A series of COX-2 inhibitors endowed with NO-releasing properties: synthesis, biological evaluation and docking analysis.

Sara Consalvi,*[a] Giovanna Poce,[a] Rino Ragno,[a] Manuela Sabatino,[a] Concettina La Motta,[b] Stefania Sartini,[b] Vincenzo Calderone,[b] Alma Martelli,[b] Carla Ghelardini,[c] Lorenzo Di Cesare Mannelli[c] and Mariangela Biava[a]

a] Dr. S. Consalvi, Dr. G. Poce, Dr. R. Ragno, M. Sabatino, Dr. M. Biava

Dipartimento di Chimica e Tecnologie del Farmaco

Sapienza Università di Roma, Piazzale A. Moro 5, 00185 Roma (Italy)

E-mail: sara.consalvi@uniroma1.it

[b] Dr. V. Calderone, Dr. A. Martelli, Dr. C. La Motta, Dr. S. Sartini

Dipartimento di Farmacia

Università di Pisa, via Bonanno Pisano 6, 56126 Pisa (Italy)

[c] Dr. C. Ghelardini, Dr. L. Di Cesare Mannelli

Dipartimento di Neurologia, Psicologia, Area del Farmaco e Salute del Bambino

Università degli Studi di Firenze, Viale G. Pieraccini 6,50139 Firenze (Italy)

Abstract: We report herein the synthesis, the biological evaluation and the docking analysis of a class of COX-2 inhibitors endowed with NO-releasing properties. Some of the synthesized compounds displayed good activities towards COX-2, as well as vasorelaxing properties due to NO release. Overall, they exhibited a good in vivo pharmacological profile and proved to be able to reduce writhes in a murine model of inflammatory pain. Derivative 2b showed an outstanding activity, with an efficacy comparable to that of celecoxib even at very low concentrations.

Introduction

Conventional non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for treating different pathological conditions, from small injuries, headache or fever to chronic and severe pain. The efficacy of this class of drugs in relieving inflammation is related to their capability to inhibit Cyclooxygenase (COX) enzymes, thus preventing the production of prostaglandins from arachidonic acid. While the therapeutic effects are primarily mediated by Cyclooxygenase-2 (COX-2) inhibition, the inhibition of Cyclooxygenase-1 (COX-1) is responsible for NSAIDs side effects, including renal and gastrointestinal (GI) toxicities. For this reason, a class of drugs that selectively inhibit the COX-2 isoform (coxibs) has been developed and marketed[1,2]. However, the complete inhibition of COX-2 has led to a lack of a balance in the prostanoid production at cardiovascular (CV) level and as a consequence, the long-term administration of coxibs caused to many patients an increased incidence of thrombotic events and a greater CV hazard[3–6]. Therefore, different strategies have been adopted to obtain safer anti-inflammatory agents, endowed with spare gastrointestinal and cardiovascular toxicity[7,8]. A valuable option is that of designing multitarget-directed ligands, combining in the same scaffold pharmacophoric elements able to simultaneously modulate different targets, in order either to reduce side effects or enhance the anti-inflammatory

activity[9]. Dual fatty acid amide hydrolase (FAAH)/COX inhibitors are a representative example of this class of drugs[10].

Another interesting strategy to reduce the side effects due to COX inhibition is that of developing “molecular hybrids” conjugating the COX-inhibiting scaffold with a moiety able to counteract side-effects, such as a nitric oxide (NO) releasing group[11,12]. NO is a small endogenous gasotrasmitter produced by three isozymes (NOS, NO synthases) in a large number of cells and plays a pivotal role in blood pressure regulation, cell proliferation, vascular homeostasis and vasorelaxant processes[13,14]. Due to its small size and to its very high reactivity, it serves as a potent local modulator that rapidly diffuses through membranes to target cells and it is involved in cell-to-cell communication[15]. In the CV system it acts as a physiological vasodilator and mediates smooth muscle relaxation by modulating the soluble Guanylyl Cyclase-Cyclic Guanosine monophosphate (sGC-cGMP) pathway[16]. It also inhibits platelet aggregation and is likely to prevent both early and later steps of atherogenosis by reducing leucocyte adhesion to the vascular wall[17]. In addition, it can regulate gastric blood flow and contribute to gastric mucosal defence by modulating mucus and bicarbonate secretion[18]. The first example of COX-inhibiting nitric oxide donors (CINODs) is Naproxcinod (AZD3582, produced by NicOx), resulting from the conjugation of a NO-releasing moiety to a NSAID scaffold[12] (Fig.1). Hereafter, given the key role of NO at CV level, such strategy was applied to COX-2-selective inhibitors, in order to obtain more potent anti-inflammatory agents with an abated GI toxicity and an acceptable CV safety profile[19–23].

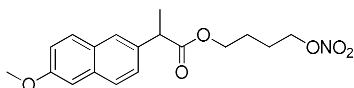


Figure 1. Chemical structure of Naproxcinod.

In this context, our research group developed a number of selective COX-2 inhibitors nitric oxide donors by conjugating a diaryl-pyrrole scaffold endowed with selective COX-2 inhibition properties[24–32] with different nitrooxyalkyl side chains, such as esters, α-amino esters, amides, α-amino amides, ethers, β-amino ethers, inverse esters and amides[33–37]. Such medicinal chemistry program led to the identification of very promising candidates (Fig. 2), with high in vitro potencies (COX-2 inhibition at 10 μM ≥ 96%), a great efficacy in determining NO-vasorelaxing responses and a good antinociceptive activity in the abdominal writhing test (% of writhes reduction between 46 and 77 at 10 mg/kg).

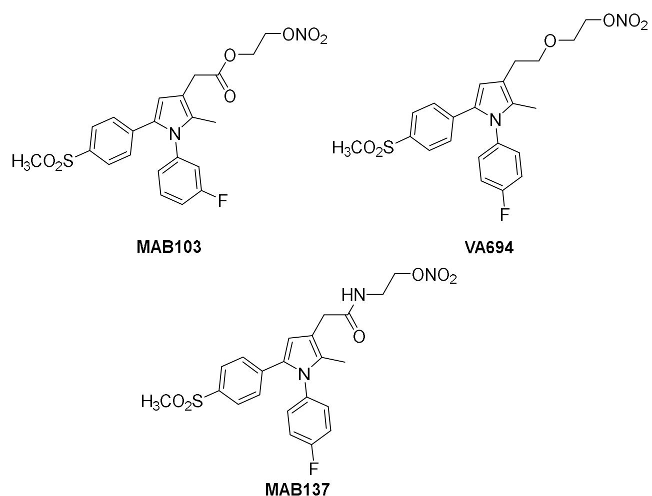


Figure 2. Chemical structures of the most promising compounds previously synthesized.

To complete the picture, we present herein the development of a new class of sulphamoyl pyrroles as COX-2 inhibitors NO donors. Indeed, assuming that the lower logP of arylsulphonamides could contribute to improved bioavailability and absorption with respect to arylsulphones, we explored the replacement of the methylsulphonyl moiety with a sulfamoyl one, to further improve in vivo activity[31,38]. Thus, we prepared the nitrooxy acetic esters and amides 1-2 bearing a C5 sulphamoylphenyl group and a fluorinated N1 phenyl ring, according to Structure Activity Relationship (SAR) data emerged from the previously synthesized compounds. Finally, considering that such compounds are subjected to in vivo metabolism and that they could be in turn converted into the corresponding alcohols after NO release, the hydroxyl-derivatives 3-4 were synthesized and tested as probable metabolites (Fig.3).

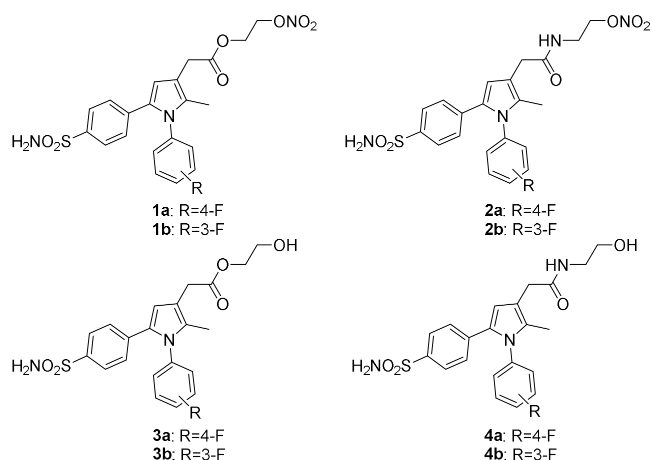
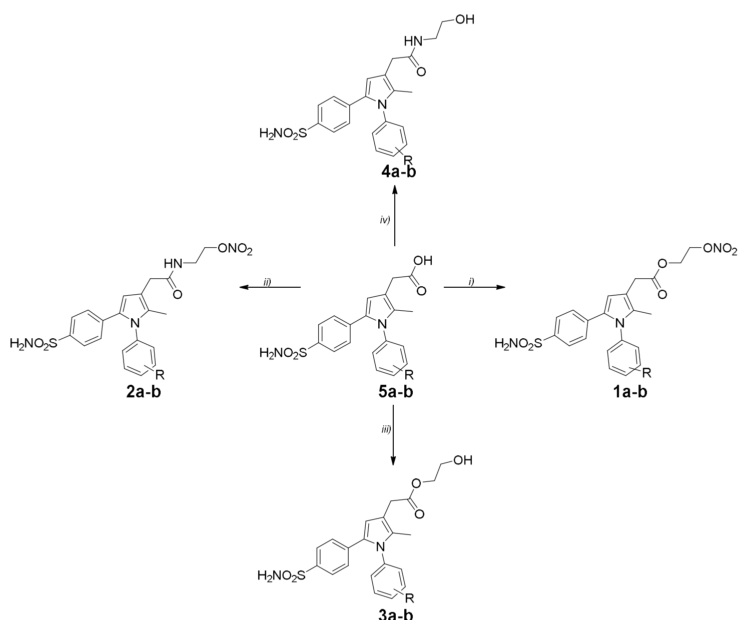


Figure 3. Chemical structures of compounds 1-4.

Results and Discussion

Chemistry

Compounds 1-4 were prepared following the synthetic pathway described in Scheme 1. The acetic acid derivatives 5a,b were synthesized according to the previously reported procedure[31]. Briefly, derivatives 1-3 were obtained through a coupling reaction between 2-hydroxyethyl nitrate, 2-aminoethyl nitrate or ethylene glycol and the suitable acid 5a,b in the presence of DMAP, EDCi and TEA. On the other hand, the hydroxyl derivatives 4a,b were obtained via an EDCi/HOBt coupling stage.



Reagents and conditions: *i)*: 2-nitrooxy ethanol, DMAP, EDCi, TEA, DCM/DMF 10:1 (v/v), 15 h, rt; *ii)*: 2-nitrooxyethyl amine, DMAP, EDCi, TEA, DCM/DMF 10:1 (v/v), 15h, rt; *iii):* ethylene glycol, DMAP, EDCi, TEA, DCM/DMF 10:1 (v/v), 15h, rt; *iv)*: ethanolamine, HOBt, EDCi, DCM/DMF 10:1 (v/v), 15h, rt.

Scheme 1. Synthesis of compounds 1-4.

In vitro studies

Inhibition of COX-1/COX-2 was assessed through an enzyme immunoassay and the prostanoids were quantified using the commercially available COX Inhibitor Screening Assay (Cayman Chemical Company, Ann Arbor, MI, USA). Data are reported as % of inhibition for all the tested compounds and as IC50 for the two most active ones (1a and 1b, Table 1).

Table 1. In vitro activities of compounds 1-4 and Dup-697 used as reference compound towards COX-1 and COX-2.

Table 1. In vitro activities of compounds **1-4** and Dup-697 used as reference compound towards COX-1 and COX-2.

Compound	COX-1 % of inhibition (10 μM)[a]	COX-2 % of inhibition (10 μM)[a]	IC_{50} COX-2 (μM)[d]
1a	n.a[b]	86	1.17±0.06
1b	n.a[b]	89	0.41±0.01
2a	n.t[c]	16	n.d.[e]
2b	n.t[c]	45	n.d.[e]
3a	n.t[c]	16	n.d.[e]
3b	n.t[c]	n.a[b]	n.d.[e]
4a	n.t[c]	18	n.d.[e]
4b	n.t[c]	15	n.d.[e]
Dup-697	-	100	-

[a] Each value is the mean of at least three determination, carried out in triplicate. SEM ≤5%. [b] Not active: no inhibition was observed at 10 μM of tested compound. [c] Not tested.[d] IC_{50} values, means±SD, represent the concentration required to produce 50% enzyme inhibition. [e] Not determined.

As reported in Table 1, nitrated acetic esters 1a,b displayed the best activities against COX-2, with IC50 values of 1.17±0.06 and 0.41±0.01, respectively. Such derivatives were found to be much more active than the corresponding hydroxyl derivatives 3a,b, confirming the same trend observed for the methylsulphonyl analogues[33]. On the other hand, the presence of the amide fragment was responsible for a drop in activity; in fact, both the acetic amides 2a and 2b proved to be less active than the corresponding acetic esters, proving that the combination of a nitroxyalkyl chain with an ester moiety guarantees the best interactions within the binding site.

Ex vivo NO releasing studies

The capability of nitrooxy derivatives 1-2 in determining vasorelaxing responses was assessed using rat endothelium-denuded aortic rings precontracted with 30 mM KCl as an experimental model of vascular smooth muscle. Since NO induces vasodilator responses by activating GC and consequently increasing cGMP levels, the experiments were also performed in the presence of a GC inhibitor (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, ODQ), to confirm their mechanism of action. As reported in Table 2, all the tested compounds proved to be effective in inducing vasorelaxing responses. In particular, the nitrooxy amides 2a,b showed the best activity, with values of efficacy (Emax) and potency (pIC50) comparable to that of Naproxcinod, used as a control. Moreover, all the tested compounds were found to be inactive in the presence of ODQ, proving that their efficacy was due to NO release. Notably, no vasorelaxing effect was observed at low concentrations, suggesting that these compounds gradually release NO (Fig. 4); as a consequence, they are not supposed to be responsible for a massive hypotensive effect in vivo, that is an essential feature for hybrid drugs.

Table 2. Nitric oxide releasing efficacy and potency for compounds **1-2** and Naproxcinod used as reference compound. The vasorelaxing efficacy was evaluated as the maximal vasorelaxing response (E_{max}), expressed as a percentage of the contractile tone induced by KCl 30 mM. The potency was expressed as pIC_{50} that was calculated as negative logarithm of the molar concentration of the tested compounds evoking a half reduction of the contractile tone induced by KCl 30 mM.

Compound	E_{max}	pIC_{50}	+ ODQ 1 μM
1a	47±0.5	n.c[a]	+
1b	59.1±0.4	5.55±0.07	+
2a	74.9±2.5	5.82±0.04	+
2b	67.5±2.3	5.49±0.00	+
Naproxcinod	68.0±3.0	6.33±0.06	+

[a] Not calculated.

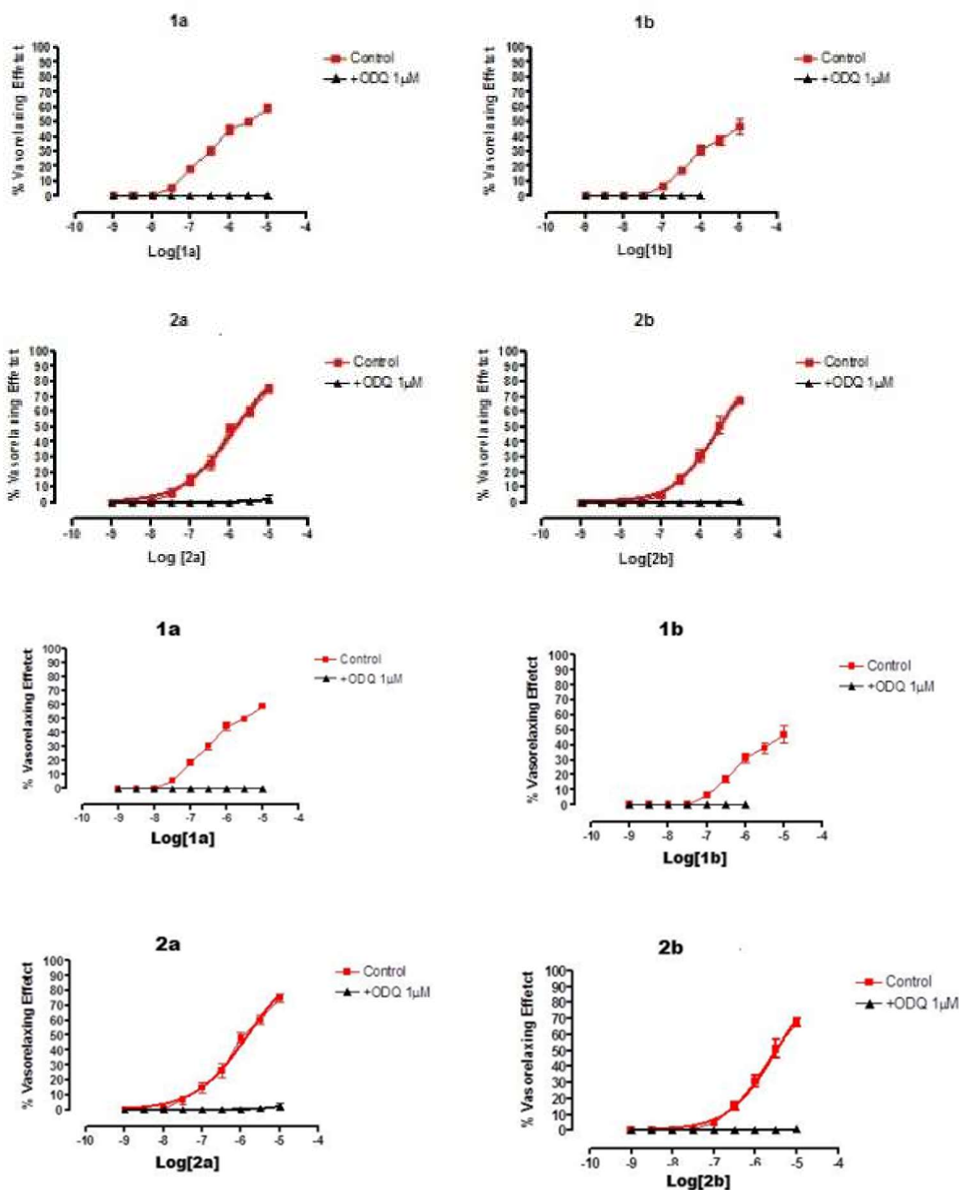


Figure 4. Concentration-response curve for the vasorelaxing effects exerted by compounds 1-2. The experiments were performed both in the presence (control, black triangles) and in the absence (red squares) of 1 µM ODQ. The vertical bars indicate the standard error.

In vivo studies: writhes reduction in the acetic acid-induced abdominal constrictions in mice.

Based on their in vitro potencies, compounds 1-2 and 4b were selected to assess their analgesic efficacy in a murine model of inflammatory pain. Such derivatives were orally administered 30 minutes before inducing writhes by means of an acetic acid intraperitoneal injection. Afterward, the number of writhes was measured and the results were expressed as a percentage of writhes reduction with respect to the vehicle-treated mice (Table 3). All the tested compounds proved to be effective in reducing the number of writhes. However, the nitrooxy amides 2a,b exhibited a better pharmacological profile with respect to the acetic esters, differently from what emerged from the in vitro results. Indeed, the acetic esters 1a,b were almost inactive at low doses, showing an appreciable efficacy only when administered at doses higher than 10 mg/kg. Interestingly, acetic ester 1b was as active as the methylsulphonyl analogue MAB 103 only in the 20 mg/kg range (MAB 103 % of writhes inhibition at 20 mg/kg= 47.5), while it was even active at 10 mg/kg (MAB 103 is inactive below 20 mg/kg)[33]. On the other hand, the acetic amides showed good activities

also in the 1-10 mg/kg range, probably because they are endowed with both a greater solubility and a higher stability, as previously demonstrated for the methylsulphonyl analogues[37]. Therefore, their improved pharmacokinetic properties might counterbalance the lower affinity for the enzyme. Derivative 2b exhibited a unique profile: being the most active one in the 3-10 mg/kg range, its efficacy was also evaluated at lower doses and it proved to be significantly effective even at 1 mg/kg (35% of writhes reduction), with an efficacy comparable to that of celecoxib and better than that of the methylsulphonyl analogue MAB137 (inactive below 3 mg/kg)[37]. Finally, the hydroxyl compound 4b was found to be less active than the corresponding nitrooxyl analogue, confirming the trend observed for the previously synthesized compounds.

Table 3. Effects of 1-2, 4b and celecoxib in the mouse abdominal constriction test (acetic acid 0.6%).

Table 3. Effects of **1-2**, **4b** and celecoxib in the mouse abdominal constriction test (acetic acid 0.6%).

Treatment	No. of mice	Dose (mg/kg po)	No.of writhes	Writhes reduction (%)
CMC	28	-	33.6±2.2	-
1a	5	10	27.6±3.5	17.8
1a	6	30	19.0±2.9	43.4
1b	10	5	30.6±3.7	8.9
1b	10	10	18.9±3.0*	43.7
1b	8	20	17.8±3.4*	47.0
2a	10	5	26.7±3.3	20.5
2a	10	10	18.5±4.2*	44.9
2a	8	20	15.8±3.7*	53.0
2a	9	40	16.7±2.5*	50.3
2b	5	1	21.8±3.5*	35.1
2b	5	3	19.4±2.9*	42.3
2b	6	10	16.7±3.5*	50.3
4b	7	3	26.4±2.5^	21.4
4b	8	10	23.8±3.1	29.2
4b	6	30	21.2±2.7*	36.9
celecoxib	10	1	19.3±2.5	42.5
celecoxib	10	5	16.6±2.2	50.6
celecoxib	10	10	14.2±2.3	57.7
celecoxib	10	20	13.9±2.7	58.6
celecoxib	10	40	10.2±2.1	69.6

All drugs were administered per os 30 min before test ^P<0.05; *P<0.01 versus vehicle treated mice.

Structure-based studies

As previously reported, Vina showed the most suitable docking program to study the binding mode of COX-2 inhibitors[31]. Therefore, the newly synthesized compounds were all cross-docked by means of this program. In particular, compounds were modeled and cross-docked into all the available COX-2 experimental structures following the docking protocol previously reported[31]. The lowest energy poses were selected as the likely binding modes for the newly synthesized compounds. In general the new derivatives were found to dock into COX-2 similarly to the previously reported compounds[31]. To avoid redundancy the herein binding mode description focused on the most in vivo active compound 2b (see Table 3), which was found able to inhibit COX-2 (see Table 1). The lowest energy docked pose of 2b was found in the celecoxib/COX-2 experimental complex (pdb entry code 3LNI) displaying a binding mode highly superimposable to that of the reference compound (Fig. 5). The three aromatic portions of the two compounds are highly overlapped and only small differences are visible for the 3-fluorophenyl group of 2b and the 4-methylphenyl group of celecoxib, being the former slightly rotated of about 30 degrees to allow accommodation of the 3-fluoro substituent. Differently from celecoxib, the 2b side chain fills a pocket formed mainly by hydrophobic residues (Met113, Val116, Leu117, Val344, Ile345, Val349, Leu359, Ser530, Leu531 and Leu534) and a moderate hydrogen bond distance is observable among the distal nitro group and the Ser530 side chain hydroxyl (Ser513-NO2•••HOSer513= 3.02 Å).

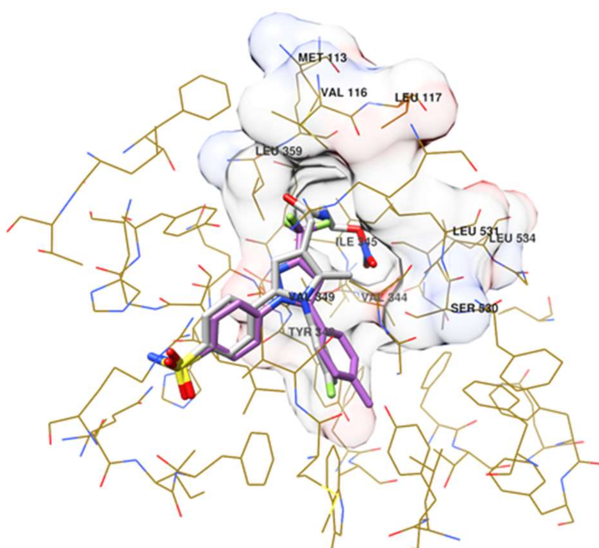


Figure 5. Docked proposed 2b (gray colored carbon atoms) binding mode into 3LNI binding site. For comparison purpose, the co-crystallized celecoxib is also depicted (light magenta colored carbon atoms). The figure was generated by means of UCSF Chimera 1.10.2. The surface of Met113, Val116, Leu117, Val344, Ile345, Val349, Leu359, Ser530, Leu531 and Leu534 residues is displayed to highlight the 2b side chain binding pocket.

Conclusions

We reported the further structural modification of a series of pharmacodynamic hybrids based on a 1,5-diarylpyrrole scaffold. Some of the newly synthesized compounds were endowed with a good affinity for COX-2, proving that the replacement of methylsulphonylphenyl moiety at the C5 of the pyrrole ring does not significantly affect the affinity for the target enzyme. The nitrooxyl derivatives 1-2 also exhibited good vasorelaxing properties, due to NO release. Overall, the tested compounds exhibited a good in vivo pharmacological profile and they all proved to be able to reduce writhes in a murine model of inflammatory

pain. In particular, derivative 2b is characterized by an outstanding in vivo potency and proved to be effective even at 1 mg/kg, showing a lower effective dose with respect to the most potent methylsulphonyl analogue previously synthesized MAB 137. Structure-based studies confirmed that it docks into COX-2 adopting a conformation highly superimposable to that of celecoxib, used as a reference compound. Being the most promising derivative of this class, further studies will be addressed to assess its pharmacokinetic profile and to evaluate which is the actual contribution of the sulphamoyl moiety to its solubility and to its bioavailability.

Experimental Section

Chemistry

All chemicals used were of reagent grade. Yields refer to purified products and are not optimized. Melting points were determined in open capillaries on a Gallenkamp apparatus and were uncorrected. Sigma–Aldrich silica gel 60 (230–400 mesh) was used for column chromatography. Merck TLC plates (silica gel 60 F 254) were used for thin-layer chromatography (TLC). 13C NMR and 1H NMR spectra were recorded with a Bruker AC 400 spectrometer in the indicated solvent (TMS as the internal standard). The values of the chemical shifts are expressed in δ. Mass spectra were recorded on an API-TOF Mariner by Perspective Biosystem (Stratford, Texas, USA). Compound purity was assessed by elemental analysis obtained with a PE 2400 analyzer (PerkinElmer). The purity of target compounds was >95%.

General procedure for the preparation of nitrooxyalkyl and hydroxyalkyl esters 1a,b and 3 a,b.

To a solution of the appropriate acid (5 a,b) (0.200 g, 0.54 mmol) in a mixture of DCM / DMF 10:1 (v/v) (7 mL), the suitable alcohol (0.8 mmol), DMAP (0.109 g, 0.65 mmol), EDCi (0.106 g, 0.87 mmol) and TEA (0.11 mL, 0.65 mmol) were added in sequence under a nitrogen atmosphere. The reaction was quenched after 15 h with water (10 mL) and extracted with DCM (50 mL X 3). The organic layer was washed with 1 N HCl (50 mL), NaHCO3 saturated solution (50 mL), brine (50 mL) and dried over Na2SO4. The organic phase was then filtrated and concentrated under reduced pressure to give a crude product that was purifyed on silica gel using a mixture of cyclohexane/ethyl acetate 2:1 (v/v) as eluent to give derivatives 1a,b and 3 a,b as yellowish and white powders, respectively.

2-(Nitrooxy)ethyl 2-(1-(4-fluorophenyl)-2-methyl-5-(4-sulfamoylphenyl)-1H-pyrrol-3-yl)acetate (1a): Yellowish powder, (0.162 g, 63% yield); mp 109 °C; 1H NMR (400 MHz CDCl3) δ (ppm) = 2.05 (s, 3 H, CH3 pyrrolic), 3.51 (s, 2H, C-CH2-CO), 4.43 (t, J=3.18 Hz, 2 H, CO-O-CH2-CH2-ONO2), 4.75 (t, J=3.18 Hz, 2 H, CO-O-CH2-CH2-ONO2), 4.78 (s broad, 2 H, SO2NH2), 6.48 (s, 1H, CH pyrrolic), 7.17-7.28 (m, 6H, H aromatic), 7.68 (d, J=7.34 Hz, 2H, H aromatic); 13C-NMR (100 MHz, CDCl3) δ (ppm): 169.5, 158.8, 146.6, 143.4, 142.5, 134.9, 127.8, 126.4, 123.1, 122.6, 121.8, 117.0, 114.4, 66.2, 62.3, 34.8, 10.3; IR (KBr): ν˜=3390, 3300, 1752, 1630 cm−1; ESI-Mass: m/z 500.09 [M + Na]+; HRMS-FAB m/z [M+H]+ calcd for C21H20FN3O7S: 477.1006, found: 477.1003; Anal. calcd for C21H20FN3O7S: C 52.83, H 4.22, N 8.80, O 23.46, found: C 52.80, H 4.25, N 8.82, O 23.47.

2-(Nitrooxy)ethyl 2-(1-(3-fluorophenyl)-2-methyl-5-(4-sulfamoylphenyl)-1H-pyrrol-3-yl)acetate (1b): Yellowish powder, (0.149 g, 58% yield); mp 102 °C; 1H NMR (400 MHz CDCl3) δ (ppm) = 2.09 (s, 3 H, CH3 pyrrolic), 3.55 (s, 2 H, C-CH2-CO), 4.43 (t, J=3.18 Hz, 2 H, CO-CH2-CH2-ONO2), 4.71 (t, J=3.18 Hz, 2 H, CO-CH2-CH2-ONO2), 4.75 (s broad, 2 H, SO2NH2), 6.48 (s, 1H, CH pyrrolic), 6.90 (d, J=7.34 Hz, 1H, H aromatic), 6.96 (d, J=7.34 Hz, 1H, H aromatic), 7.07-7.19 (m, 3H, H aromatic), 7.39-7.37 (m, 1H, H aromatic), 7.68 (d, J=7.34 Hz, 2H, H aromatic); 13C-NMR (100 MHz, CDCl3) δ (ppm): 169.5, 163.8, 144.6, 143.4, 142.5, 134.9, 131.2, 127.5, 127.0, 126.4, 122.6, 117.0, 114.4, 111.1, 106.5, 69.2, 62.3, 34.8, 10.3; IR (KBr): ν˜=3366, 3255, 1749, 1620 cm−1; ESI-Mass: m/z 500.09 [M + Na]+; HRMS-FAB m/z [M+H]+ calcd for C21H20FN3O7S: 477.1006, found: 477.1003; Anal. calcd for C21H20FN3O7S: C 52.83, H 4.22, N 8.80, O 23.46 found: C 52.80, H 4.25, N 8.78, O 23.47.

2-Hydroxyethyl 2-(1-(4-fluorophenyl)-2-methyl-5-(4-sulfamoylphenyl)-1H-pyrrol-3-yl)acetate (3a): White powder, (0.107 g, 43% yield); mp 131 °C; 1H NMR (400 MHz, DMSO-d6) δ ppm 1.98 (s, 3H, CH3 pyrrolic), 3.50 (s, 2H, C-CH2-CO), 3.58 (t, J= 3.20 Hz, 2H, CO-O-CH2-CH2-OH), 4.05 (t, J= 3.20 Hz, 2H, CO-O-CH2-CH2-OH), 4.81 (s broad, 1H, OH), 6.46 (s, 1H, CH pyrrolic), 7.11 (d, J=8.07 Hz, 2H, H aromatic) 7.22-7.27 (m, 6H, H aromatic and SO2NH2), 7.57 (d, J=8.07 Hz, 2H, H aromatic); 13C-NMR (100 MHz, DMSO-d6) δ (ppm): 169.5, 158.8, 146.6, 143.4, 142.5, 134.9, 127.8, 126.4, 123.1, 122.6, 121.8, 117.0, 114.4, 66.2, 62.3, 34.8, 10.3; IR (KBr): ν˜=3350, 3256, 1760 cm−1; ESI-Mass: m/z 455.10 [M + Na]+; HRMS-FAB m/z [M+H]+ calcd for C21H21FN2O5S: 432.1155, found: 432.1150; Anal. calcd for C21H21FN2O5S: C 58.32, H 4.89, N, 6.48, O 18.50 found: C 58.35, H 4.91, N, 6.53, O 18.45.

2-Hydroxyethyl 2-(1-(3-fluorophenyl)-2-methyl-5-(4-sulfamoylphenyl)-1H-pyrrol-3-yl)acetate (3b): White powder, (0.107 g, 46% yield); mp 121 °C; 1H NMR (400 MHz, DMSO-d6) δ (ppm) = 2.01 (s, 3H, CH3 pyrrolic), 3.50 (s, 2H, C-CH2-CO), 3.58 (t, J = 3.16 Hz, 2H, CO-O-CH2-CH2-OH), 4.06 (t, J = 3.16 Hz, 2H, CO-O-CH2-CH2-OH), 4.81 (s broad, 1H, OH), 6.48 (s, 1H, CH pyrrolic), 7.01 (d, J=7.37 Hz, 1H, H aromatic) 7.12 (d, J=7.37 Hz, 2H, H aromatic), 7.25-7.30 (m, 4H, H aromatic and SO2NH2), 7.41-7.45 (m, 1H, H aromatic), 7.51 (d, J=7.37 Hz, 2H, H aromatic); 13C-NMR (100 MHz, DMSO-d6) δ (ppm): 169.5, 158.8, 146.6, 143.4, 142.5, 134.9, 131.7, 127.8, 126.4, 123.1, 121.8, 117.0, 114.4, 11.8, 108.5, 66.2, 62.3, 34.8, 10.3; R (KBr): ν˜= IR (KBr): ν˜=3383, 3350, 1765 cm−1; ESI-Mass: m/z 455.10 [M + Na]+; HRMS-FAB m/z [M+H]+ calcd for C21H21FN2O5S: 432.1155, found: 432.1159; Anal. calcd for C21H21FN2O5S: C 58.32, H 4.14, N 6.48, O 18.76 found: C 58.35, H 4.20, N 6.45, O 18.73.

General procedure for the preparation of nitrooxyalkyl amides 2a,b.

2-aminoethylnitrate salt (0.203 g, 1.2 mmol), DMAP (0.164 g, 0.97 mmol) and EDCI (0.159 g, 1.3 mmol) were added in sequence to a solution of the acid partner (5a,b) (0.300 g, 0.81 mmol) dissolved in a mixture of DCM/DMF 10:1 (v/v) (10 mL), under a nitrogen flow. An excess of TEA (0.17 mL, 1.3 mmol) was then added dropwise and the reaction was stirred at rt for 12 h. The mixture was then quenched with water (10 mL) and extracted with DCM (50 mL X 3). The organic layer was washed with 1 N HCl (50 mL), NaHCO3 saturated solution (50 mL), brine (50 mL) and dried over Na2SO4. After filtration and concentration of the organic phase a crude material was obtained and was purified by chromatography on silica gel using as eluent a mixture of cyclohexane/ethyl acetate 1:1 (v/v), affording compounds 2a,b as yellowish powders.

2-(2-(1-(4-Fluorophenyl)-2-methyl-5-(4-sulfamoylphenyl)-1H-pyrrol-3-yl)acetamido)ethyl nitrate (2a): Yellowish powder, (0.237 g, 50 % yield); mp 127°C ; 1H NMR (400 MHz, DMSO-d6) δ ppm 1.97 (s, 3H, CH3 pyrrolic), 3.26 (s, 2H, C-CH2-CO) 3.37 (t, J=5.01 Hz, 2 H, CO-NH-CH2-CH2-ONO2), 4.54 (t, J=5.01 Hz, 2H, CO-NH-CH2-CH2-ONO2), 6.43 (s, 1H, CH pyrrolic), 7.09 (d, J=8.07 Hz, 2H, H aromatic), 7.21-7.29 (m, 6H, aromatic and SO2NH2), 7.56 (d, J=8.07 Hz, 2H, H aromatic), 8.11 (s broad, 1H, CONH); 13C-NMR (100 MHz, DMSO-d6) δ (ppm) = 169.5, 158.8, 146.6, 143.4, 142.5, 134.9, 127.8, 126.4, 123.1, 122.6, 121.8, 117.0, 114.4, 72.5, 36.2, 34.8, 10.3; IR (KBr): ν˜=2967, 2923, 1735, 1637 cm−1; ESI-Mass: m/z 499.11 [M + Na]+; HRMS-FAB m/z [M+H]+ calcd for C21H21FN4O6S: 477.1166, found: 477.1158; Anal. calcd for: C21H21FN4O6S: C 52.94, H 4.44, N 11.76, O 20.15, found: C 53.00, H 4.43, N 11.70, O 20.13.

2-(2-(1-(3-Fluorophenyl)-2-methyl-5-(4-sulfamoylphenyl)-1H-pyrrol-3-yl)acetamido)ethyl nitrate (2b): Yellowish powder, (0.242 g, 51 % yield); mp 125°C; 1H NMR (400 MHz, DMSO-d6) δ (ppm) = 2.03 (s, 3H, CH3 pyrrolic), 3.28 (s, 2H, C-CH2-CO), 3.43 (t, J=5.14 Hz, 2H, CO-NH-CH2-CH2-ONO2), 4.57 (t, J=5.14 Hz, 2H, CO-NH-CH2-CH2-ONO2), 6.46 (s, 1H, CH pyrrolic) 7.02 (d, J=8.31 Hz, 1H, H aromatic), 7.13 (d, J=8.31 Hz, 2 H, H aromatic), 7.18-7.33 (m, 4H, H aromatic and SO2NH2), 7.51-7.57 (m, 1 H, H aromatic), 7.61 (d, J=8.31 Hz, 2H, H aromatic), 8.15 (s broad, 1H, CONH); 13C-NMR (100 MHz, DMSO-d6) δ (ppm): 169.5, 158.8, 146.6, 143.4, 142.5, 134.9, 131.6, 127.8, 126.3, 125.2, 121.4, 117.0, 114.4, 109.4, 11.9, 76.2, 37.3, 34.8, 10.3. IR (KBr): ν˜=3182, 3079, 1731, 1635 cm−1; ESI-Mass: m/z 499.11 [M + Na]+; HRMS-FAB m/z [M+H]+ calcd for:

477.1166, found: 477.1158; Anal. calcd for: $C_{21}H_{21}FN_4O_6S$: C 52.94, H 4.44, N 11.76, O 20.15, found: C 53.00, H 4.43, N 11.83, O 20.13.

General procedure for the preparation of hydroxyalkyl amides 4a,b.

Ethanolamine (0.067 g, 1.1 mmol), HOBt (0.168 g, 1.1 mmol), and EDCi (0.159 g, 1.3 mmol) were added in sequence to a solution of the suitable 1,5-diarylpyrrole-3-acetic acid (5a,b) (0.300 g, 0.8 mmol) in a mixture of DCM/DMF 10:1 (v/v) (10 mL), under a nitrogen atmosphere. An excess of TEA (0.17 mL, 1.3 mmol) was then added dropwise and the reaction was stirred at rt. After 12 h, the mixture was quenched with water (10 mL) and extracted with DCM (50 mL X 3). The organic layer was washed with 1 N HCl (50 mL), $NaHCO_3$ saturated solution (50 mL), brine (50 mL) and dried over Na_2SO_4. After filtration and concentration of the organic phase the so obtained crude material was purified by chromatography on silica gel using ethyl acetate as eluent to give the desired products as white powders.

2-(1-(4-Fluorophenyl)-2-methyl-5-(3-sulfamoylphenyl)-1H-pyrrol-3-yl)-N-(2-hydroxyethyl)acetamide (4a): White powder, (0.271 g, 71 % yield); mp 155°C; 1H NMR (400 MHz, DMSO-d6) δ (ppm)= 2.06 (s, 3H, CH_3 pyrrolic), 3.12 (t, J=5.14 Hz, 2H, CO-NH-CH_2-CH_2-OH), 3.25 (s, 2H, C-CH_2-CO), 3.41 (t, J=5.14 Hz, 2H, CO-NH-CH_2-CH_2-OH), 4.68 (s broad, 1H, OH), 6.46 (s, 1H, CH pyrrolic), 7.11 (d, J=7.89 Hz, 2H, H aromatic), 7.23-7.31 (m, 6H, H aromatic and SO_2NH_2), 7.57 (d, J=7.89 Hz, 2H, H aromatic), 7.83 (s broad, 1H, CONH); 13C-NMR (100 MHz, DMSO-d6) δ (ppm):171.9, 160.2, 147.0, 144.0, 142.9, 134.6, 128.0, 127.3, 126.2, 121.9, 121.5, 116.0, 113.7, 66.9, 41.8, 33.9,10.1; IR (KBr): ν˜=3358, 3342, 1752 cm^{-1}; ESI-Mass: m/z 454.12 $[M + Na]^+$; HRMS-FAB m/z $[M+H]^+$ calcd for $C_{21}H_{22}FN_3O_4S$: 431.1315, found: 431.1318; Anal. calcd for $C_{21}H_{22}FN_3O_4S$: C 58.46, H 5.14, N 9.74, O 14.83 found: C 58.40, H 5.20, N 9.67, O 14.80.

2-(1-(3-Fluorophenyl)-2-methyl-5-(3-sulfamoylphenyl)-1H-pyrrol-3-yl)-N-(2-hydroxyethyl)acetamide (4b): White powder, (0.256 g, 67% yield); mp 175°C; 1H NMR (400 MHz, DMSO-d6) δ (ppm)= 2.04 (s, 3H, CH_3 pyrrolic), 3.15 (t, J=5.62 Hz, 2H, CO-NH-CH_2-CH_2-OH), 3.27 (s, 2H, C-CH_2-CO), 3.46 (t, J=5.62 Hz, 2H, CO-NH-CH_2-CH_2-OH), 4.69 (s broad, 1H, OH), 6.48 (s, 1H, CH pyrrolic), 7.04 (d, J=7.82 Hz, 1H, H aromatic) 7.13 (d, J=8.07 Hz, 2H, H aromatic), 7.19-7.33 (m, 4H, H aromatic and SO_2NH_2), 7.49-7.51 (m, 1H, H aromatic), 7.60 (d, J=8.07 Hz, 2H, H aromatic), 7.90 (s broad, 1H, CONH); 13C-NMR (100 MHz, DMSO-d6) δ (ppm): 171.3, 162.9, 142.5, 142.0, 141.6, 133.6, 129.2, 127.8, 126.5, 125.0, 120.9, 116.8, 112.6, 111.9, 106.8, 60.9, 40.8, 33.2, 9.6; IR (KBr): ν˜=3346, 3300, 1750 cm^{-1}; ESI-Mass: m/z 454.12 $[M + Na]^+$; HRMS-FAB m/z $[M+H]^+$ calcd for $C_{21}H_{22}FN_3O_4S$: 431.1315, found: 431.1309; Anal. calcd for $C_{21}H_{22}FN_3O_4S$: C 58.46, H 5.14, N 9.74, O 14.83 found: C 58.90, H 5.17, N 9.77, O 14.86.

In vitro anti-inflammatory studies

The inhibitory activity of compounds 1-4 against both COX-1 and COX-2 was determined by the commercially available COX Inhibitor Screening Assay (Cayman Chemical, Ann Arbor, MI, USA, catalogue no. 560131), which exploits an enzyme immunoassay to measure PGE_2 α produced by stannous chloride reduction of $PGEH_2$, derived in turn by reaction between the target enzyme and the substrate, arachidonic acid. According to manufacture's protocol, test compounds (10 µL) were incubated for 10 min at 37 °C with assay buffer (0.1 M Tris-HCl, pH 8, 160 µL), Heme (10 µL), and either ovine COX-1 or human recombinant COX-2 enzyme solution (10 µM). Arachidonic acid (10 µL) was then added, and the resulting mixture was incubated for 2 min at 37°C. Enzyme catalysis was stopped by adding HCl (1M, 10 mL) and the obtained $PGEH_2$ were converted to $PGE_2α$ with saturated stannous chloride solution (20 µL). Prostanoids were finally quantified by EIA and their amount was determined through interpolation from a standard curve. The % inhibition of target enzyme by test compounds was calculated by comparing $PGE_2α$ produced in compound-treated samples with that of the compound-free, control sample. The highly selective COX-2 inhibitor Dup-697 was used as the reference compound. All the test compounds were dissolved into dilute assay buffer, and their solubility was facilitated by using DMSO, whose concentration never exceeded 1% in the final reaction mixture. The inhibitory effect of test compounds was routinely estimated at a

concentration of 10 µM. Results obtained are the mean of at least three determination, carried out in triplicate (Table 1).

Ex vivo vasorelaxing activity

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609. The effects of the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g). After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding. The aortae were immediately excised, freed of extraneous tissues and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five mm wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; glucose 5.5), thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with a computerised system (Biopac). After an equilibration period of 60 min, the endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 lM) to KCl (30 mM)-precontracted vascular rings. A relaxation <10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation P10% (i.e. significant presence of the endothelium), were discarded. From 30 to 40 min after the confirmation of the endothelium removal, the aortic preparations were contracted by a single concentration of KCl (30 mM) and when the contraction reached a stable plateau, 3-fold increasing concentrations of the tested compounds (1 nM–10 µM) were added. Preliminary experiments showed that the KCl (30 mM)-induced contractions remained in a stable tonic state for at least 40 min. The same experiments were carried out also in the presence of a well-known GC inhibitor: ODQ 1 µM which was incubated in aortic preparations after the endothelium removal confirmation. The vasorelaxing efficacy was evaluated as maximal vasorelaxing response (E_{max}), expressed as a percentage (%) of the contractile tone induced by KCl 30 mM. When the limit concentration 10 µM (the highest concentration, which could be administered) of the tested compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM, evoked by this limit concentration. The parameter of potency was expressed as pIC_{50}, calculated as negative logarithm of the molar concentration of the tested compounds evoking a half reduction of the contractile tone induced by KCl 30 mM. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as mean ± standard error, for 6-10 experiments. Two-way ANOVA was selected as statistical analysis, $P < 0.05$ was considered representative of significant statistical differences. Experimental data were analysed by a computer fitting procedure (software: Graph-Pad Prism 4.0).

In vivo studies

All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines[39]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Male Swiss albino mice (23–25 g) and Sprague-Dawley or Wistar rats (150–200 g) were used. The animals were fed with a standard laboratory diet and tap water ad libitum and kept at 23 (1 °C with a 12 h light/dark cycle, light on at 7 a.m.) The analgesic activity of compounds was assessed by performing the abdominal constriction test, using mice into which a 0.6% solution of acetic acid (10 mL/kg) had been injected intra-peritoneal (ip). The number of stretching movements was counted for 10 min, starting 5 min after administration.

New COX-inhibitor preparation and docking

All calculations were performed using a blade (Intel-Xeon CPU) running Debian GNU/Linux “Jessie” 8.1 64 bit operating system. Ligand’s 3D conformations were generated from scratch. Marvin was used for drawing, displaying and characterizing chemical structures, Marvin 15.1.12.0, 2015, ChemAxon (http://www.chemaxon.com). OpenBabel was used to generate random conformation to run the cross-docking experiments by Vina COX-2. Docking procedure and detail were the same as previously reported[31].

Keywords: CINODs • drug discovery • 1,5-diarylpyrroles • inflammation • pharmacodynamics hybrids.

[1] P. Patrignani, C. Patrono, Biochim. Biophys. Acta 2015, 1851, 422–432.

[2] G. A. FitzGerald, C. Patrono, N. Engl. J. Med. 2001, 345, 433–442.

[3] Y. Cheng, S. C. Austin, B. Rocca, B. H. Koller, T. M. Coffman, T. Grosser, J. A. Lawson, G. A. FitzGerald, Science 2002, 296, 539–541.

[4] J.-M. Dogné, C. T. Supuran, D. Pratico, J. Med. Chem. 2005, 48, 2251–2257.

[5] R. S. Bresalier, R. S. Sandler, H. Quan, J. A. Bolognese, B. Oxenius, K. Horgan, C. Lines, R. Riddell, D. Morton, A. Lanas, et al., N. Engl. J. Med. 2005, 352, 1092–1102.

[6] P. McGettigan, D. Henry, JAMA 2006, 296, 1633–1644.

[7] B. H. McCarberg, B. Cryer, Am. J. Ther. 2015, 22, e167–178.

[8] S. Consalvi, M. Biava, G. Poce, Expert Opin. Ther. Pat. 2015, 1–15.

[9] S. H. Hwang, A. T. Wecksler, K. Wagner, B. D. Hammock, Curr. Med. Chem. 2013, 20, 1783–1799.

[10] G. Palermo, A. D. Favia, M. Convertino, M. De Vivo, ChemMedChem 2015, DOI 10.1002/cmdc.201500507.

[11] A. Martelli, M. C. Breschi, V. Calderone, Curr. Pharm. Des. 2009, 15, 614–636.

[12] J. L. Wallace, L. J. Ignarro, S. Fiorucci, Nat. Rev. Drug Discov. 2002, 1, 375–382.

[13] R. M. Palmer, A. G. Ferrige, S. Moncada, Nature 1987, 327, 524–526.

[14] L. J. Ignarro, J. Physiol. Pharmacol. Off. J. Pol. Physiol. Soc. 2002, 53, 503–514.

[15] J. O. Lundberg, M. T. Gladwin, E. Weitzberg, Nat. Rev. Drug Discov. 2015, 14, 623–641.

[16] Y. Zhao, P. M. Vanhoutte, S. W. S. Leung, J. Pharmacol. Sci. 2015, 129, 83–94.

[17] U. Förstermann, W. C. Sessa, Eur. Heart J. 2012, 33, 829–837.

[18] J. L. Wallace, Physiol. Rev. 2008, 88, 1547–1565.

[19] K. R. A. Abdellatif, A. Moawad, E. E. Knaus, Bioorg. Med. Chem. Lett. 2014, 24, 5015–5021.

[20] A. Bhardwaj, Z. Huang, J. Kaur, E. E. Knaus, ChemMedChem 2012, 7, 62–67.

[21] M. A. Chowdhury, K. R. A. Abdellatif, Y. Dong, G. Yu, Z. Huang, M. Rahman, D. Das, C. A. Velázquez, M. R. Suresh, E. E. Knaus, Bioorg. Med. Chem. Lett. 2010, 20, 1324–1329.

[22] K. Chegaev, L. Lazzarato, P. Tosco, C. Cena, E. Marini, B. Rolando, P.-A. Carrupt, R. Fruttero, A. Gasco, J. Med. Chem. 2007, 50, 1449–1457.

[23] E. Del Grosso, D. Boschi, L. Lazzarato, C. Cena, A. Di Stilo, R. Fruttero, S. Moro, A. Gasco, Chem. Biodivers. 2005, 2, 886–900.

[24] M. Biava, G. C. Porretta, A. Cappelli, S. Vomero, F. Manetti, M. Botta, L. Sautebin, A. Rossi, F. Makovec, M. Anzini, J. Med. Chem. 2005, 48, 3428–3432.

[25] M. Biava, G. C. Porretta, G. Poce, S. Supino, S. Forli, M. Rovini, A. Cappelli, F. Manetti, M. Botta, L. Sautebin, et al., J. Med. Chem. 2007, 50, 5403–5411.

[26] M. Biava, G. C. Porretta, G. Poce, S. Supino, F. Manetti, S. Forli, M. Botta, L. Sautebin, A. Rossi, C. Pergola, et al., Bioorg. Med. Chem. 2008, 16, 8072–8081.

[27] M. Anzini, M. Rovini, A. Cappelli, S. Vomero, F. Manetti, M. Botta, L. Sautebin, A. Rossi, C. Pergola, C. Ghelardini, et al., J. Med. Chem. 2008, 51, 4476–4481.

[28] M. Biava, G. C. Porretta, G. Poce, C. Battilocchio, F. Manetti, M. Botta, S. Forli, L. Sautebin, A. Rossi, C. Pergola, et al., J. Med. Chem. 2010, 53, 723–733.

[29] M. Biava, G. C. Porretta, G. Poce, C. Battilocchio, M. Botta, F. Manetti, M. Rovini, A. Cappelli, L. Sautebin, A. Rossi, et al., Curr. Med. Chem. 2011, 18, 1540–1554.

[30] C. Battilocchio, G. Poce, S. Alfonso, G. C. Porretta, S. Consalvi, L. Sautebin, S. Pace, A. Rossi, C. Ghelardini, L. Di Cesare Mannelli, et al., Bioorg. Med. Chem. 2013, 21, 3695–3701.

[31] S. Consalvi, S. Alfonso, A. Di Capua, G. Poce, A. Pirolli, M. Sabatino, R. Ragno, M. Anzini, S. Sartini, C. La Motta, et al., Bioorg. Med. Chem. 2015, 23, 810–820.

[32] A. Di Capua, C. Sticozzi, S. Brogi, M. Brindisi, A. Cappelli, L. Sautebin, A. Rossi, S. Pace, C. Ghelardini, L. Di Cesare Mannelli, et al., Eur. J. Med. Chem. 2016, 109, 99–106.

[33] M. Biava, G. C. Porretta, G. Poce, C. Battilocchio, S. Alfonso, M. Rovini, S. Valenti, G. Giorgi, V. Calderone, A. Martelli, et al., J. Med. Chem. 2011, 54, 7759–7771.

[34] M. Biava, C. Battilocchio, G. Poce, S. Alfonso, S. Consalvi, G. C. Porretta, S. Schenone, V. Calderone, A. Martelli, L. Testai, et al., Eur. J. Med. Chem. 2012, 58, 287–298.

[35] M. Anzini, A. Di Capua, S. Valenti, S. Brogi, M. Rovini, G. Giuliani, A. Cappelli, S. Vomero, L. Chiasserini, A. Sega, et al., J. Med. Chem. 2013, 56, 3191–3206.

[36] A. Martelli, L. Testai, M. Anzini, A. Cappelli, A. Di Capua, M. Biava, G. Poce, S. Consalvi, A. Giordani, G. Caselli, et al., Pharmacol. Res. 2013, 78, 1–9.

[37] M. Biava, C. Battilocchio, G. Poce, S. Alfonso, S. Consalvi, A. Di Capua, V. Calderone, A. Martelli, L. Testai, L. Sautebin, et al., Bioorg. Med. Chem. 2014, 22, 772–786.

[38] I. K. Khanna, Y. Yu, R. M. Huff, R. M. Weier, X. Xu, F. J. Koszyk, P. W. Collins, J. N. Cogburn, P. C. Isakson, C. M. Koboldt, et al., J. Med. Chem. 2000, 43, 3168–3185.

[39] C. Kilkenny, W. J. Browne, I. C. Cuthill, M. Emerson, D. G. Altman, J. Pharmacol. Pharmacother. 2010, 1, 94–99.