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Research paper

Therapeutic potential for coxibs-nitric oxide releasing hybrids in cystic fibrosis

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A B S T R A C T

This review discusses the rational for further studies of COX-2 inhibitors-NO releaser hybrids (NO-Coxibs) in the pharmacological treatment of the airway inflammation in Cystic Fibrosis (CF). Our research group developed several classes of NO-Coxibs for the pharmacological treatment of arthritis, and among them several compounds showed an outstanding *in vivo* efficacy and good pharmacokinetic properties. The good antiinflammatory properties displayed by these compounds during the previous screening could, by itself, suggest appropriate candidates for further testing in CF.

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1. Introduction

Cystic fibrosis (CF) is one of the most common genetic inherited life-shortening disease affecting Caucasian population. It is caused by mutations in the gene of the cystic fibrosis transmembrane regulator (CFTR), a cAMP-dependent anion channel mostly expressed in the apical membrane of epithelia cells [1,2]. Therefore, the primary defect in CF patients is a reduction in chloride and bicarbonate transport capacity across the apical membrane of epithelial cells, which in turn impacts on water secretion and sodium concentration, altering the characteristics of the secreted fluids [3]. The most common CFTR mutation found in CF patients is F508CFTRdel. This deletion results in a loss of the phenylalanine at position 508 of the protein, which prevents the protein from folding efficiently and affects the function and processing of the CFTR molecules [4]. Although, the shortened life expectancy accompanying the disease can most often be attributed to pulmonary complications [5], patients with CF also suffer from a variety of systemic complications including dysfunction of the gastrointestinal (pancreas and liver), renal, immune, endocrine and male genital systems [6,7]. In the lungs CFTR dysfunction is associated with loss of the critical hydration of the airway surface, abnormal mucin secretion, epithelial cell vulnerability and with dysregulation of the local inflammatory responses, resulting in excessive airway neutrophilic inflammation and pathogen growth [8]. The resulting dysregulation of defence function followed by persistent bacterial infections gives rise to airway damage and remodelling, leading to severe lung deficiency [9,10].

1.1. Inflammation in CF

In the past, the combination of mucus obstruction and infection was believed to drive the exaggerated and dysfunctional inflammatory response, which leads to irreversible airway destruction and fibrosis and hence to severe lung damage, the main cause of morbidity and mortality in CF patients. More recently, it has been

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Abbreviations

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CINODs	COX-inhibiting nitric oxide donors
COX	cyclooxygenase
CV	cardiovascular
GSNO	S-nitrosoglutathione
GI	gastrointestinal
LPS	lipopolysaccharide
NO	nitric oxide
NOS	nitric oxide synthase
NOBA	4-(nitroxy) butanol
NO-coxibs	NO-releasing coxibs
NSAIDs	nonsteroidal anti-inflammatory drugs
ODQ	1H-[1,2,4]-oxadiazole[4,3-a] quinoxalin-1-one
PGH2	prostaglandin H2
PGI_2	prostacyclin 2
PBS	phosphate buffered saline
SAR	structure-activity relationships
SBP	systolic blood pressure
SGF	simulated gastric fluid
SHRs	spontaneous hypertensive rats
SNOs	S-nitrosothiols
t-NSAIDs	*traditional*-NSAIDs
WHB	whole human blood

shown that neutrophilic inflammation is already present in young children with CF, even in the absence of respiratory infections, suggesting that the disease itself might be related to anomalous inflammatory responses [11]. Moreover, it has been reported how CFTR defects in CF contribute to endogenous activation of NF-kB, and to the exaggerated production of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α, even in the absence of bacterial infections [12–14]. In addition to increased release of pro-inflammatory cytokines, it was also demonstrated an over-production of PGE_2 and other prostanoids in patients with CF [15]. Cyclooxygenase (COX) represents the key enzyme in arachidonic acid metabolism, converting arachidonic acid to prostaglandin H2 (PGH2). COX consists of two isoforms: COX-1 and COX-2. The constitutively expressed COX-1 exerts housekeeping functions in most tissues maintaining the homeostasis. Inhibition of COX-1 reduces platelet thromboxane B_2 and PGE_2 production in several tissues. Even though COX-1 may contribute to the total pool of prostaglandins at the site of inflammation, its contribution to inflammation is at the present not fully understood. Conversely, COX-2 is induced, in response to inflammatory cytokines, in various cell types (including monocytes/macrophages). Since this isoform is notably activated by pro-inflammatory stimuli and presents mainly a pro-inflammatory function, it was highlighted as the most important player in inflammatory processes. COX-2 is considered the main target for NF-kB activation, a key factor in the dysregulation of the innate immune and host inflammatory response [16]. It is well known how the mutations in CFTR influence the lung epithelial innate immune function leading to exaggerated and ineffective airway inflammation that fails to abolish pulmonary pathogens and gives rise to structural damage of the airways impairing lung function and ultimate leading to respiratory failure and death [10]. It was recently shown, how the reduction of COX protein levels (mainly COX-2) had a positive effect on all analysed clinical parameters. This suggests an important role of the inhibition of arachidonic acid conversion into prostaglandins which, by reducing the inflammatory process, acts as protective modifier of pulmonary disease in CF patients [17,18].

With the recognition that airway inflammation plays a significant pathogenic role in CF, modulation of the inflammatory response has become a significant therapeutic target and drugs that target inflammation have been shown to slow the decline in lung function and improve survival [19,20]. Corticosteroid administration showed positive effects on lung function; however, the accompanying adverse effects led to premature discontinuation of clinical trials. Ibuprofen, a COX-1/COX-2 inhibitor, is the only anti-inflammatory drug currently recommended for the long-term treatment of CF airway inflammation [21]. In animal models of chronic infection, high-dose ibuprofen was demonstrated to reduce inflammation without hindering bacterial clearance. This led to clinical trials which demonstrated a benefit in slowing the progression of lung disease in CF. However, concerns about adverse effects have limited the use of high-dose ibuprofen in CF patients. Interestingly, it was observed that ibuprofen at the high clinically relevant doses can suppress transcriptional activity of NF-κB and other pro-inflammatory transcription factors [22]. High-dose ibuprofen can also enhance the function of F508delCFTR and it corrects CFTR function [23].

1.2. CFTR correctors and potentiators

In addition to therapeutics that have been developed to treat the symptoms of CF, more recent research has been focused on the development of therapies targeting the root cause of CF, identifying suitable compounds that could reverse the CFTR molecular defect and prevent the progression of CF disease [24]. Recent studies have revealed that inhibition of F508delCFTR ubiquitination and proteasomal degradation with chemical or pharmacological chaperones promotes its correct folding and channel function. Thus, pharmacological strategies that increase the maturation of the mutant CFTR (correctors) [25] and/or potentiate channel conductance (potentiator) [26] should be beneficial to the majority of CF patients. The knowledge of the CFTR molecular target, the development of appropriate pharmacological tests along with high-throughput screening led to the success in partial pharmacologic restoration of CFTR activity. However, the currently available clinical data suggest that this therapy will require combinations of CFTR correctors and potentiator exploiting different mechanisms of action with additive/synergistic efficacies. A few small molecules targeting mutant CFTR are available for CF patients: lumacaftor (VX-809) (corrector) and ivacaftor (VX-770) (potentiator) along with the corresponding combination therapy [27,28]. Unwanted side effects and drug interactions of lumacaftor have recently been overcame with the development of tezacaftor (VX-661) [29,30]. Recently, a triple combination of two correctors, elexacaftor (VX-445) and tezacaftor, and a potentiator (ivacaftor) has been approved for the treatment of CF patients carrying the F508del-CFTR mutation [31–33]. Despite these progresses many CF patients still await more effective drug treatments [34].

1.3. Nitric oxide and CF

Nitric Oxide (NO), formed from L-arginine by NO synthases (NOS-1,2,3) has been shown to play an essential role in a variety of biological processes in the lung, including bronchodilation, inflammation and host-defence against pathogens [35–37]. NOS1 derived NO is a neurotransmitter and is involved in human

bronchomotor control. Its anomalous activity contributes to the development of airway obstruction. In the lung NOS2 is expressed by macrophages, neutrophils and bronchial epithelial cells and manly acts as antimicrobial agent. NOS3 is constitutively expressed in pulmonary blood vessels, airway epithelial cells and neutrophils. There is increasing evidence that NOS3 contributes to NO related patho-physiology in the airways [38].

Although NO concentration in exhaled air is generally increased in inflammatory lung diseases like asthma and bronchiectasis, in CF patients it is not increased, or even decreased. Moreover, in upper airways NO is significantly lower in CF patients than in controls [39]. The reasons for this NO deficiency may include reduced expression of NOS, as well as lower availability of L-arginine [40]. Therapeutic interventions aiming at correcting the NO deficiency in CF patients are therefore currently being explored as new therapies for this disease. A clinical study assessing the safety and efficacy of inhaled NO as adjuvant therapy in CF patients was reported [41]. However, the hazards of pressurized cylinders and toxicity concerns have led researchers to develop and study the utility of NO donors. For example, the cephalosporin (NO)-donor prodrug DEA-C3D designed to deliver NO to bacterial infection sites, selectively releases NO in response to contact with bacterial β-lactamase [42]. Several carbonic anhydrase inhibitors NO-donors have shown good ability in reducing elevated intraocular pressure in an animal model of ocular hypertension for the treatment of glaucoma [43–45]. Alginate biopolymers can be chemically modified to store and release NO. The resulting N-diazenium diolate alginate NO donors showed in aqueous solution NO-release kinetics able to produce therapeutic NO levels. In particular, the generated NO showed bactericidal activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* and other strains relevant to CF [46,47]. S-nitrosothiols (SNOs), which can be formed by reaction of NO with thiols, are endogenous cell signalling molecules present in the lung [48–50]. Despite the physiological importance, low concentrations of SNOs were found in CF patients [51]. It was reported that S-nitrosoglutathione (GSNO) increases cellular expression, maturation, and function of CFTR in human airway epithelial cell cultures expressing mutant F508del CFTR [52]. These actions reflect in a clinical efficacy in CF patients treated with GSNO [53,54]. More recently, it was reported how this effect is common also with other SNOs [55]. Therefore, the ability of SNOs to augment the maturation and stability of the CFTR could be helpful on the treatment of CF, and there is growing interest in more bioavailable and stable NOSs compounds or corresponding precursors as a novel class of corrector therapies for CF (Fig. 1).

1.4. COX-inhibiting nitric oxide donors (CINODs)

Among all the mediators participating in the inflammation process, prostaglandins remain the major target of anti-inflammatory therapies since nonsteroidal anti-inflammatory drugs (NSAIDs) have been discovered. NSAIDs are also widely used to treat the inflammatory pain and act by inhibiting COX isoforms. As discussed in paragraph 1.2, the COX isoforms catalyse the conversion of arachidonic acid to prostanoids, but with differences in terms of expression, function and structure. The constitutively expressed COX-1 exerts housekeeping functions in most tissues maintaining the homeostasis. It is also important for gastric cytoprotection. COX-2 is induced in response to inflammatory cytokines giving rise to a massive release of PGE_2, and it is the main player in inflammation. However, COX-2 is also constitutively expressed in some tissues (e.g. kidney, endothelial cells and brain) where its products PGE_2 and prostacyclin (PGI_2) are involved in diverse physiological functions including renal haemodynamic and the control of blood pressure along with endothelial thromboresistance. COX-1 and COX-2 enzymes share about 60% sequence identity and are characterized by similar three-dimensional structures. However, the COX active sites are not identical, the COX-2 binding site is more flexible and 25% larger than the one of COX-1, with a side-pocket not present in the COX-1 isoform. These differences in the binding site size and structure enable to develop ligands "selective" for COX-2. All NSAIDs can inhibit COX-1 and COX-2 in a concentration-dependent fashion. The ratio between the 50% inhibitory concentration (IC_{50}) values for COX-1 and COX-2 is used to describe the COX-2 selectivity of these drugs and to characterize selective vs. nonselective inhibitors. The selective COX-2 inhibitors have been named *coxibs* while COX inhibitors with scanty selectivity for COX-2 are referred to as *traditional-NSAIDs* (*t*-NSAIDs). Therapy with *t*-NSAIDs is hampered by some side-effects including gastrointestinal erosions and renal insufficiency. Such critical adverse reactions are mostly dependent on COX-1 inhibition [56]. Due to its main pro-inflammatory function, COX-2 was

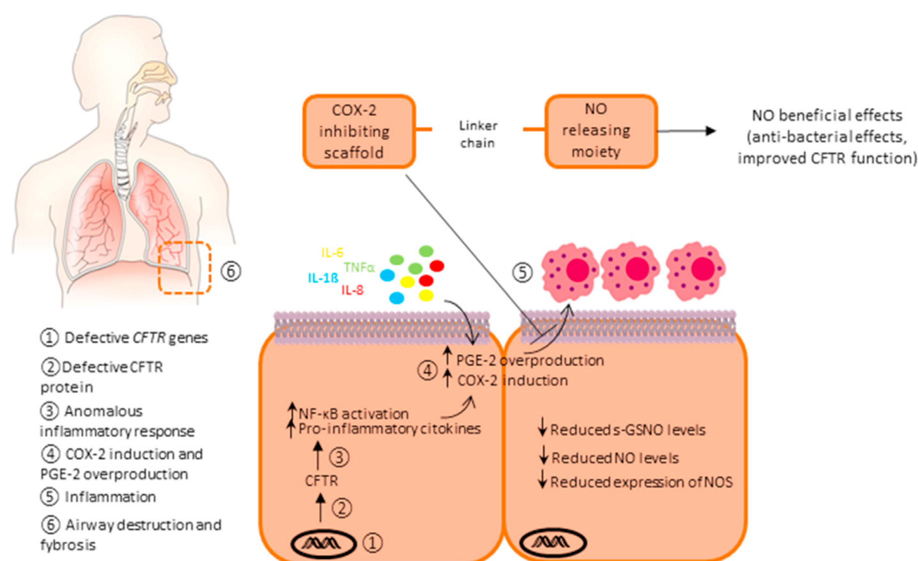


Fig. 1. Perspective on the rationale of using NO-Coxibs in Cystic Fibrosis.

highlighted as a suitable target for treating inflammation, possibly without gastrointestinal (GI) side-effects [57]. Consequently, coxibs were developed to overcome the GI and renal side effects associated with the use of *t*-NSAIDs [58]. Later on, numerous studies of clinical pharmacology and pharmacoepidemiology showed that preferential inhibition of COX-2 translates into a cardiovascular (CV) hazard [59]. The pattern of the cardiovascular effects of COX-2 inhibitors is consistent with the increased risk of coronary events being related to a diminished restraining effect of endothelial PGI_2 on platelet activation, as suggested by in-depth studies of experimental thrombogenesis in COX-2-deficient mice [60]. This CV hazard is shared with *t*-NSAIDs through the same mechanism and it is more relevant as more is pronounced the preference for COX-2 inhibition of the drug [61].

COX-inhibiting nitric oxide donors (CINODs) was a class of drugs initially developed to combine the pharmacological actions of a COX inhibitor with the protective actions of NO, with the aim of reducing the GI toxicity of the drug, while maintaining its analgesic and anti-inflammatory effects. Naproxcinod (AZD3582) was the first in class of this group of compounds formerly developed by NicOx [62]. As NO is endowed with remarkable CV protective actions, being involved in vascular smooth muscle relaxation, platelet aggregation and thrombosis inhibition, it was speculated that NSAIDs endowed with NO-releasing properties could also mitigate the corresponding CV hazard. Aiming to design COX-2 selective inhibitors with improved CV safety, NO releasing coxibs have been synthesised, in order to compensate for the COX-2 effects on prostacyclin with NO-mediated CV effects. Our past research has been focused on the development of a new class of potent and selective coxibs based on the diarylpyrrole scaffold [63–65]. Later on, we developed a class of NO-releasing coxibs (NO-coxibs) endowed with anti-inflammatory properties and characterized by a better cardiovascular safety profile, due to NO antithrombotic and cardioprotective effects [66–68]. The pharmacokinetic of the compounds and the NO releasing properties were also optimized in order to provide compounds with the appropriate duration of action along with slow NO releasing properties [69–71]. Within the same timeframe, an interesting NO-releasing prodrug of rofecoxib [72], was reported as well as nitroxy-substituted 1,5-diarylimidazoles [73] and corresponding analogues of celecoxib [74]. In this review we discuss the rational for further studies of COX-2 inhibitors-NO releaser hybrids (NO-Coxibs) in the pharmacological treatment of the airway inflammation in CF. Our research group developed several classes of NO-Coxibs for the pharmacological treatment of arthritis, and among them several compounds showed an outstanding *in vivo* efficacy and good pharmacokinetic properties. The good antiinflammatory properties displayed by these compounds during the previous screening could suggest, by itself, appropriate candidates for further testing in CF.

2. NO-coxibs based on diarylpyrrole scaffold: nitrooxyalkyl esters

Due to their high COX-2 inhibitory potency and selectivity, 1,5-diarylpyrrole-3-acetic acid (**1a-d**) and esters (**2a-m**) (Fig. 2) were the starting point for this work. As structure-activity relationships (SAR) of these compounds was investigated in previous works [63–65], the substitution patterns on N1-phenyl that provided better results were selected for this study (representative substituents: H, 3-F, 4-F, 4-OMe) as well as the preference for the *p*-methylsulfonyl-phenyl moiety in the C-5 position of the pyrrole ring. CINOD structures are characterized by a central core responsible for COX inhibition with a linked NO donating moiety. In particular, naproxcinod is the ester of naproxen with 4-(nitroxy) butanol (NOBA) (Fig. 2). Among the possible approaches for "hybridization" of our scaffold, the one consisting in the manipulation at position-3 of pyrrole nucleus, allowed us to retain COX-2 inhibitory properties and to introduce the NO-releasing moiety. Conversely, other scaffold manipulations led to the loss of COX-2 inhibition.

In order to test the pharmacological hypothesis of the study, the straightforward preparation of 1,5-diarylpyrrole-3-acetic nitrooxyalkyl esters (**3a-h**) (Table 1) [66] as NO-releasing hybrids was chosen. These compounds were designed by combining the 1,5-diarylpyrrole moiety (compounds **1a-d**) with NO-donor alkyl chains of different length, on the basis of previous molecular modelling considerations on different length and hindrance chains of analogue esters **2a-m** [63]**.** Since the nitrooxyalkyl groups are metabolized *in vivo*, we also considered the corresponding hydroxyl derivatives (**4a–h**) (Table 1) as possible metabolites. All compounds herein described were evaluated for *in vitro* potency and selectivity for COX-1 and COX-2 inhibition in a J774 murine macrophage cell line. For selected compounds the human whole-blood assays (WHB) was used to assess COX-1/-2 activities and selectivity. This test is represented by WHB assay of COX isoforms activities that exploit the constitutive and lipopolysaccharide (LPS)-inducible COX activities of circulating platelets and monocytes, as reflected by serum TXB_2 and plasma PGE_2 levels, respectively [66]. In order to evaluate NO-releasing properties the compounds were evaluated assessing their efficacy and potency in determining NO-vasorelaxing responses, using a model of vascular smooth muscle relaxation (endothelium denuded rat aortic rings) [66]. In order to confirm that the vasorelaxing effects observed were actually due to the NO release, the experiments were also carried out in the presence of the guanylate cyclase inhibitor 1H-[1,2,4]-oxadiazole [4,3-a] quinoxalin-1-one (ODQ). The time dependent determination of nitrate and nitrite formation by incubation with liver homogenate was assessed as well [67,70]. For selected compounds, *in vivo* anti-inflammatory and analgesic activities were evaluated in animal models of inflammation, using the chemical visceral inflammatory model (pain induced by the intraperitoneal injection of acetic acid, writhing test), and the carrageenan-induced inflammatory pain model [66]. Intraperitoneal injection of acetic acid induces abdominal irritation characterized by abdominal stretching, flinching, licking, and motor incoordination. The writhing response is due to the release of cytokines, prostaglandins, and bradykinin, factors capable of sensitizing visceral nociceptive

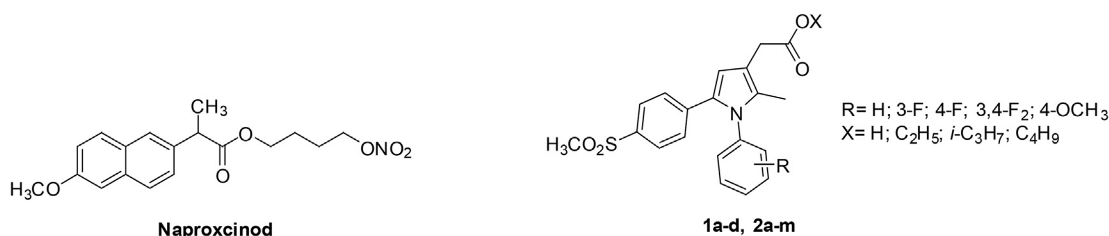
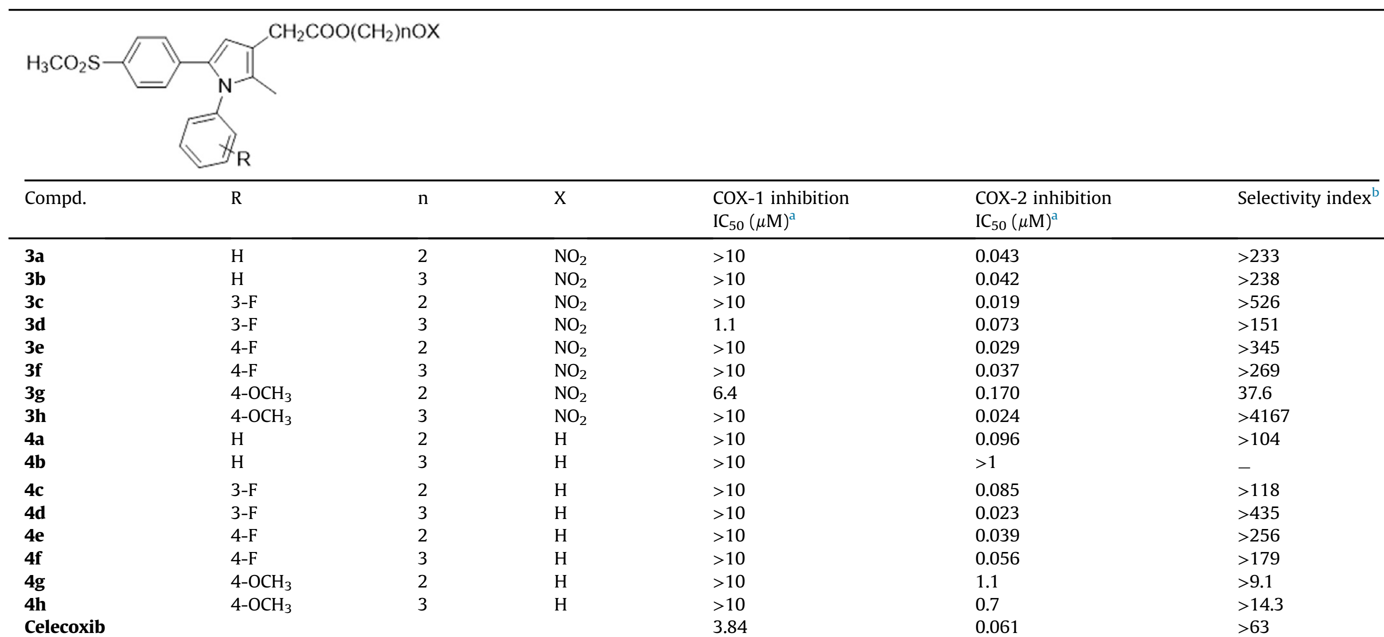


Fig. 2. Chemical structures of Naproxcinod and compounds **1a-d** and **2a-m**.

Table 1
COX-1 and COX-2 inhibitory activity of **3a**−**h, 4a**−**h,** and Celecoxib.



Compd.	R	n	X	COX-1 inhibition IC_{50} (μM)[a]	COX-2 inhibition IC_{50} (μM)[a]	Selectivity index[b]
3a	H	2	NO_2	>10	0.043	>233
3b	H	3	NO_2	>10	0.042	>238
3c	3-F	2	NO_2	>10	0.019	>526
3d	3-F	3	NO_2	1.1	0.073	>151
3e	4-F	2	NO_2	>10	0.029	>345
3f	4-F	3	NO_2	>10	0.037	>269
3g	4-OCH_3	2	NO_2	6.4	0.170	37.6
3h	4-OCH_3	3	NO_2	>10	0.024	>4167
4a	H	2	H	>10	0.096	>104
4b	H	3	H	>10	>1	_
4c	3-F	2	H	>10	0.085	>118
4d	3-F	3	H	>10	0.023	>435
4e	4-F	2	H	>10	0.039	>256
4f	4-F	3	H	>10	0.056	>179
4g	4-OCH_3	2	H	>10	1.1	>9.1
4h	4-OCH_3	3	H	>10	0.7	>14.3
Celecoxib				3.84	0.061	>63

[a] Results are expressed as the mean (n = 3 experiments) of the % inhibition of PGE_2 production by test compounds with respect to control samples and the IC_{50} values were calculated by GraphPad Instat program; data fit was obtained using the sigmoidal dose−response equation (variable slope) (GraphPad software).
[b] *In vitro* COX-2 Selectivity Index [IC_{50} (COX-1)/IC_{50}(COX-2)].

afferents [75]. The carrageenan-induced inflammatory pain model is widely used to assess the anti-inflammatory activity of several compounds. Carrageenan is a non-antigenic phlogistic agent with the devoid of any visible systemic effects, it contains sulphated sugars that are liable for the activation of complement system and the inflammatory mediators [76]. Compounds **3a-h** and **4a-h** were then evaluated according to the above pharmacological protocol. Nitrooxyalkyl esters **3a-f** and **3h** resulted very active and selective COX-2 inhibitors (Table 1). They displayed better potencies and selectivity than corresponding acids **2a-g** and esters **1a-g.** Dependence on the side-chain length with the COX-2 inhibition potency was highlighted in this series. Hydroxylated derivatives (**4a**−**h**) displayed a similar trend. The side-chain length seems to be crucial also in determining NO-dependent vasorelaxing responses. For compounds **3a-h** strong vasorelaxing effects (pIC_{50} ranging between 5.47 and 6.75) (Table 2) were found only for the two-carbon chain nitroesters (**3a**, **3c**, **3e**, **3g**), while a weak activity characterized the three-carbon chain derivatives. The test with ODQ confirmed these vasorelaxing effects were due to the NO release. The total loss of vasorelaxing activity of the hydroxylated derivatives **4a**−**h** further supported the absence of contribution from other moieties of the molecule to the CV effect**.** Additional experiments suggested that these compounds ensured a slow NO release suitable for the expected pharmacological action [66]. The *meta*-fluorine subclass of compounds (**3c-d**) was selected for *ex vivo* and *in vivo* studies. The COX-2 selectivity was assessed *ex vivo* for derivatives **3c** and the expected metabolite **4c**, by the HWB assay. Compounds **3c** and **4c** inhibited platelet COX-1 in a concentration-dependent fashion, with IC_{50}: 9.6 μM and 12.2 μM respectively. Compounds **3c** and **4c** displayed similar inhibitions of monocyte COX-2 with IC_{50}: 3.2 μM and 1.7 μM, respectively (Celecoxib COX-1 12.5 μM; COX-2 0.54 μM). Thus, **3c** was 3-fold and **4c** was 7.3-fold more potent toward COX-2 than toward COX-1; the drop in COX-2 potency and selectivity in HWB assay versus J774 model can be explained by the different sensitivities of mice and human COX

Table 2
Efficacy and potency in determining NO-dependent vasorelaxing responses of **3a**−**h** and GTN[a].

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

[a] GTN: glyceryl trinitrate.
[b] Emax represents the vasorelaxing efficacy, expressed as a % of the vasoconstriction induced by the pre-administration of KCl.
[c] Parameter of potency is expressed as pIC_{50}, representing −log of the molar concentration capable of inducing a vasorelaxing effect = 50% of E_{max}.

Table 3
Effect of **3c-d, 4c-d,** Celecoxib, and vehicle (CMC) in the mouse abdominal constriction test (Acetic acid 0.6%).

Compd.[a]	No. mice	dose po, mg/kg (μmol/kg)[b]	no. writhes
CMC	43	_	32.6 ± 2.1
3c	12	20 (42)	17.1 ± 2.3*
3c	12	40 (84)	9.4 ± 2.5*
3d	18	20 (41)	25.7 ± 2.8∧
3d	10	40 (82)	24.9 ± 2.2*
4c	8	3 (7)	26.9 ± 3.0*
4c	8	10 (23)	17.4 ± 2.9*
4c	8	20 (46)	16.2 ± 2.8*
4c	8	30 (70)	8.9 ± 2.1*
4d	10	20 (45)	25.3 ± 2.8
4d	10	40 (90)	15.6 ± 2.2*
Celecoxib	10	10 (26)	13.4 ± 2.6*

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

enzymes. The writhing test confirmed the *in vivo* anti-inflammatory activity for all the compounds tested (Table 3). Despite the higher *in vitro* potency, all the compounds were *in vivo* less potent than Celecoxib, though similar effects could be obtained at higher doses. Findings reported for carrageenan induced hyperalgesia and edema showed that all compounds were endowed with a satisfactory activity (Table 4). Overall the activity of the compounds was remarkable, albeit not comparable to celecoxib. Noteworthy, compound **3c** (*in vitro* more active than celecoxib) was less effective and had a shorter duration of action in the *in vivo* test. The shorter duration of the pharmacological effect suggested a possible metabolic deactivation for compound **3c** and/or other pharmacokinetic issues.

2.1. NO-coxibs based on diarylpyrrole scaffold: esters, the solubility issue

Since the pharmacokinetic issues affecting the *in vivo* effects seen with compounds **3c-d**, in addition to metabolism, can also be due to absorption, the compounds' solubility was investigated as well. Simulated gastric fluid (SGF-without pepsin) and phosphate buffered saline (PBS) were chosen to evaluate the solubility in acid (pH 1.5 for SGF) and neutral conditions (7.4 pH for PBS). The solubility of **3c** was < 1μM and 1.6 μM respectively in SGF and PBS; similarly, the solubility for compound **4c** was 1<μM and 1.1 μM respectively in SGF and PBS [69]. These molecules were then characterised by low solubility and this feature could heavily affect their absorption. In order to increase solubility an ionisable moiety was added to the scaffold, thus an amino group was introduced at the α-position of the acetic side chain, since previous SAR studies highlighted this as a favourable position for such a change [64]. Compounds **5a-d** along with their corresponding derivatives **6a-d** were prepared for exploring the approach (Table 5) [69]. The NO-release properties of the compounds were appropriate (**5a**: $E_{max} = 84.0 \pm 2.0$; $pIC_{50} = 5.66 \pm 0.03$; **5c:** $E_{max} = 93.0 \pm 1.0$; $pIC_{50} = 5.81 \pm 0.03$) [69]. As expected, the ionisable moiety determined a remarkable increase of solubility. Solubility of **5a** was >200 μM and 70.8 μM, respectively in SGF and PBS, and solubility for compound **5b** was >200 μM and 80.0 μM, respectively in SGF and PBS. Despite the cell-based assay for COX activity showed a reduction in the potency for compounds **5a-d** and **6a-d** in comparison with **3c-d** and **3e-f** (Table 1), the writhing test highlighted for compounds **5a** and **5c** (Table 6) [69] activities comparable to those of compounds **3c-d**. This finding strongly pointed out the solubility among the causes responsible for the drop of the *in vivo* activity seen with compounds **3c-3d**. Compound **5a** was selected

Table 4
Effect of **3c-d, 4c-d,** Celecoxib, and vehicle (CMC) on hyperalgesia and edema induced by carrageenan in the rat paw pressure test[a].

paw pressure (g)							
pretreatment, ipl	treatment, po	dose mg/kg	before treatment	after treatment			volume (mL)
				30 min	60 min	120 min	60 min
saline	**CMC**		62.6 ± 2.1	62.5 ± 1.8	63.4 ± 2.2	62.7 ± 2.5	1.33 ± 0.07
carrageenan	**CMC**		61.9 ± 2.0	33.7 ± 2.6	35.1 ± 2.3	34.8 ± 2.3	2.44 ± 0.08
carrageenan	**3c**	20	32.6 ± 2.7	40.1 ± 3.4	35.3 ± 2.8	ND	2.52 ± 0.06
carrageenan	**3c**	40	29.8 ± 3.1	54.1 ± 3.7*	38.7 ± 3.1	ND	2.17 ± 0.08*
carrageenan	**3c**	100	31.9 ± 3.0	57.2 ± 3.5*	36.5 ± 3.8	ND	1.88 ± 0.08*
carrageenan	**3d**	20	62.5 ± 3.1	43.2 ± 3.2	45.1 ± 3.7∧	40.7 ± 2.6	2.05 ± 0.08∧
carrageenan	**3d**	40	63.2 ± 3.3	45.5 ± 3.7*	44.9 ± 4.2∧	36.8 ± 3.8	1.96 ± 0.09*
carrageenan	**4c**	30	31.8 ± 2.5	55.7 ± 3.1*	52.3 ± 2.8*	39.3 ± 3.5	2.51 ± 0.05
carrageenan	**4d**	20	62.6 ± 3.1	41.2 ± 3.0	45.8 ± 3.6∧	36.4 ± 3.1	ND
carrageenan	**4d**	40	61.3 ± 3.0	43.1 ± 3.6	48.8 ± 3.6*	44.4 ± 3.3∧	ND
carrageenan	**Celecoxib**	10	61.5 ± 3.4	54.3 ± 3.9*	57.1 ± 4.0*	54.9 ± 3.6*	1.36 ± 0.07*

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

Table 5
COX-1 and COX-2 inhibitory activity of **5a-d, 6a-d**, and Celecoxib.

Compd.	R	n	X	IC_{50} (COX-1) (μM)[a]	IC_{50} (COX-2) (μM)[a]	Selectivity index[b]
5a	4-F	1	NO_2	>10	0.82	>12.20
5b	3-F	1	NO_2	>10	1.51	>6.62
5c	4-F	2	NO_2	>10	1.00	>10.00
5d	3-F	2	NO_2	>10	1.41	>1.35
6a	4-F	1	H	>10	0.76	>13.16
6b	3-F	1	H	>10	1.19	>8.40
6c	4-F	2	H	>10	0.22	>45.45
6d	3-F	2	H	>10	1.50	>6.67
Celecoxib				3.84	0.061	>63

[a] Results are expressed as the mean (n = 3 experiments) of the % inhibition of PGE_2 production by test compounds with respect to control samples and the IC_{50} values were calculated by GraphPad Instat program; data fit was obtained using the sigmoidal dose−response equation (variable slope) (GraphPad software).
[b] *In vitro* COX-2 Selectivity Index [IC_{50} (COX-1)/IC_{50}(COX-2)].

Table 6
Effect of **5a** and **5c** and vehicle (CMC) in the mouse abdominal constriction test (Acetic acid 0.6%).

Compound[a]	Dose per p.o.[b]		N. writhes	% of writhes reduction
	mg/kg	μmol/kg		
CMC			33.4 ± 2.5	–
5a	3	5.5	26.9 ± 2.6	19.0
	10	18.4	21.3 ± 2.5*	36.2
	20	36.8	18.1 ± 3.0*	46.1
	40	73.6	13.5 ± 2.8*	59.6
5c	20	35.9	24.5 ± 3.2	26.9
	40	71.8	19.8 ± 2.4*	40.7

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

for the carrageenan test: its *in vivo* activity (Table 7) was equivalent to the one of compound **3c**, more potent *in vitro*. However, the duration of action of **5a** is still shorter than celecoxib, pointing out the presence of a metabolic issue as well.

2.2. NO-coxibs based on diarylpyrrole scaffold: amides

Though the metabolism of the nitroxy derivatives may be complex, the pathway leading to the hydroxylated derivatives is widely considered as the most usual metabolic destiny of organic nitrates [77]. However, for naproxcinod and related CINODs, the esterase mediated hydrolysis into the NSAID scaffold and NOBA, followed by further metabolic conversion of the nitroester was proved to be the major metabolic pathway [78]. Accordingly, the hydrolysis of alkyl esters into the corresponding acid and nitroxy alcohol could be expected also for compounds **3a-h.** Since the enzymatic liability of the esters, was considered to be a remarkable factor in the gap between *in vitro* and *in vivo* pharmacological profiles of the compounds discussed at paragraph 2.1, four different groups of compounds (Table 8) were generated through replacement of the ester moiety with the amide group, in order to address the structural issues impacting both stability and solubility [68,71].

Firstly, a class of "simple" amides obtained through the replacement of the ester moiety with the amide group was explored. The diarylpyrrole-3-acetic scaffold was retained and the substitution pattern as well as the side-chain length were based on the previously discussed SAR. Compounds **7a-d** and the expected metabolites **8a-d** (Table 8) were synthesised and tested. Amide derivatives (**7a-d**) displayed a COX-2 inhibition ranging from 0.25 to 0.31 μM, and the corresponding hydroxylated analogues (**8a-d)** highlighted a COX-2 inhibition ranging from 0.045 to 0.30 μM. The NO-releasing properties for compounds **7a-c** are reported in Table 9. While compound **7a** showed low vasorelaxing effect, compounds **7b** and **7c** were endowed with good efficacy, with pIC_{50} spanning from 5.78 (**7b**) to 5.31 (**7c**). ODQ antagonized the vasorelaxing activity of these compounds.

In the second group of compounds (Table 8), in addition to the replacement of the ester moiety with the amide group, a carboxylate group was added to the α-position of the acetic moiety in order to increase solubility. The corresponding serine/homoserine derivatives **9a-d** and **10a-d** were synthesised and tested [71]. The homoserine derivatives were in general more potent in COX-2 inhibition than the corresponding serine derivatives, with IC_{50} ranging from 0.14 to 1.6 μM, the corresponding hydroxylated derivatives (**10a-d**) were effective in the range IC_{50} 0.068–0.16 μM. However, **9a-d** produced unsatisfactory vasorelaxing effects (Table 9), probably due to the presences of the unfavourable carboxylate moiety. In the third group of compounds (Table 8), in addition to the introduction of the amide group, an amino group was added to the α-position of the acetic moiety to increase solubility. The derivatives **11a-b** and corresponding possible metabolites **12a-b** were synthesised [71]. A good activity for COX-2 inhibition was found for both the compounds with the higher potency for **11a** (IC_{50} = 0.054 μM). Interestingly, the hydroxylated derivative **12a** lacked any relevant COX inhibitory activity, while **12b** was effective (IC_{50} = 0.047 μM). The introduction of the amide group within the glycine skeleton, did not compromise the NO-mediated vasorelaxing effects. Compounds **11a** and **11b** exhibited high levels of vasorelaxing efficacy and good potencies, with pIC_{50} = 6.38 for both compounds (Table 9). ODQ significantly antagonized the vasorelaxing activity.

Compounds **13a-b** and **14a-b** (Table 8) form the fourth group: in addition to the replacement of the ester moiety with the amide group, the compounds were manipulated in the 5-phenyl ring where the 4-sulfonyl moiety was replaced with the 4-sulfamoyl group to increase solubility [68]. However, COX-2 inhibitory potency for these compounds was rather low (IC_{50} > 10 μM) although the NO releasing properties were appropriate.

The selectivity for COX-2 inhibition was also assessed using the HWB assays for the most effective compounds **7c** and **11a**, along with the derivatives **8c** and **12a** [71]**.** Compound **7c** displayed a concentration-dependent inhibition of COX-1 and COX-2 with IC_{50} of 0.6 and 1.7 μM, respectively. Its derivative **8c**, inhibited COX-2 and COX-1 activities with similar IC_{50}: 1.4 and 1.9 μM, respectively. Thereby, in the HWB assay **7c** and its metabolite **8c** proved to be *non*-selective COX inhibitors (SI = 0.3). On the other hand, compound **11a** inhibited COX-2 and COX-1 activities in a concentration-dependent fashion with IC_{50}: 21 and 140 μM, respectively. Compound **11a** was then significantly more potent towards COX-2 than COX-1 (IC_{50}, ratio: 6.7). The corresponding derivative **12a** inhibited COX-2 activity with an IC_{50} value of 13.7 and was less potent in COX-1 inhibition (IC_{50} = 300 μM) (SI = 22) [71].

The kinetics of NO-release for compounds **7c** and **11a** were compared with naproxcinod [71]. The time-dependent increase of

Table 7
Activity of compound **5a** and Celecoxib in the carrageenan-induced inflammation[a].

Pre-treatment ipl	Treatment p.o.	Dose	Before treatment	Paw-pressure				Edema volume (mL)
				After treatment				
				15 min	30 min	45 min	60 min	
Saline	**CMC**	–	61.2 ± 2.5	65.6 ± 3.4	58.9 ± 3.8	61.4 ± 4.1	63.6 ± 4.0	1.44 ± 0.05
Carrageenan	**CMC**	–	36.1 ± 3.3	32.8 ± 2.9	30.6 ± 3.2	31.8 ± 2.2	34.8 ± 3.1	2.71 ± 0.08
Carrageenan	**5a**	20	33.8 ± 2.8	40.2 ± 3.6	43.7 ± 3.7	38.3 ± 3.1	37.1 ± 3.0	2.58 ± 0.05
Carrageenan	**5a**	40	32.9 ± 3.0	45.4 ± 3.2	50.2 ± 4.4	45.6 ± 4.3	37.5 ± 2.8	2.11 ± 0.09
Carrageenan	**Celecoxib**	10	31.7 ± 2.7	45.7 ± 4.2	52.9 ± 3.1	48.3 ± 3.4	42.5 ± 2.9	2.53 ± 0.05

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

Table 8
COX-1 and COX-2 inhibitory activity of **7a–d**, **8a–d**, **9a-d**, **10a–d**, **11a–b, 12a-b, 13a-b, 14a-b** and Celecoxib.

Compd.	R	n	X	COX-1 inhibition IC_{50} (μM)[a]	COX-2 inhibition IC_{50} (μM)[a]	Selectivity index[b]
7a	3-F	2	NO_2	>10	0.300	>33
7b	3-F	3	NO_2	>10	0.250	>40
7c	4-F	2	NO_2	>10	0.249	>42
7d	4-F	3	NO_2	>10	0.310	>32
8a	3-F	2	H	>10	0.290	>34
8b	3-F	3	H	>10	0.045	>222
8c	4-F	2	H	>10	0.300	>33
8d	4-F	3	H	>10	0.068	>147
9a	3-F	1	NO_2	>10	0.140	>71
9b	3-F	2	NO_2	>10	ND	>6
9c	4-F	1	NO_2	>10	0.310	>32
9d	4-F	2	NO_2	>10	1.60	>6.2
10a	3-F	1	H	>10	0.068	>167
10b	3-F	2	H	>10	ND	>10
10c	4-F	1	H	>10	0.160	>62.5
10d	4-F	2	H	>10	0.086	>16
11a	3-F	2	NO_2	>10	0.054	>185
11b	3-F	3	NO_2	>10	0.140	>71
12a	3-F	2	H	>10	>10	ND
12b	3-F	3	H	>10	0.047	>213
13a	4-F	2	NO_2	>10	16%@ 10 μM	ND
13b	3-F	2	NO_2	>10	45%@ 10 μM	ND
14a	4-F	2	H	ND	18%@ 10 μM	ND
14b	3-F	2	H	ND	15%@ 10 μM	ND
Celecoxib				3.84	0.061	>63

[a] Results are expressed as the mean (n = 3 experiments) of the percentage inhibition of PGE2 production by test compounds with respect to control samples and the IC_{50} values were calculated by GraphPad Instat program; data fit was obtained using the sigmoidal dose−response equation (variable slope) (GraphPad software).
[b] In vitro COX-2 selectivity index [IC_{50} (COX-1)/IC_{50}(COX-2)].

the concentrations of inorganic nitrites and nitrates (stable NO metabolites) was measured after incubation (2 h) of the compounds in rat liver homogenates. The nitrite concentration for **7c** was 27.0 ± 12.1 μM, while corresponding nitrate concentration was 38.3 ± 4.3 μM. Compound **11a**, in the same conditions, showed 42.9 ± 6.6 μM and 56.1 ± 16.0 μM for nitrites and nitrates respectively. These data indicate that both compounds are endowed with a slow NO-releasing rate, which is a fundamental feature for the development of well-balanced hybrids. Conversely, Naproxcinod exhibited after 2 h, the highest concentration (367.7 ± 11.4 μM), of nitrate and nitrites (NOx). Interestingly, the concentration of NOx was largely due to the released nitrates (340.1 ± 5.0 μM) while a lower concentration of nitrites was detected (27.6 ± 6.2 μM). Such a lower ratio nitrites/NOx would suggest that only a small part of the nitro-oxy group of naproxcinod is converted to NO and that a direct hydrolysis to inorganic nitrate is prevalent. Indeed, NOBA is a relatively fast NO-releasing compound, and the CV effects are detected for a maximum of 3 h after the naproxcinod administration. Conversely, the analgesic and anti-inflammatory effects of naproxcinod are more extended because of the long half-life (i.e., 17 h) of naproxen, thus not assuring the GI/CV protection for the whole duration of the COX inhibition.

Solubility of compounds was assessed in SGF and PBS (Table 10) [71]. The replacement of the ester functionality with the amide moiety provided in general more soluble molecules. The best solubility profile was displayed by the glycine amide **11a** (>200 μM in both SGF and PBS). Comparative stability studies of the esters **3c** and **5c** with amides **7c**, **9a**, **11a,** revealed liability of the esters to

Table 9
Vasorelaxing properties for compounds **7a–c**, **9a–d**, **11a-b** and **13a-b**.

Compd.	E_{max}[a]	pIC_{50}[b]
7a	49.0 ± 2.0	NC
7b	69.0 ± 8.0	5.78 ± 0.07
7c	68.0 ± 0.5	5.31 ± 0.05
9a	18.0 ± 9.0	NC
9b	25.0 ± 2.0	NC
9c	25.0 ± 1.9	NC
9d	42.0 ± 9.0	NC
11a	74.1 ± 1.0	5.38 ± 0.20
11b	86.0 ± 2.0	5.38 ± 0.03
13a	74.9 ± 2.5	5.82 ± 0.04
13b	67.5 ± 2.3	5.49 ± 0.00
Naproxcinod	68.0 ± 3.0	6.33 ± 0.06

[a] Emax represents the vasorelaxing efficacy, expressed as a % of the vasoconstriction induced by the pre-administration of KCl.
[b] Parameter of potency is expressed as pIC_{50}, representing −log of the molar concentration capable of inducing a vasorelaxing effect = 50% of E_{max}.

Table 10
Solubility in SGF and PBS for compounds **3c**, **5c**, **7a**, **7c**, **9c**, **11a**.

Compd.	SGF (pH 1.5) (μM)	PBS (pH 7.4) (μM)
3c	<1	1.6
5c	>200	80
7a	150	>200
7c	147	>200
9c	112	90.5
11a	>200	200

Table 11
Stability in SGF, PBS and rat plasma for compounds **3c**, **5c**, **7a**, **9a** and **11a.**

Compd.	Time (min)	Parent molecule remained (%)		
		PBS (pH 7.4)	SGF (pH 1.5)	Rat plasma
3c	30	74.1	80.2	0.00
	60	53.6	65.7	0.00
	120	0.15	42.8	0.00
5c	30	75.0	100	0.00
	60	75.0	100	0.00
	120	75.0	100	0.00
7c	30	100	100	100
	60	100	100	100
	120	100	100	100
9a	30	75	100	71
	60	50	93.8	57
	120	37.5	87.5	28.6
11a	30	100	100	94
	60	100	100	93
	120	100	100	77

hydrolysis in comparison with amides (Table 11). Compound **7c** was completely resistant to hydrolysis, whereas compound **11a** was relatively more liable in rat plasma (even though after 120 min of incubation 77% of the compound was still detected).

All the nitrooxyamides were tested in the writhing test, and highlighted a good and dose-dependent activity (Table 12) [71]. In particular, compound **7c,** and glycine derivative **11a** were endowed with the higher potency, displaying respectively 77% and 79% of reduction at 20 mg/kg. Moreover, compound **11a** displayed writhes reduction of 56% at the dose of 10 mg/kg. The most active nitro-oxy compounds **7c** and **11a** were also evaluated for their activity in the carrageenan induced edema and hyperalgesia (Table 13) [71]. The carrageenan induced inflammation test showed that **7c** was associated with a good but not outstanding activity. Conversely, compound **11a** was proved to be highly active, with a reduction of hyperalgesia at 10 mg/kg of 80% after 30 min. The activity was maintained for 60 min (70%) and even after 90 min a satisfactory activity (50%) was still found.

The pharmacokinetics of the best performing compounds **7c** and **11a** were assessed in rats after po and iv administration at 10 mg/kg [71]. After iv administration, **7c** was detected in plasma up to 6 h. Both **7c** and **11a** appear to be characterized by moderate to high rate of clearance and high volume of distribution. Compound **7c** showed absolute bioavailability (24%) higher than **11a** (9%). The presence of the metabolites **8c** and **12a** was also

Table 12
Effect of **1a**–**d**, **4c**, **2a**–**d**, **5c**, and **3a,b** and vehicle (CMC) in the mouse abdominal constriction test (Acetic acid 0.6%).

Compd.	n. mice	Dose po mg/kg	n. Writhes after 30 min	Writhes reduction (%)
CMC	23	_	32.6 ± 2.1	_
7a	10	10	26.8 ± 2.1**	18
	10	20	20.6 ± 3.0*	37
	10	40	14.7 ± 2.1*	55
7b	9	10	33.2 ± 3.5	0
	9	20	29.8 ± 3.6	8.6
	10	40	22.9 ± 2.2*	30
7c	9	10	12.2 ± 2.3*	63
	9	20	7.4 ± 1.3*	77
	10	40	8.1 ± 1.2*	75
7d	8	3	27.2 ± 2.7	17
	8	10	20.4 ± 2.2*	37
	8	20	21.6 ± 2.8*	34
8c	10	10	20.7 ± 3.5*	37
	10	20	12.6 ± 2.7*	61
9a	10	10	28.3 ± 2.5	12
	10	20	17.1 ± 3.1*	47
	10	40	16.4 ± 2.9*	50
9b	8	20	29.2 ± 2.4	10
	10	40	25.2 ± 3.3**	23
9c	8	10	33.1 ± 3.5	0
	9	20	23.2 ± 2.3*	29
	10	40	16.3 ± 3.2*	50
9d	10	3	33.7 ± 3.0	0
	10	10	22.8 ± 2.7*	30
	11	20	14.9 ± 2.8*	54
10c	14	10	23.7 ± 3.0	27
	15	20	13.4 ± 2.8*	59
	15	40	12.9 ± 2.1*	60
11a	13	3	25.1 ± 2.3**	23
	10	10	14.2 ± 2.9*	56
	13	20	6.7 ± 2.0*	79
11b	9	10	30.5 ± 2.6	6
	10	20	21.4 ± 3.7*	34
	10	40	18.8 ± 3.1*	42
13a	10	5	26.7 ± 3.3*	20.5
	10	10	18.5 ± 4.2*	44.9
	8	20	15.8 ± 3.7*	53.0
	9	40	16.7 ± 2.5*	50.3
13b	5	1	21.8 ± 3.5*	35.1
	5	3	19.4 ± 2.9*	42.3
	6	10	16.7 ± 3.5*	50.3
Celecoxib	13	10	13.5 ± 3.0*	59

*P < 0.01.
**P < 0.05 in comparison with CMC treated animals.

Table 13
Hyperalgesia and edema reduction in the carrageenan induced inflammation for compounds **7c** and **11a** in comparison with celecoxib.

pretreatment, ipl	paw pressure (g)							
	treatment po	dose mg/kg	before treatment	after treatment[b]				volume (mL)[b]
				30 min	60 min	90 min	120 min	60 min
Saline	**CMC**		61.6 ± 3.4	58.9 ± 3.8	61.4 ± 4.1	61.4 ± 4.1	61.4 ± 4.1	1.44 ± 0.05
Carrageenan	**CMC**		36.1 ± 3.3	32.8 ± 2.9	30.6 ± 3.2	31.8 ± 2.2	31.8 ± 2.2	2.71 ± 0.08
Carrageenan	**7c**	20	33.8 ± 2.9	40.7 ± 3.5**	42.8 ± 3.4**	37.8 ± 2.6	37.8 ± 2.6	2.63 ± 0.05
Carrageenan		40	32.9 ± 3.3	44.2 ± 4.0*	48.3 ± 4.6	41.5 ± 3.7*	31.5 ± 2.6	2.50 ± 0.09
Carrageenan	**11a**	20	33.8 ± 2.9	55.2 ± 3.3*	52.1 ± 4.2*	47.3 ± 3.7*	39.5 ± 3.0	1.79 ± 0.09*
Carrageenan	**Celecoxib**	10	31.7 ± 2.7	45.7 ± 4.2*	52.9 ± 3.1*	48.3 ± 3.4*	42.5 ± 2.9	2.53 ± 0.05*

*P < 0.01.
**P < 0.05 in comparison with CMC treated animals.

measured. Compound **8c** was detected in rat plasma up to 6 h after both iv and po administrations, while **12a** was detected in rat plasma at least up to 6 h after intravenous administrations and only up to 2 h after oral administration. For **7c**, after intravenous administration at least 39% of the parent compound was converted into the metabolite **8c** while after oral administration 99% of the parent compound was converted into the metabolite, suggesting that after oral administration **7c** is readily metabolized to **8c**. For **11a**, after intravenous administration only 10% of the parent compound was converted into the metabolite **12a** during the 6 h of the experiment.

2.3. *NO-coxibs based on diarylpyrrole scaffold: ethers*

In order to further increase the stability of the linker between the scaffold and the NO releasing moiety, the corresponding ethers **15a-f** (Table 14) [64,67] were prepared. The conversion of the nitrooxyalkyl ester into the nitrooxyalkyl ether moiety led to potent COX-2 inhibitors. Results for compounds **15a-f**, and corresponding metabolites **16a-f**, in the COX inhibition (J774 cells assay) are reported in Table 14. Some of them were endowed with good NO-donating properties (Table 15) [67] along with quite good and selective COX-2 inhibitory activity ranging from low nanomolar to micromolar values. Nitrooxyalkyl ethers **15a-f** highlighted a better COX-2 inhibitory activity with respect to derivatives **16a-f,** most of the compounds were as active as corresponding nitrooxyalkyl esters. Compounds **15a** (n = 2, H), **15b** (n = 3, H), **15c** (n = 2, 3-F), **15d** (n = 3, 3-F) emerged as low nanomolar COX-2 inhibitors. Compounds **15a, 15c** and **15e** showed better NO-vasorelaxing responses in comparison with the other compounds of the group (Table 15),

Table 15
Efficacy and potency in determining NO-dependent vasorelaxing responses of **15a–f** and GTN.[a]

Compd.	E_{max}[b]	pIC_{50}[c]
15a	65 ± 3	5.22 ± 0.03
15b	48 ± 5	≤5
15c	60 ± 4	5.32 ± 0.05
15d	ND	–
15e	58 ± 5	5.47 ± 0.07
15f	41 ± 2	≤5
GNT	93 ± 2	6.90 ± 0.07

[a] GTN: glyceryl trinitrate.
[b] Emax represents the vasorelaxing efficacy, expressed as a % of the vasoconstriction induced by the pre-administration of KCl.
[c] Parameter of potency is expressed as pIC_{50}, representing −log of the molar concentration capable of inducing a vasorelaxing effect = 50% of E_{max}.

Table 14
COX-1 and COX-2 inhibitory activity Compounds **15 a-f, 16a-f,** and Celecoxib.

Compd.	R	n	X	COX-1 inhibition IC_{50} (μM)[a]	COX-2 inhibition IC_{50} (μM)[a]	Selectivity index[b]
15a	H	2	NO_2	>10	0.017	>588.2
15b	H	3	NO_2	>10	0.015	>666.7
15c	3-F	2	NO_2	>10	0.027	>357.1
15d	3-F	3	NO_2	2.9	0.023	126.1
15e	4-F	2	NO_2	>10	0.014	>714.3
15f	4-F	3	NO_2	>10	0.190	>52.6
16a	H	2	H	>10	0.027	>370.4
16b	H	3	H	>10	8.990	1.11
16c	3-F	2	H	>10	0.046	>217.4
16d	3-F	3	H	3.7	0.240	15.4
16e	4-F	2	H	>10	0.089	>112.3
16f	4-F	3	H	>10	0.940	>10.6
Celecoxib				3.84	0.061	>63

[a] Results are expressed as the mean (n = 3 experiments) of the % inhibition of PGE_2 production by test compounds with respect to control samples and the IC_{50} values were calculated by GraphPad Instat program; data fit was obtained using the sigmoidal dose−response equation (variable slope) (GraphPad software).
[b] *In vitro* COX-2 Selectivity Index [IC_{50} (COX-1)/IC_{50}(COX-2)].

Table 16
Dose−response results of compounds **15a**−**e**, **16a**−**d**, and Celecoxib in the Acetic Acid Writhing Test.[a].

Compd.	n mice	dose per os (mg/kg)	no. writhes	Writhes reduction (%)
Saline	6		32.4 ± 1.9	-
15a	10	10	21.2 ± 3.0∧	35
	8	20	15.5 ± 3.6*	53
15b	9	10	29.4 ± 3.0	9
	10	20	21.2 ± 3.0*	35
	8	40	26.8 ± 3.2∧	17
15c	8	10	32.5 ± 3.7	0
	8	20	30.3 ± 2.1	6
	8	40	25.3 ± 3.6*	22
15e	8	3	28.5 ± 3.2	12
	8	10	19.1 ± 2.7*	41
	8	20	15.2 ± 3.3*	53
16a	8	10	21.2 ± 3.0*	35
	8	20	15.5 ± 3.6*	52
16b	9	10	29.7 ± 3.4	8
	9	20	22.3 ± 2.5*	31
	7	40	21.5 ± 2.3*	34
16c	8	10	29.4 ± 3.0	9
	8	20	26.8 ± 3.2∧	17
	8	40	17.3 ± 3.5*	47
16e	7	10	31.3 ± 2.7	3
	8	20	27.9 ± 3.6	14
	8	40	18.1 ± 3.1*	44
Celecoxib	10	1	25.6 ± 3.1*	21
	11	3	15.4 ± 2.5*	52
	15	10	11.3 ± 2.9*	65

[a]Each value represents the mean of at least seven mice: (∧) $P < 0.05$.
(*) $P < 0.01$ in comparison with CMC treated group.

confirming what previously shown in terms of dependence of the activity with side chain length. Experiments with ODQ confirmed the vasorelaxing effects were due to the NO release. COX-2 inhibition was assessed *ex vivo* by the HWB assay for compounds **15e** and **16e** [66]**.** Concentration−response curves for inhibition of COX-1 and COX-2 in HWB showed compound **15e** and its derivative **16e** inhibited LPS induced PGE_2 generation (COX-2 assay) in a concentration-dependent fashion with IC_{50} values respectively of 0.64 and 0.44 μM. Compounds **15e** and **16e** also showed comparable IC_{80} values (respectively 2.26 and 2.31 μM). These results pointed out that **15e** and its metabolite **16e** gave a comparable and potent inhibitory effect on COX-2 activity in HWB. Furthermore, **15e** and **16e** inhibited platelet COX-1 activity in a concentration-dependent fashion; **16e** was slightly more potent ($IC_{50} = 20.0$; $IC_{80} = 37.14$) in COX-1 inhibition than **15e** ($IC_{50} = 31.24$; $IC_{80} = 63.3$). Thus, both compounds were proved to be selective, being 50-fold more potent toward COX-2 than COX-1 at IC_{50}. Compounds **15a-f,** and corresponding hydroxylated derivatives were evaluated *in vivo*, both in the writhing test and in the carrageenan-induced inflammatory pain model [66]. In the writhing test the minimal dose able to revert the painful condition was 10 mg/kg po for **15a**, **15e** and **16a**, 20 mg/kg for **15b**, **16b,** 40 mg/kg for **15c** and **16e** (Table 16). In the carrageenan inflammatory pain model all the tested molecules were effective in the range between 40 and 20 mg/kg po, at 120 min, and a significant paw edema decrease was observed 60 min after the administration for all the investigated compounds (Table 17) [67]. At the higher tested dose, the antihyperalgesic activity lasted up to 120 min for all the compounds, except for **15a** and **15b.**

The incubation of **15e** in rat plasma was followed by a negligible formation of nitrites and nitrates (3.2 ± 0.7 μM and 0.24 ± 0.24 μM, respectively, at 120 min) while the corresponding experiment with naproxcinod showed a massive formation of nitrate [70]. The incubation of **15e** in liver homogenate was followed by a slow and time-dependent production of nitrites and nitrates; after 120 min of incubation, the recorded concentrations were 4.6 ± 0.5 μM and 26.9 ± 3.4 μM, respectively. Naproxcinod showed a more rapid accumulation of larger amounts of these metabolites (28.0 ± 6.0 μM of nitrites and 340.1 ± 5.0 μM of nitrates, at 120 min) [70]. Therefore, **15e** exhibited the feature of "NO-reservoir", stable in plasma and slowly converted to NO by the cell metabolism (liver, vascular smooth muscle, endothelium, etc.). The **15e** NO-releasing properties were confirmed by the endothelium-independent vasorelaxing effects evoked in aortic rings of normotensive rats; these effects were antagonized by ODC. In order to determine the NO generation *in vivo* and its possible effects on the progression of systolic blood pressure (SBP), **15e** (20 mg/kg/day) was chronically administered to young male spontaneous hypertensive rats (SHRs) [70] The effect of the metabolite **16e** was studied as well. The coxib-associated CV adverse effects are increased when other CV disorders are already present; therefore, **15e** effect was studied when chronically

Table 17
Effect of **15a-c**, **16a-c**, **16e** and Celecoxib on hyperalgesia and edema induced by carrageenan in the Rat Paw Pressure Test.

paw pressure (g)								
pretreatment ip	Compd.	dose mg/kg	before treatment	after treatment			Paw volume (mL)	
				30 min	60 min	120 min	before treatment	60 min
Saline	**CMC**	_	62.6 ± 2.4	61.5 ± 3.1	60.2 ± 3.3	62.9 ± 3.5	1.46 ± 0.05	1.42 ± 0.07
Carrageenan	**CMC**		31.4 ± 3.4	34.8 ± 3.0	33.9 ± 3.7	31.4 ± 3.7	1.47 ± 0.07	2.48 ± 0.06
Carrageenan	**15a**	20	33.9 ± 3.1	54.2 ± 3.1*	55.3 ± 3.5*	48.7 ± 3.7∧	1.31 ± 0.09	1.45 ± 0.10*
Carrageenan	**15b**	10	34.1 ± 2.7	55.1 ± 4.7*	52.6 ± 4.0*	48.3 ± 5.2∧	1.49 ± 0.08	1.87 ± 0.07*
Carrageenan		20	32.0 ± 3.5	56.8 ± 3.9*	49.2 ± 4.6*	43.8 ± 4.5∧	1.53 ± 0.08	1.92 ± 0.08*
Carrageenan	**15c**	20	34.6 ± 3.0	43.9 ± 3.1∧	40.7 ± 3.0	35.2 ± 3.5	1.58 ± 0.08	2.16 ± 0.09∧
Carrageenan		40	31.6 ± 2.7	47.3 ± 4.2*	41.0 ± 4.4	34.3 ± 4.1	1.55 ± 0.09	2.08 ± 0.07*
Carrageenan	**16a**	20	32.7 ± 3.7	44.6 ± 4.0∧	46.9 ± 4.2∧	38.8 ± 3.5	1.54 ± 0.06	2.19 ± 0.07∧
Carrageenan		40	35.2 ± 3.1	49.5 ± 3.8*	42.5 ± 4.1	35.3 ± 3.8	1.48 ± 0.07	2.10 ± 0.08*
Carrageenan	**16b**	20	33.8 ± 3.8	52.9 ± 3.6*	50.3 ± 2.9*	46.6 ± 3.1∧	1.47 ± 0.05	1.86 ± 0.04*
Carrageenan	**16c**	20	32.7 ± 3.9	46.9 ± 3.8∧	48.6 ± 3.5∧	37.2 ± 3.9	1.55 ± 0.08	2.46 ± 0.08
Carrageenan		40	33.5 ± 3.9	51.4 ± 3.0*	45.3 ± 4.4∧	39.5 ± 4.0	1.49 ± 0.09	2.12 ± 0.06∧
Carrageenan	**16e**	20	32.5 ± 3.4	46.8 ± 3.7*	38.7 ± 3.3	38.3 ± 3.9	1.57 ± 0.07	2.01 ± 0.09*
Carrageenan		40	30.5 ± 3.5	57.2 ± 4.6*	58.3 ± 4.5*	47.6 ± 4.4∧	1.55 ± 0.09	1.95 ± 0.08*
Carrageenan	**Celecoxib**	3	31.7 ± 2.7	44.3 ± 3.8*	41.6 ± 3.2	40.7 ± 2.6	1.59 ± 0.08	2.35 ± 0.07
Carrageenan		10	33.5 ± 2.6	52.9 ± 3.1*	48.3 ± 3.4*	39.8 ± 3.1	1.50 ± 0.05	1.45 ± 0.06*

(∧) $P < 0.05$, (*) $P < 0.01$ versus the carrageenan/saline-treated group.

administered to SHRs, a suitable experimental model of heavy alteration of the CV function. Indeed, in young SHRs, an age-related progression of blood pressure is always observed and highly selective COX2 inhibitors, such as rofecoxib, accelerate and worsen it. After one month of pharmacological treatments of SHRs, serum concentrations of nitrites and nitrates were measured [70]. Though, treatment with **16e** did not influence the levels of plasma nitrites and nitrates, in the **15e**-treated animals the concentration of nitrites was not significantly changed but a significant increase in nitrates concentration of was observed. In the Langendorff-perfused isolated hearts of vehicle-treated SHRs, the coronary flow was like that recorded in normotensive animals. The coronary flow in SHR rats treated with **15e** was significantly increased while the one of **16e**-treated animals was not significantly changed. The SBP development in **15e**-treated SHRs was completely equivalent to that of vehicle treated SHRs, while treatment with **16e** (devoid of NO releasing properties) did not accelerate the age-related increase of SBP in young SHRs. Overall this indicates that the NO-releasing property of **15e** can significantly improve the overall CV function and thus is a useful complementary aspect for coxibs.

3. Conclusions

Cystic fibrosis, a life-shortening disease due to mutations in CFTR ion channel, among other complications can lead to severe lung deficiency, characterized by chronic respiratory disease and lung damage with persistent and exaggerated inflammation that gives rise to irreversible airway destruction leading to morbidity and mortality in CF patients. Therapies targeting the root cause of CF have been recently developed, and results from several clinical trials are available. These therapies are based on compounds that in principle could reverse the CFTR molecular defect, preventing the CF progression. However, the available results are not completely satisfying and at least two of these therapies must be simultaneously carried out in order to obtain relevant clinical results. On the other hand, the treatment of the dysfunctional inflammatory response underling the irreversible airway destruction and fibrosis in CF has been proved difficult with t-NSAIDs or steroidal antiinflammatory drugs. Though several evidences suggest that COX-2 could be a remarkable target for treating inflammation in CF, the efficacy of coxibs has not been investigated yet, probably owing to the related side effects. Nitric oxide plays an essential role in a variety of biological processes in the lung. In addition, NO can stabilize CFTR and correct its disfunction by protein nitrosylation. Despite this physiological importance, low concentrations of NO were found in CF patients, and therapeutic interventions aiming to correct the nitric oxide deficiency in CF patients are investigated at the present. There is a growing interest in the class of bioavailable and stable nitrosothiols and corresponding precursors as a novel class of corrector therapies for CF. Herein we discussed how a suitable NO-releasing moiety endowed with appropriate releasing rates can be conjugated to a coxib, according to a strategy aimed at both improving drug effectiveness and moderating those side effects linked to the mechanism of action of the native drug. The efforts aimed at overcoming pharmacokinetics issues related to bioavailability and stability of the compounds have been discussed along with the characteristics of the products that are suitable candidates for further development. Taking together the topics discussed above it appears that the best performing compounds (**11a** in the amide and **15e** in the ether classes) can be good candidates to treat CF according to a new and promising approach.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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