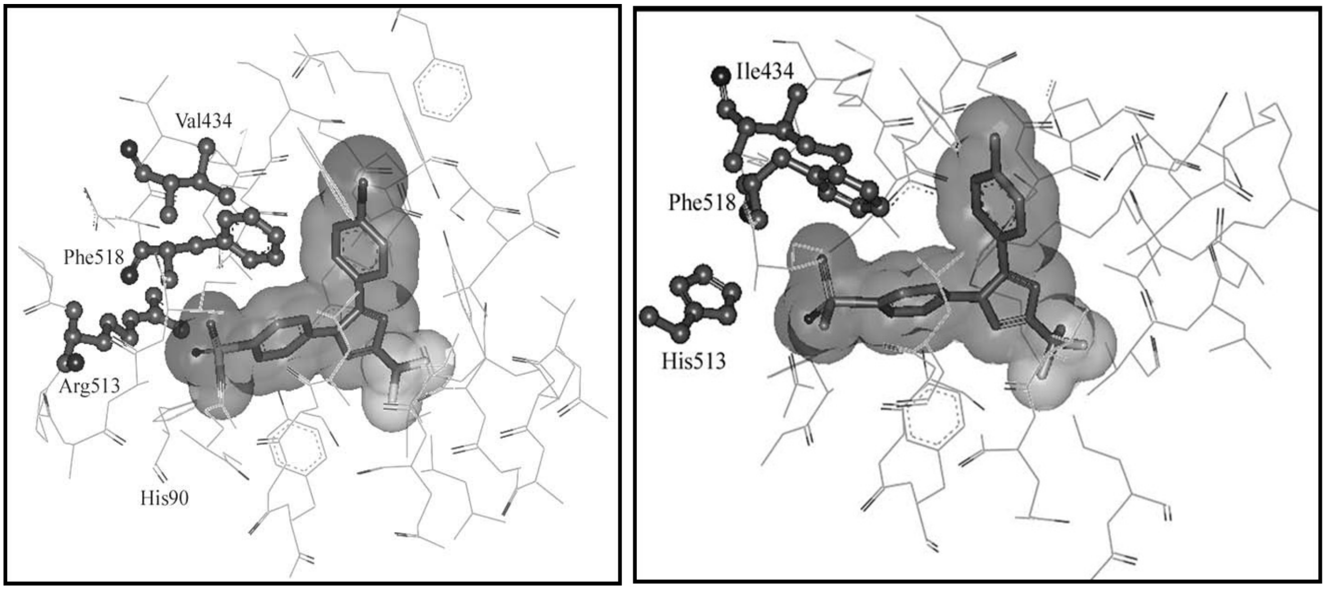


Fig. (1). Left panel. Graphical representation of the COX-2 binding site accommodating the inhibitor SC-558 (represented as van der Waals surfaces) in its crystallographic pose (entry 6cox of the protein data bank). Arg513 and His90 are able to form hydrogen bond contacts with the sulfonamide moiety of the inhihitor, differently to that found in the complex between celecoxib and COX-1. Right panel. Graphical representation of the COX-1 binding site accommodating celecoxib (represented as van der Waals surfaces) in its crystallographic pose (entry 3kk6 of the protein data bank). Distance between His513 and the sulfonamido moiety of celecoxib is higher than that found between Arg513 of COX-2 and SC-558. As a consequence, His513 does not make any hydrogen bond contacts with the sulfonamide moiety of the inhihitor, differently to that found in the complex between SC-558 and COX-2. A different spatial location of Ile434-Phe518 was found in comparison to that of Val434-Phe518 of COX-2.

Molecular docking simulations and structural optimization of the docked complexes showed the vicinal diaryl system and the central heterocyclic nucleus of both SC558 (**7**) and the new pyrrole derivatives located within the same regions of the COX-2 binding site. In general, the N1 aryl moiety of pyrroles and the bromophenyl group of the co-crystallized inhibitor share a region of the binding site thereafter referred to as the hydrophobic pocket (following the notation applied by Kurumbail and co-workers) comprising the side chains of lipophilic (Lue384) and aromatic (Tyr385, Trp387, and Phe518) amino acids (Fig. **2**) [42]; the sulphur-containing groups were located within the selectivity site; the trifluoromethyl group of SC558 (**7**) and the side chain at C3 of pyrrole derivatives occupied the classical NSAID's carboxylate binding site. Several hydrophobic contacts (between the N1 substituent and the hydrophobic pocket, between the C5 phenyl ring and Leu352 and Val523 of the selectivity site, between the terminal alkyl group of the C3 side chain and Val116 and Tyr355 of the carboxylate site, between the C2 methyl substituent and the side chains of Val349 and Ala527) and a network of hydrogen bonds (between the sulfone oxygens and both Phe518 NH and Gln192 NH_2, between the ester/ether oxygens and the Arg120 guanidino group) stabilized the complexes between COX-2 and pyrrole derivatives.

To further support the results of molecular docking simulations, the software Grid was also applied to evaluate the regions of best interactions between inhibitors and macromolecule. Grid probes able to mimic the physicochemical properties of the atom types and the substituents commonly found in ligands were used to calculate the interaction energy with amino acids constituting the COX-2 binding site. The carbonyl Grid map (generated with the O probe) was in agreement with the docking results, showing that the Arg120 side chain is surrounded by regions of profitable interactions with a carbonyl group, also including the position occupied by the carbonyl ester and ether groups of pyrrole derivatives. Moreover, it is important to note that, although additional space was available for alkyl groups longer than an ethyl substituent in the region where the ester alkyl chain was accommodated, the C3 grid (generated with the methyl probe) seemed to suggest that a two-carbon atom group was the optimal substituent to be used as the terminal portion of the ester chain. In fact, a region of profitable interactions between a methyl group and the protein was found in correspondence of the terminal methyl group of the ethyl ester chain of pyrrole inhibitors. Finally, inspection of the grid generated with the methyl probe

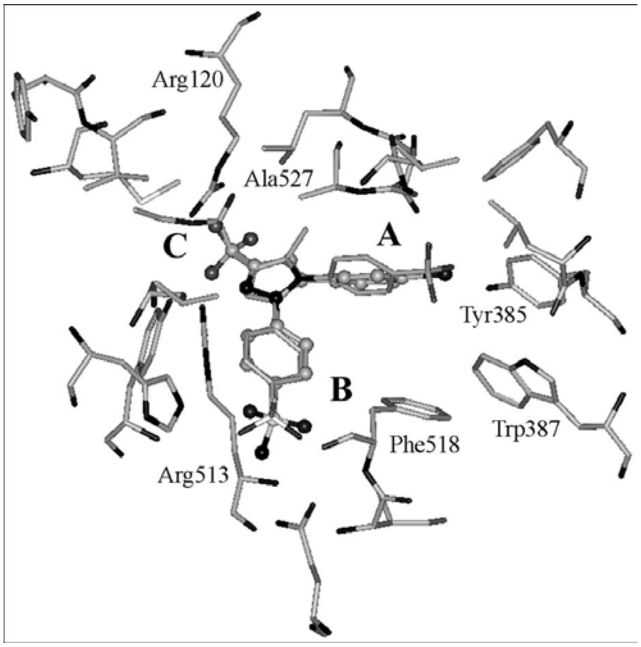


Fig. (2). Binding pose of SC-558 (ball and stick) and **28** into the COX-2 binding site. The halophenyl moieties lie into the hydrophobic pocket (region **A**), the sulphonyl groups are located into the selectivity site (**B**), and the trifluoromethyl group of SC-558 is superposed to the ester side chain of 6 within the carboxylate site (region **C**).

showed positive interactions between the 2-methyl group and the pocket defined by the side chains of Val349 and Ala527, in agreement with docking results and with previous findings suggesting that such a substituent at this position yields the optimal potency [32]. Analysis of the C3 grid showed additional stabilizing interactions involving the two para substituents of the 1,5-diaryl system of the inhibitor. This result was in agreement with the presence of a methyl group at the para position of the 5-phenyl ring of several COX-2 inhibitors, such as celecoxib. The Grid map generated for a trifluoromethyl group also gave interesting results. In detail, the best interaction with COX-2 was found in the region where the trifluoromethyl group of SC-558 (**7**) was located in the crystallographic structure. Changing the CF_3 probe into a fluorine probe, three major interaction sites were found. However, one of them was worthy of further consideration, suggesting that a fluorine substituent at the para positions of the 1-phenyl ring (similarly to the bromine of SC558) and, additionally, at the meta position of the same ring could improve the interaction with COX-2, involving Trp387, Tyr348, and Tyr385 of the hydrophobic cavity. On the basis of such considerations, molecular modeling simulations suggested structural modifications of the parent compounds aimed at synthesizing a second generation of pyrrole derivatives endowed with an improved biological profile. In agreement, fluorinated derivatives were found to have a very low IC_{50} toward COX-2 (up to a 10 nM concentration) with a selectivity index [expressed as the ratio between IC_{50}(COX-1)/IC_{50}(COX-2)] higher than 10000, further validating the reliability of the computational protocol in finding the binding mode of 1,5-diaryl-2-methyl-3-substituted pyrrole derivatives within the COX-2 binding site.

Moreover, molecular docking simulations were also able to account for the marked decrease of activity toward COX-2 (up to 270-fold) due to the inversion of the substitution pattern at positions 1 and 5 of the pyrrole ring. In fact, a reorientation of the inhibitors within the binding site led to a different and less profitable interaction pattern with the enzyme. The *p*-methylsulfonylphenyl moiety at N1 was accommodated within the carboxylate site and the ester side chain at C3 matched the selectivity site, while the remaining aryl moiety at C5 filled the hydrophobic pocket, as found for the N1 aryl substituent of the corresponding isomer. Although these poses showed electrostatic and hydrophobic interactions that contributed to the stabilization of the complex with COX-2, they lacked additional contacts found in the corresponding isomers, such as hydrogen bonds at both the carboxylate and the selectivity site (with Arg120 and Gln192, respectively), as well as hydrophobic interactions involving the methyl group at the pyrrole C2.

Previous results of modeling calculations, as well as biological data and SAR considerations were taken into account for further optimizing pyrrole compounds into better hits. In particular, the nature and the mutual position of the substituents at both the phenyl rings at C5 and N1 positions was found to influence affinity toward COX-2: the marked difference in activity among the ester derivatives suggested the importance of the SO_2Me pharmacophoric portion in the phenyl ring when placed at C5 instead of N1, and SAR data indicate that the SO_2Me at the C5 phenyl ring and a fluorine substituent at the N1 phenyl ring are cooperative determinants of both potent and selective COX-2 inhibition (Table **1**). Moreover, inspection of the binding mode of pyrrole derivatives bearing an acetic acid ethyl ester moiety at C3 led to the suggestion that its terminal alkyl moiety was very important for hydrophobic interactions with Val116 and Tyr355, and substituents larger than an ethyl group could be accommodated within the carboxylate site. Accordingly, the methylsulfonyl group was kept fixed at the para position of the C5 phenyl ring, several small substituents were used to decorate the N1 phenyl ring, while the size and length of the ester chain at C3 were modified by insertion of different alkyl groups. In particular, butyl and isopropyl groups were chosen as the terminal portion of the ester moiety with the aim of satisfying the geometrical constraint suggested by Grid calculations with the methyl probe. Docking simulations on pyrrole derivatives bearing bulkier ester functions showed that the isopropyl moiety, having the same length of an ethyl group and a larger size, occupied the Grid minimum point of the C3 probe. On the other hand, the butyl chain assumed a folded conformation with its carbon atoms accommodated in a region corresponding to the Grid C3 best interaction point, and thus making similar hydrophobic contacts with enzyme residues. As expected, such compounds showed a very impressive inhibition of COX-2 (the best value being 0.007 μM) and a selectivity index higher than 2300 (Table **4**), resulting in a significant improvement with respect to parent compounds.

Transforming the ester side chain at position 3 of the pyrrole nucleus into the corresponding acidic group (Table **1**) did not significantly influence orientation of compounds within the COX-2 binding site. Acidic compounds were docked in both their protonated and unprotonated forms, but no substantial difference was found in their binding mode. In principal, the best orientations of both forms of acidic compounds filled the binding site of COX-2 similarly to the corresponding ester derivatives. Moreover, both forms of the optimized complexes were able to form a network of three hydrogen bonds with the guanidino group of Arg120 (carboxylate site), while one of the sulfone oxygens interacted by a hydrogen bond with the NH backbone group of Phe518 within the selectivity site (similarly to the ester derivatives). However, a trend in activity was found showing that esters were in general more active than the corresponding acidic analogues, because of hydrophobic interactions involving the terminal alkyl chain of the ester function and residues of the carboxylate site (such as Leu93, Val116, Tyr355, and Leu359).

On the contrary, the conformational rigidity introduced into keto-ester derivatives (Table **1**) significantly influenced the binding mode of such compounds within the COX-2 binding site. In fact, the best orientation of these derivatives showed an interaction pattern characterized by the methylsulfone group located within the hydrophobic pocket, while the C2 methyl group matched the carboxylate site. In such an orientation, although the ester carbonyl group of the inhibitor was able to make the usual hydrogen bond with the NH backbone group of Phe518 (selectivity site), additional interactions (i.e., the hydrogen bond between the ester chain and Arg120 and the hydrophobic interactions with the hydrophobic pocket) important for a profitable binding within the active site, were lost. On the basis of their lower affinity with respect to the corresponding ester and acidic derivatives (and consequent lower selectivity between the two forms of the enzyme), probably due to the different structural properties introduced into the side chain at the position 3 on the pyrrole nucleus, keto-esters were considered not much attractive compounds and were not investigated further.

Transformation of the ester moiety into an ether substituent led to compounds that retained an unexpected very good affinity toward COX-2 (Table **3**). In fact, since the oxygen atoms of the ester chain were involved in a network of hydrogen bonds with Arg120 of the carboxylate site, it was in principle difficult to understand why ether derivatives could have highly selective and potent COX-2 inhibitory activity, even in the absence of the carbonyl group. An analysis of the interaction pattern with COX-2 binding site showed that the ether oxygen atom contacted the guanidino moiety of Arg120, while the alkyl portion of the ether chain was accommodated within a hydrophobic region, defined by Leu93, Val116, and Leu359, where the best interaction point with a C3 probe was found by Grid. This means that the sole ether oxygen could serve as a profitable anchor point for the electrostatic interactions with the carboxylate site, reinforced by hydrophobic contacts between the more lipophilic alkoxyethyl moiety and the hydrophobic environment of the carboxylate site itself.

Biological data showing that replacement of the ester function at the C3 position of the pyrrole nucleus with the corresponding acidic and ether groups led to compounds with an important affinity toward COX-2, suggested that position 3 of pyrrole was permissive and tolerant to a number of substituents and functional groups, as already reported in the literature [42]. However, C3 substituents obtained by transformation of the methylenic bridge of ethyl esters into a chiral center bearing an alcohol or ether substituent, were not optimal for interacting with the COX-2 binding site. In fact, although the new chiral derivatives adopted a binding mode very similar to that previously described for the corresponding achiral parent compounds and several groups of the ligands (such as the sulfonylmethyl and the carbonyl moieties) were confirmed as important anchor points for the binding of COX-2 inhibitors, their interactions with the amino acids of the COX-2 binding site were less profitable in comparison to those found for the corresponding ester, acid, and ether derivatives, thus accounting for their lower affinity toward COX-2.

CONCLUSIONS

Based on the results elicited from the compounds reported in this review, some considerations can be drawn:

- the position of the *p*-methylsulfonyl moiety was very important for the activity (as reported before);
- substituents and substitution pattern on the phenyl ring at N1 affected the activity, establishing a scale of activity;
- the branched side chains at C3 of the pyrrole could better interact with the corresponding pocket of the enzyme allowing an increased penetration into the pocket C, and, as a consequence, a shorter distance from the residue of Leu359, responsible for interaction.

In general, the esters proved to be more active than the corresponding acids and ketoesters. The discrepancy detected between the HWB and the cell culture assays could be due to the related different inhibitory sensitivity of the mouse and human COX isozymes. The compounds showed a very good activity against carrageenan-induced hyperalgesia, proving to be very effective and long lasting active, having been observed in this test a relevant analgesia still present at 2 hours after administration. In parallel, a very good activity was demonstrated in the carrageenan-induced oedema in the rat paw test and a dose-dependent anti-nociceptive activity in the abdominal constriction test.

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ABBREVIATIONS

t-NSAIDs = traditional Non-Steroidal Anti-inflammatory Drugs
NSAIDs = Non-Steroidal Anti-inflammatory Drugs
COX-2 = Cyclooxygenase-2
COX-1 = Cyclooxygenase-1
HWB = Human Whole Blood
PG = Prostaglandin
TX = Thromboxane
AA = Arachidonic Acid
IL-1β = interleukin-1β
GI = Gastrointestinal
RCTs = Randomized Clinical Trials
UGIB = Upper Gastrointestinal Bleed
MI = Myocardial Infarction
LSS = Laminar Shear Stress
TNF-α = Tumor Necrosis Factor-α
DFU = 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone
IP = PGI$_2$ receptor
SNPs = Single-nucleotide polymorphisms
CV = Cardiovascular
MAP = Mitogen-activated Protein
ADME = Absorption, Distribution, Metabolism, Excretion
SAR = Structure Activity Relationship
HPLC = High Performance Liquid Chromatography
SI = Selectivity Index

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