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Abstract

Nitrooxymethyl substituted analogues of Celecoxib were synthesized and tested for their COX inhibiting, vasodilator, and antiaggregatory activities, as well as for their metabolic stability in human serum and whole blood. The results showed their potency and selectivity in inhibiting the COX isoforms, evaluated in whole human blood, as well as their antiaggregatory activity, to depend closely on the position at which the NO-donor moiety is introduced. All products dilated rat aorta strips precontracted with phenylephrine in a dose dependent manner through a cGMP dependent mechanism. They were stable in human serum while in blood they were metabolically transformed, principally to the related alcohols.

Introduction. - Cyclooxygenase (COX) is one of the key enzymes implicated in the transformation of arachidonic acid into prostanoids. It exists in at least two isoforms, COX-1 and COX-2. The former is prevalently a constitutive and the latter an inducible isoform. Selective blocking of these isoforms induces specific pharmacological effects which can be exploited in therapy [1]. COX-2 selective inhibitors (Coxibs) are a new class of drugs recently introduced onto the market [2]; they induce anti-inflammatory effects without the gastrotoxic side-effects typical of the classical non-steroidal anti-inflammatory drugs (NSAIDs), which are non-selective COX inhibitors. An interesting aspect of these drugs is their potential use in treating *Alzheimer*'s disease and certain forms of cancer [3], [4]. The drawback in their use is that they increase the risk of heart attack and stroke [5], [6]. Blood vessels and platelets are the major targets of prostanoids such as prostacycline PGI_2, prostaglandin PGE_2 and thromboxane TxA_2 in the cardiovascular system. The proaggregatory and vasoconstrictor TxA_2 is mainly synthesized *via* COX-1 in the platelets, while vasodilator (PGI_2, PGE_2) and antiplatelet (PGI_2) compounds are synthesized mainly in the vascular endothelium. A strategy to improve the benefit-risk profiles of these drugs is to design a multi-target drug by combining COX-2 selective inhibition with nitric oxide (NO)-dependent activities. NO displays a variety of effects in the cardiovascular system, including vasodilation, inhibition of platelet aggregation, modulation of platelet and leukocytes adherence to vessels, and inhibition of smooth muscle cell proliferation [7]. Examples of this type of approach have already been reported [8], [9] including by our group [10], [11]. As a development of our work in this field, we now describe the synthesis and structural characterization of a new series of NO-donor COX-2 inhibitors obtained by introducing NO-donor nitrooxy functions into the well known selective COX-2 inhibitor Celecoxib

1 [12] (*Figure 1*), that has recently been placed under surveillance by FDA, following its suspected cardiotoxicity.

Figure 1

The results of a preliminary pharmacological screening on these new hybrid products and on their alcoholic metabolites show that their potency and selectivity in inhibiting the COX isoforms, evaluated in whole human blood, is closely dependent on the position at which the NO-donor moiety is introduced. This aspect is discussed, as well as the NO-dependent ability of the products to relax rat aorta strips precontracted with phenylephrine and, for selected compounds, their capacity to inhibit collagen-induced platelet aggregation of human platelet rich plasma (PRP).

Results and Discussion. – 1. *Chemistry.* The general strategy for the synthesis of the nitrooxy substituted Celecoxib derivatives **7**, **11**, **15** is outlined in *Schemes 1* and *2*. The trifluoromethyl-β-diketone **4** was obtained by refluxing 4-(hydroxymethyl)acetophenone (**2**) and ethyl trifluoroacetate in MeOH in the presence of MeONa The intermediate **4** was isolated but not purified since it was immediately condensed in refluxing EtOH with the hydrochloride of the phenylhydrazine derivative **5** to give, with a fair yield, the 1,5-diphenyl pyrazole derivative **6**, accompanied by a small amount of the 1,3-diphenyl isomer. This is in keeping with reports that 1,5-diarylpyrazole derivatives are almost exclusively produced when the hydrochloride salt of the phenylhydrazine is condensed with trifluoromethyl-β-diketones in refluxing EtOH or MeOH [13]. Treatment of **6** dissolved in MeCN with N-bromosuccinimide (NBS) in the presence of Ph_3P and $AgNO_3$ afforded a crude product that was purified by flash-chromatography to give the expected final compound **7**, mononitrooxymethyl substituted at the 5-phenyl group. When **4** underwent reaction with the hydrochloride

salt of phenylhydrazine derivative **8**, it produced the pyrazolylbenzoate **9** in a highly regiospecific manner. Reduction of the ester function of **9** with $LiAlH_4$ yielded the di-alcohol **10**, which was transformed into the final dinitrooxy derivative **11** following the same procedure used to prepare **7** from **6**. This reaction was accompanied by the formation of small amounts of a mixture of the two isomeric mononitrooxy alcohols **11a** and **11b** in a ratio of ca. 1:1 (NMR detection). When **8** was allowed to react in refluxing MeOH with the trifluoromethyl-β-diketone **12**, obtained from the commercially-available 4-methylacetophenone (**3**), the 1,5-diphenylpyrazole derivative **13** was yielded in a moderately regiospecific manner. This product was separated by flash-chromatography from a minor amount of 1,3-diphenyl isomer and transformed into the final mononitrooxy derivative **15**, through the intermediate formation of the alcohol **14**, following the sequence of reactions used to prepare **11** from **9**.

2. *Stability in human serum and in human whole blood*. It is known that nitrates can undergo chemical and enzymatic metabolism [14]. Celecoxib and its derivatives were found to be stable following 24 h incubation in human serum. By contrast, derivatives **7**, **11**, **15** were extensively metabolized when incubated in whole human blood. This transformation was monitored by RP-HPLC. The half lives ($t_{1/2}$) are reported in Table 1. Products **11** and **15** behaved similarly, while **7** surprisingly did not: its $t_{1/2}$ is about 1 h, *versus* 25-30 h of the other compounds, and after 24 h only 4.5% of the original product remained unchanged. Compounds **11** and **15** were principally transformed into the corresponding alcohols **10**, **11a/b** and **14** respectively (Table 1). These metabolites were identified by comparison of their retention times with those of alcohols **10** and **14**, which were used as intermediates to prepare the final compounds. Similarly, the mixture **11a/b** was used as reference to identify the two mononitrooxy isomeric alcohols that derive from the biotransformation of **11**. The behavior of compound **7**

again differed: HPLC analysis, using the alcohol **6** as standard, revealed only 4.5% of this product as detectable metabolite. This indicates that the other metabolites formed are not free in the cytosol but they are sequestrated by components of the blood compartment.

3. *COX Inhibition*. The ability of the products and of their alcoholic metabolites to inhibit the COX-enzymes was evaluated in human whole blood following an established procedure [15]. In order to assay the extent of COX-2 isoform inhibition, human heparinized whole blood was incubated with lipopolysaccharide (LPS) overnight in the presence of the inhibitors, and plasma reduction of PGE_2 production in comparison with control samples was assayed as a function of COX-2 inhibition. For COX-1 inhibition, whole blood samples without any anticoagulant were incubated with the inhibitors for 1 h; plasma was then collected and analyzed for TxB_2 production. The potencies of the products are expressed as IC_{50} (Table 2); when this could not be calculated because inhibition did not reach 50%, inhibition at the maximum concentration tested (100 μM) is reported.

The data show that product **7**, derived from substituting the nitrooxymethyl function for the methyl group of Celecoxib, is a feeble COX-2 inhibitor, being about fifty times less potent than the lead, but that it retains a good degree of COX-2 selectivity: it displayed negligible COX-1 activity when tested at 100 μM concentration. Its alcoholic metabolite **6** behaves similarly. When it is the aminosulfonyl group that is substituted, which is an important determinant for the COX-2 selectivity of **1** [16], **15** is obtained, that is a more potent COX-1 inhibitor than **1** and that displays a rather good COX-1 selectivity. COX-1 inhibition potency and selectivity are even more evident in its alcoholic metabolite **14**. Finally, the simultaneous substitution of both the methyl and the aminosulfonyl moiety in **1** gives rise to **11**, which is a fairly potent COX-2 inhibitor,

with negligible COX-1 activity when tested at a concentration of 100 μM. By contrast, its dihydroxy metabolite **10** shows a COX-inhibitory profile very close to that of **14**. All compounds were also tested for their ability to inhibit collagen-induced platelet aggregation of human platelet rich plasma (PRP). The products **10** and **14** display antiplatelet action in the μM range (Table 3) in keeping with their ability to inhibit the COX-1 isoform. Also the activity of the NO-donor **15** is essentially COX-1 and not NO-dependent, in agreement with the reduced ability of platelets to effect NO release from organic nitrates [17]. Indeed, the antiaggregatory potency of this compound was not modified by the presence of ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a well known inhibitor of the soluble guanylate cyclase (sGC).

4. *Vasodilation*. The in vitro vasodilating activities of this new series of COX inhibitors was assessed on rat aorta strips precontracted with phenylephrine. All products relaxed the contracted tissues in a concentration-dependent manner. Their potencies, expressed as EC_{50}, are in Table 3. All act as potent vasodilators, their potencies being closely distributed in the submicromolar range. Vasodilating potencies markedly decreased when the experiments were repeated in the presence of 1 μM ODQ. This is typical of the classical nitrooxy-containing compounds, such as isosorbide dinitrate (ISDN), which was used as reference.

Conclusions. – Introduction at the *p*-position of the phenyl rings present in Celecoxib of either the nitrooxymethyl group or the hydroxymethyl group, deriving from its metabolic transformation, gives rise to a complex behaviour. When these moieties are linked to the N-phenyl ring the selective and potent COX-1 inhibitors **14**, **15**, endowed with antiaggregatory activity, are obtained. By contrast, when they are attached to the C-phenyl group the selective but weak COX-2 inhibitors **6**, **7** are generated. Interesting results occurred following the simultaneous introduction of either two nitrooxy or two

hydroxymethyl functions on the two phenyl groups. In the former case the fairly potent and selective COX-2 inhibitor **11**, endowed with a feeble antiaggregatory activity, was obtained while in the latter case compound **10** was obtained, which is a potent and selective COX-1 inhibitor endowed with a high antiaggregatory activity. All of the products containing nitrooxy moieties displayed NO-dependent vasodilatory activity. Compound **11** emerges as the most interesting product arising from these chemical manipulations of compound **1**, in terms of the possibility of producing a COX-2 selective inhibitor potentially possessing low cardiotoxicity.

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Experimental Part.

General. M.p. were measured with a capillary apparatus (*Büchi 540*). M.p. with decomposition were determined after placing the sample in a bath at a temperature 10° below the M.p.; a heating rate of 1° min^{-1} was used. All compounds were routinely checked by FT-IR (*PerkinElmer SPECTRUM BXII*), ^{1}H and ^{13}C-NMR (*Bruker Avance 300*) and mass spectrometry (*Finnigan-Mat TSQ-700*). Flash column chromatography was performed on silica gel (*Merck* Kieselgel 60, 230-400 mesh ASTM) using the eluents indicated. Thin layer chromatography (TLC) was carried out on 5 x 20 cm plates with 0.25 mm layer thickness. Anhydrous $MgSO_4$ was used as drying agent for the org. phases. Analysis (C, H, N) of the new compounds was performed by REDOX (*Monza*) and the results are within ± 0.4% of the theoretical values. Compounds **2** [18], **5** [19], **8** [20], and **12** [12], were synthesized following methods described in the literature. Tetrahydrofuran (THF) was distilled immediately before use from Na and benzophenone.

4,4,4-Trifluoro-1-[4-(hydroxymethyl)phenyl]butane-1,3-dione (**4**)**.** 1.5 M MeONa in MeOH (5 ml, 7.5 mmol) was added to a soln. of 4-(hydroxymethyl)acetophenone (**2**)

(0.72 g, 4.8 mmol) in dry MeOH (4 ml). The mixture was stirred for 5 min, after which ethyl trifluoroacetate (0.7 ml, 7.0 mmol) was added. After refluxing for 24 h, the mixture was cooled to r. t. and concentrated *in vacuo*; 10% HCl (10 ml) was added and the mixture extracted with AcOEt. The extracts were dried and concentrated to afford **4** as a brown oil (0.99 g); this was used without further purification.

*4-{5-[4-(Hydroxymethyl)phenyl]-3-(trifluoromethyl)-1*H*-pyrazol-1-yl}benzensulfonamide* (**6**)**.** 4-(Sulfamoylphenyl)hydrazine hydrochloride (**5**)(0.93 g, 4.2 mmol) was added to a stirred soln. of **4** (0.99 g) in EtOH (50 ml). The mixture was heated to reflux for 24 h. After cooling to r. t., the mixture was concentrated *in vacuo*, the residue was taken up in AcOEt, washed with H_2O and brine, dried and concentrated *in vacuo* to give an oil that was purified by flash chromatography (eluent PE/*i*-PrOH 9/1) to give **6** as a pale yellow solid (0.95 g, 57% yield). M.p. 157-158° (from benzene). ^{1}H-NMR ($CDCl_3$): 7.84 (*d*, 2H, *J* = 8.6, Ar); 7.41 (*d*, 2H, *J* = 8.6, Ar); 7.34 (*d*, 2H, *J* = 8.1, Ar); 7.20 (*d*, 2H, *J* = 8.1, Ar); 6.76 (*s*, 1H, CH pyrazol); 5.31 (*s*, 2H, NH_2); 4.69 (*s*, 2H, CH_2); 2.06 (*s*, br, 1H, OH). ^{13}C-NMR ($CDCl_3$): 144.9; 144.2 (q, ^2J = 38); 142.4; 142.3; 141.5; 129.0; 127.7; 127.5; 127.4; 125.6; 121.0 (q, ^1J = 268); 106.6 (q, ^3J = 2); 64.5. CI-MS (*i*-Bu): 398 $(M+1)^+$. Anal. calc. for $C_{17}H_{14}F_3N_3O_3S$ (397.38): C 51,38, H 3.55, N 10.57; found C 51.68, H 3.66, N 10.52.

*Methyl 4-{5-[4-(hydroxymethyl)phenyl]-3-(trifluoromethyl)-1*H*-pyrazol-1-yl}benzoate* (**9**)**.** 4-(Methoxycarbonyl)phenylhydrazine hydrochloride (**8)** (0.84 g, 4.2 mmol) was added to a stirred soln. of **4** (0.99 g) in MeOH (40 ml). The mixture was heated to reflux for 3 h. After cooling to r. t., the reaction mixture was concentrated *in vacuo*, the residue was taken up in AcOEt, washed with H_2O and brine, dried and concentrated *in vacuo* to give an oil that was purified by flash chromatography (eluent CH_2Cl_2/AcOEt 99/1) to give **9** as a yellow solid (0.73 g, 46% yield). M.p. 95-97° decomp. (*i*-Pr_2O). ^{1}H-NMR ($CDCl_3$): 8.02 (*d*, 2H, *J* = 8.6, Ar); 7.39 (*d*, 2H, *J* = 8.6, Ar);

7.34 (*d*, 2H, *J* = 8.1, Ar); 7.21 (*d*, 2H, *J* = 8.1, Ar); 6.76 (*s*, 1H, CH pyrazol); 4.71 (*s*, 2H, CH_2); 3.92 (*s*, 3H, CH_3); 2.15 (*s*, br, 1H, OH). ^{13}C-NMR ($CDCl_3$): 166.1; 144.8; 143.9 (q, ^2J = 38); 142.7; 142.3; 130.6; 130.0; 129.8; 128.0; 127.2; 125.0; 121.1 (q, ^1J = 267); 106.3 (q, ^3J = 2); 64.5; 52.4. CI-MS (*i*-Bu) 377 $(M+1)^+$. Anal. calc. for $C_{19}H_{15}F_3N_2O_3$ (376.34): C 60.64, H 4.02, N 7.44; found C 60.87, H 4.40, N 7.40.

*Methyl 4-{5-[4-(methyl)phenyl]-3-(trifluoromethyl)-1*H*-pyrazol-1-yl}benzoate* (**13**)**.** 4-(Methoxycarbonyl)phenylhydrazine hydrochloride (**8**) (3.41 g, 17.0 mmol) was added to a stirred soln. of **12** (3.87 g, 17.0 mmol) in MeOH (100 ml). The mixture was heated to reflux for 20 h. After cooling to r. t., the mixture was concentrated *in vacuo*, the residue was taken up in AcOEt, washed with H_2O and brine, dried and concentrated *in vacuo* to give an oil that was purified by flash chromatography (eluent PE/AcOEt 97/3) to give **13** as a white solid (2.06 g, 34% yield). M.p. 78.5-79.5° (hexane). ^{1}H-NMR ($CDCl_3$): ,8.03 (*d*, 2H, *J* = 8.6, Ar); 7.39 (*d*, 2H, *J* = 8.6, Ar); 7.16-7.08 (*m*, 4H, Ar); 6.73 (*s*, 1H, CH pyrazol); 3.92 (*s*, 3H, OCH_3); 2.36 (*s*, br, 3H, CH_3), ^{13}C-NMR ($CDCl_3$): 166.2; 145.2; 143.9 (q, ^2J = 38); 142.8; 139.6; 130.6; 129.8; 129.7; 128.8; 126.1; 125.1, 121.3 (q, ^1J = 267); 106.1 (q, ^3J = 2); 52.5; 21.4.EI-MS: 360 (100%, M^+), 329 (50%). Anal. calc. for $C_{19}H_{15}F_3N_2O_2$ (360.34): C 63.33, H 4.20, N 7.77; found C 63.95, H 4.13, N 7.81.

*4-{1-[4-(Hydroxymethyl)phenyl]-3-(trifluoromethyl)-1*H*-pyrazol-1-yl}phenylmethanol* (**10**)**.** A soln. of **9** (1.26 g, 3.4 mmol) in dry THF (10 ml) was added dropwise to a stirred mixture of $LiAlH_4$ (0.13 g, 3.4 mmol) in dry THF (10 ml) maintained under N_2. After 30 min H_2O (1 ml), NaOH 15% (1 ml) and H_2O (1 ml) were added sequentially to the mixture, after which the solid was filtered and washed with MeOH. The filtrate was concentrated *in vacuo*, the residue was taken up in AcOEt, washed with H_2O and brine, dried and concentrated *in vacuo* to give an oil that was purified by flash chromatography (eluent CH_2Cl_2/AcOEt 8/2) to give **10** as a white solid

(0.70 g, 59% yield). M.p. 107-111° (*i*-Pr_2O). ^{1}H-NMR ($CDCl_3$): 7.35-7.26 (*m*, 6H, Ar); 7.21 (*d*, 2H, *J* = 8.1, Ar); 6.74 (*s*, 1H, CH pyrazol); 4.70 (*s*, 4H, two CH_2); 1.98 (*s*, br, 1H, OH); 1.90 (*s*, br, 1H, OH). ^{13}C-NMR ($CDCl_3$): 144.5; 143.2 (q, ^2J = 38), 141.8; 141.3; 138.3; 128.9; 128.3; 127.4; 127.1; 125.6; 121.2 (q, ^1J = 267); 105.6 (q, ^3J = 2); 64.6; 64.4. CI-MS (*i*-Bu) 349 $(M+1)^+$. Anal. calc. for $C_{18}H_{15}F_3N_2O_2$ (348.32): C 62.07, H 4.34, N 8.04; found C 62.45, H 4.42, N 7.94.

*4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]phenylmethanol* (**14**). A soln. of **13** (2.18 g, 6.0 mmol) in dry THF (25 ml) was added dropwise to a stirred mixture of $LiAlH_4$ (0.23 g, 6.0 mmol) in dry THF (25 ml) maintained under N_2. After 30 min H_2O (1 ml), NaOH 15% (1 ml) and H_2O (1 ml) were added sequentially to the mixture, then the solid was filtered and washed with MeOH. The filtered soln. was concentrated *in vacuo*, the residue was taken up in AcOEt, washed with H_2O and brine, dried and concentrated *in vacuo* to give a solid (1.91 g, yield 96%) that was purified by crystallization from EtOH/H_2O to give **14** as a white solid. M.p. 109-111° (EtOH/H_2O). ^{1}H-NMR ($CDCl_3$): 7.35-7.27 (*m*, 4H, Ar); 7.11-7.09 (*m*, 4H, Ar); 6.71 (*s*, 1H, CH pyrazol); 4.70 (*s*, 2H, CH_2); 2.34 (*s*, 3H, CH_3);.1.93 (*s*, br, 1H, OH). ^{13}C-NMR ($CDCl_3$): 144.8, 143.2 (q, ^2J = 38), 141.2, 139.1, 138.5, 129.4, 128.7, 127.3, 126.2, 125.5, 121.3 (q, ^1J = 267), 105.3 (q, ^3J = 2), 64.4, 21.3. EI-MS:332 (100%, M^+). Anal. calc. for $C_{18}H_{15}F_3N_2O$ (332.32): C 65.06, H 4.55, N 8.43; found C 65.12, H 4.53, N 8.33.

4-{1-[4-(Aminosulfonyl)phenyl]-3-(trifluoromethyl)-1H-pyrazol-5-yl}benzyl nitrate (**7**). $AgNO_3$ (1.00 g, 5.8 mmol) and Ph_3P (0.75 g, 2.9 mmol) were added to a soln. of **6** (1.00 g, 2.5 mmol) in MeCN (30 ml) maintained under N_2. The mixture was cooled to 0°, and NBS (0.50 g, 2.8 mmol) was added portion-wise. Stirring was continued at 0° for 1 h and then at r. t. for 3 h. AcOEt was added to the mixture and the solid was filtered; the filtrate was washed with H_2O, brine, dried and concentrated *in vacuo* to give a solid that was purified by flash chromatography (eluent PE/AcOEt 7/3) to give **7**

as a white solid (0.76 g, 70% yield). ^{1}H-NMR ((D$_6$)DMSO): 7.89 (*d*, 2H, *J* = 8.6, Ar); 7.58-7.51 (*m*, 6H, Ar and NH$_2$); 7.39 (*d*, 2H, *J* = 8.3, Ar); 7.30 (*s*, 1H, CH pyrazol); 5.60 (*s*, 2H, CH$_2$). ^{13}C-NMR ((D$_6$)DMSO): 144.5; 144.5; 142.2 (q, ^{2}J = 37); 140.9; 133.4; 129.5; 129.2; 129.0; 126.8; 126.0; 121.2 (q, ^{1}J = 267); 106.7 (q, ^{3}J = 2); 74.3. EI-MS: 442 (40%, M^{+}), 397 (40%), 302 (100%). Anal. calc. for $C_{17}H_{13}F_3N_4O_5S$ (442.38): C 46.16, H 2.96, N 12.66; found C 45.96, H 2.89, N 12.29.

*4-{{1-[4-(Nitrooxy)methyl]phenyl}-3-(trifluoromethyl)-1*H*-pyrazol-5-yl}benzyl nitrate* (**11**). $AgNO_3$ (1.03 g, 6 mmol) and Ph_3P (1.31 g, 5 mmol) were added to a soln. of **10** (0.7 g, 2 mmol) in MeCN (20 ml) maintained under N_2. The mixture was cooled to -15° under N_2, and NBS (0.89 g, 5 mmol) was added portion-wise. Stirring was continued at -15° for 1 h and then at r. t. for 12 h. AcOEt was added to the mixture and the solid was filtered; the filtrate was washed with H_2O, brine and dried and concentrated *in vacuo* to give an oil that was purified by flash chromatography (eluent Hexane/AcOEt 9/1). The first eluted compound was **11** which was obtained as a white solid (0.42 g, 48% yield). M.p. 52-53° (*i*-Pr_2O/PE). ^{1}H-NMR ($CDCl_3$): 7.43-7.25 (*m*, 8H, Ar); 6.78 (*s*, 1H, CH pyrazol); 5.44, 5.43 (2 *s*, 4H, CH_2ONO_2). ^{13}C-NMR ($CDCl_3$): 143.9; 143.7 (q, ^{2}J = 38); 139.8; 133.4; 132.7; 130.0; 129.8; 129.4; 129.4; 125.7; 121.1 (q, ^{1}J = 268); 106.3 (q, ^{3}J = 2); 73.9; 73.6..EI-MS: 438 (30%, M^{+}), 345 (48%), 316 (100%). Anal. calc. for $C_{18}H_{13}F_3N_4O_6$ (438.32): C 49.32, H 2.99, N 12.78; found C 49.38, H 2.95, N 12.56. The second eluted fraction was the mixture of the two mononitrate products **11a** and **11b**, obtained as a transparent oil. ^{1}H-NMR ($CDCl_3$): 7.41- 7.20 (*m*, 16H, Ar); 6.75, 6.77 (2*s*, 2H, CH pyrazol); 5.42, 5.43 (2 *s*, 4H, CH_2ONO_2); 4.73, 4.72 (2 *s*, 4H, CH_2OH); 1.79 (*s*, br, 2H, OH). ^{13}C-NMR ($CDCl_3$): 144.6; 143.8; 143.4 (q, ^{2}J = 38); 142.1; 141.6; 140.0; 138.2; 133.1; 132.5; 130.3; 129.7; 129.3; 129.2; 129.0; 128.1; 127.5; 127.2; 125.7; 125.6; 121.4 (q, ^{1}J = 268); 106.0 (q, ^{3}J = 2); 105.9 (q, ^{3}J = 2); 73.9; 73.7; 64.6; 64.4.EI-MS: 393 (97%, M^{+}), 347 (100%), 317

(99%). Anal. calc. for $C_{18}H_{14}F_3N_3O_4$ (393.32): C 54.97, H 3.59, N 10.68; found C 55.44, H 3.94, N 10.30.

*4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzyl nitrate* (**15**). $AgNO_3$ (0.68 g, 4 mmol) and Ph_3P (0.92 g, 3.5 mmol) were added to a soln. of **14** (0.66 g, 2 mmol) in MeCN (30 ml) maintained under N_2. The mixture was cooled to -15° under N_2, and NBS (0.62 g, 3.5 mmol) was added portion-wise. Stirring was continued at -15° for 1 h and then at r. t. for 4 h. AcOEt was added to the mixture and the solid was filtered; the filtrate was washed with H_2O, brine, dried and concentrated *in vacuo* to give a residue that was purified by flash chromatography (eluent PE/AcOEt 9.5/0.5) to give **15** as a white solid (0.52 g, 68% yield). M.p. 106-107° (MeOH). ^{1}H-NMR ($CDCl_3$): 7.41-7.34 (*m*, 4H, Ar) 7.16-7.09 (*m*, 4H, Ar), 6.72 (*s*, 1H, CH pyrazol), 5.43 (*s*, 2H, CH_2), 2.37 (*s*, 3H, CH_3). ^{13}C-NMR ($CDCl_3$): 144.9; 143.5 (q, ^2J = 38); 140.2; 139.4; 132.3; 130.0; 129.7; 128.7; 126.1; 125.7; 121.2 (q, ^1J = 267); 105.8 (q, ^3J = 2); 73.8; 21.3. EI-MS: 377 (100%, M^+), 331 (85%), 300 (90%). Anal. calc. for $C_{18}H_{14}F_3N_3O_3$ (377.32): C 57.30, H 3.74, N 11.14; found C 57.30, H 3.91, N 10.82.

Evaluation of stability in human serum and in whole human blood. Blood samples for both stability, COX-selectivity and platelet aggregation assays were obtained from healthy volunteers who had not taken any drug for at least two weeks. Volunteers, who were treated according to the Helsinki protocol for biomedical experimentation, gave their informed consent to the use of blood samples for research purposes.

Compounds **7, 11** and **15** were incubated in human serum (sterile-filtered from human male AB plasma, *Sigma Aldrich*) and in heparinized human blood at 100 μM concentration. The resulting soln.s were maintained at 37 ± 0.5 °C and at appropriate time intervals the mixtures were diluted 1:2 with MeCN containing 0.1% trifluoroacetic acid in order to deproteinize and precipitate cells, proteins and enzymes. Samples were

sonicated, vortexed and then centrifuged for 15' at 2150 *g*. The clear supernatant was filtered by 0.45 μm PTFE filters (*Alltech*) and analyzed by RP-HPLC.

The reverse-phase HPLC procedure allowed separation and quantitation of remaining compounds and metabolites. HPLC analyses were performed with an HP1100 chromatograph system (*Agilent Technologies*, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (model G1379A), a diode-array detector (DAD) (model G1315B) integrated in the HP1100 system. Data were analyzed using an HP ChemStation system (*Agilent Technologies*). The analytical column was a ZORBAX Eclipse XDB-C8 (4.6 x 150mm, 5μm) *Agilent*. The mobile phase consisted of MeCN /H_2O (70/30) with 0.1% trifluoroacetic acid and the flow-rate was 1.2 ml/min. The injection volume was 20 μl (*Rheodyne*, Cotati, CA). The column effluent was monitored at 226 nm and 254 nm. Quantitation was done by comparison of peak areas with standards chromatographed under the same conditions.

Cyclooxygenase Inhibition Studies. A whole blood assay [15] was performed to evaluate the ability of synthesised compounds to inhibit COX-1 and COX-2.
Blood samples were divided in two aliquots to test COX-1 and COX-2 inhibition. The COX-2 aliquots were treated with 10 IU/ml of sodium heparine, 10 μg/ml acetylsalicylic acid, and 10 μg/ml lipopolysaccaride from *E.coli* (LPS). Methanolic soln.s of the tested compounds at different concentrations were prepared, 10 μl aliquots were distributed in incubation tubes and the solvent was evaporated. The residues were dissolved by vortexing either in 1ml heparinized blood to test COX-2 inhibition, or in 1 ml untreated blood to test COX-1 inhibition. The final concentrations of the tested compounds were therefore diluted 100 times in the incubation tubes. The COX-1 aliquots were incubated in glass tubes for 1 h at 37°, which is sufficient to complete coagulation, then centrifuged at 2000 *g* for 10 min, after which the serum was ready to be tested for platelet TxB_2 production. % Inhibition in samples treated with the test

compounds was evaluated in comparison with control samples with basal TxB_2 production.

The COX-2 aliquots were incubated in polyethylene tubes for 24 h at 37° to allow COX-2 expression in monocytes and maximal PGE_2 production. They were then centrifuged at 2000 *g* for 10 min after which the plasma was ready to be tested for PGE_2 production. Basal PGE_2 production in blood untreated with LPS was subtracted from values for each sample, and % inhibition in samples incubated with tested compounds was calculated *versus* control samples with maximal PGE_2 production.

Prostanoid production was evaluated by enzyme immunoassay, following the specific instructions provided by *Cayman Chemical*, based on a competitive reaction, for COX-1, between TxB_2 and a TxB_2-acetylcholinesterase conjugate (TxB_2 tracer) for a specific TxB_2 antiserum, and, for COX-2, between PGE_2 and PGE_2-acetylcholinesterase (PGE_2-tracer) for a specific PGE_2 antiserum. Standard curves with known concentrations of TxB_2 and of PGE_2 were used to determine prostanoid concentrations in the sample wells. Percent inhibition in compound-treated samples was calculated by comparison with untreated controls. The concentration of the tested compounds causing 50% inhibition (IC_{50}) was calculated from the concentration-inhibition response curve (5-6 experiments).

Inhibition of Platelet Aggregation in vitro. Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 200 *g* for 20 minutes. Aliquots (500 μl) of PRP were added into aggregometer (Chrono-log 4902D) cuvettes and aggregation was recorded as increased light transmission under continuous stirring (1000 rpm) at 37° for 10 minutes after addition of the stimulus. Collagen (1.0 μg/ml) was used as platelet activator in PRP. The inhibitory activity of the compounds was tested by addition of drug to PRP 10 min before addition of the stimulus (collagen). Drug vehicle (≤ 0.5 % DMSO) added to PRP did not affect platelet function in control samples. At least 5 experiments were

performed for each compound. The potential role of NO and sGC in the inhibitory effect of **15** was verified using the sGC inhibitor, ODQ (100 μM).

The antiaggregatory activity of the test compounds was evaluated as % inhibition of platelet aggregation compared to controls (5-6 experiments) and IC_{50} values were calculated by non-linear regression analysis.

Vasodilating activity assay. Thoracic aortas were isolated from male Wistar rats weighing 180-200 g. The endothelium was removed and the vessels were helically cut: three strips were obtained from each aorta. The tissue was placed in organ baths containing 30 ml of Krebs-bicarbonate buffer of the following composition (mM): NaCl 111.2, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.0, $NaHCO_3$ 12, glucose 11.1 maintained at 37° and continuously gassed with 95% O_2 – 5% CO_2 (pH = 7.4). The aortic strips were allowed to equilibrate for 120 min and then contracted with 1 μM (-) phenylephrine. When the response to the agonist reached a plateau, cumulative concentration–response curves to compounds **7**, **11** and **15** were determined. The effect of 1 μM ODQ was evaluated in a separate series of experiments in which the ODQ was added 5 min before contraction. EC_{50} values are means of 5-9 determinations. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab®.

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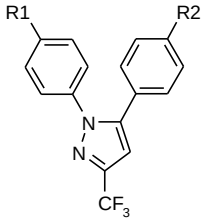
Table 1. *Whole human blood stability*

Compound	% of unchanged compound after 24 h (± SEM)	% of alchoholic metabolites after 24 h (± SEM)	$t_{1/2}$ (hours)
1	100		
7	4.5 ± 1.1	4.5 ± 0.5 (**6**)	0.8
11	59.4 ± 4.4	16.2 ± 0.7 of **11a/b** 11.7 ± 1.0 of **10**	30.0
15	54.4 ± 2.3	41.2 ± 1.7 (**14**)	25.4

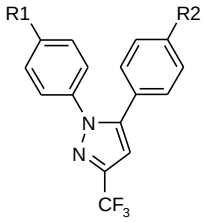
Table 2. *COX-1 and COX-2 inhibition data for compounds and Celecoxib **1**, taken as reference.*



Compound	R_1	R_2	COX-1		COX-2
			IC_{50} ± SE μM	% inhibition ± SE (100 μM)[a])	IC_{50} ± SE μM
1	SO_2NH_2	Me	14 ± 2	-	1.3 ± 0.4
6	SO_2NH_2	CH_2OH	[b])	20 ± 13	52 ± 3
7	SO_2NH_2	CH_2ONO_2	[b])	6.7 ± 3.8	67 ± 19
10	CH_2OH	CH_2OH	0.51 ± 0.14	-	12 ± 3
11	CH_2ONO_2	CH_2ONO_2	[b])	15 ± 7	19 ± 1
14	CH_2OH	Me	0.41 ± 0.08	-	11 ± 3
15	CH_2ONO_2	Me	4.6 ± 1.6	-	31 ± 10

[a]) Maximum concentration tested. [b]) Inhibition of control TxB_2 production did not reach 50%.

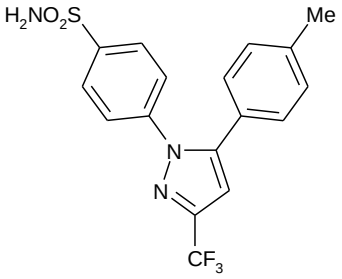
Table 3. *Anti-aggregatory and vasodilating properties of derivatives.*



Compound	R_1	R_2	Anti-aggregatory activity		Vasodilating activity
			IC_{50} (95%CL) μM	% inhibition ± SE (100 μM)[a])	EC_{50} ± SE μM
1	SO_2NH_2	Me	31 (25 – 39)	-	-
6	SO_2NH_2	CH_2OH	[b])	5.8 ± 2.1	-
7	SO_2NH_2	CH_2ONO_2	[b])	3.4 ± 2.6	0.26 ± 0.05 7.0 ± 1.3 [c])
10	CH_2OH	CH_2OH	8.4 (7.0 - 10)	-	-
11	CH_2ONO_2	CH_2ONO_2	[b])	7.4 ± 4.3	0.10 ± 0.03 26 ± 9 [c])
14	CH_2OH	Me	11 (10 - 12)	-	-
15	CH_2ONO_2	Me	48 (44 – 52)[d]	-	0.12 ± 0.01 > 100 [c])
ISDN					4.7 ± 0.6 > 100 [c])

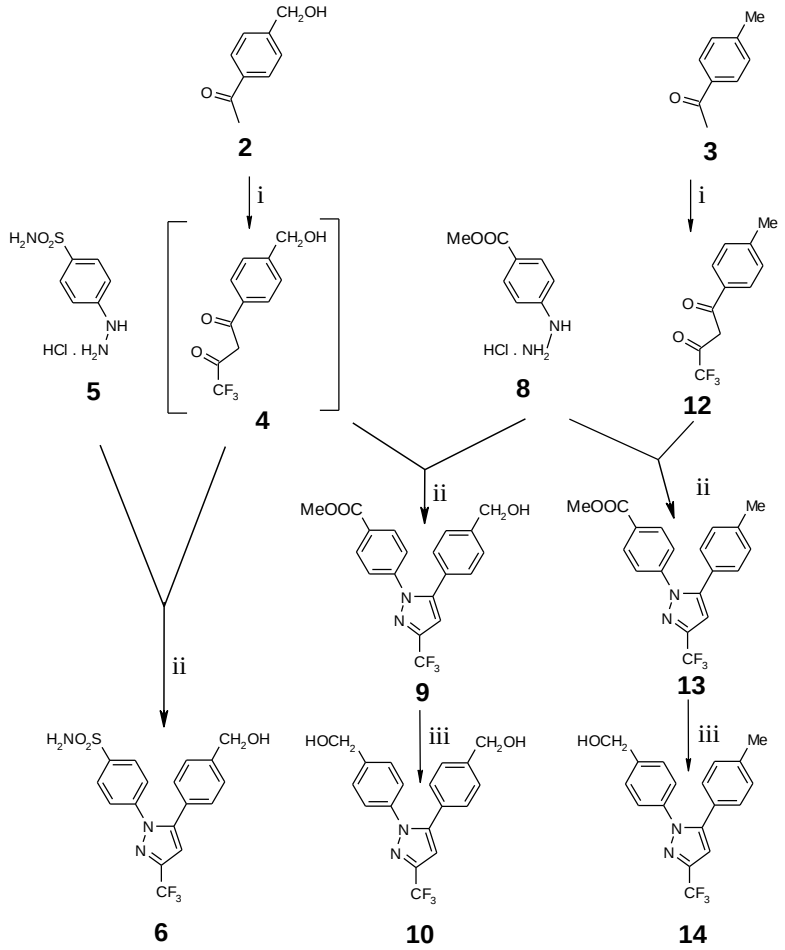
[a]) Maximum concentration tested. [b]) Inhibition of control aggregation effect did not reach 50 %. [c]) In the presence of 1 μM ODQ. [d]) Unchanged in the presence of 100 μM ODQ.

Figure 1.



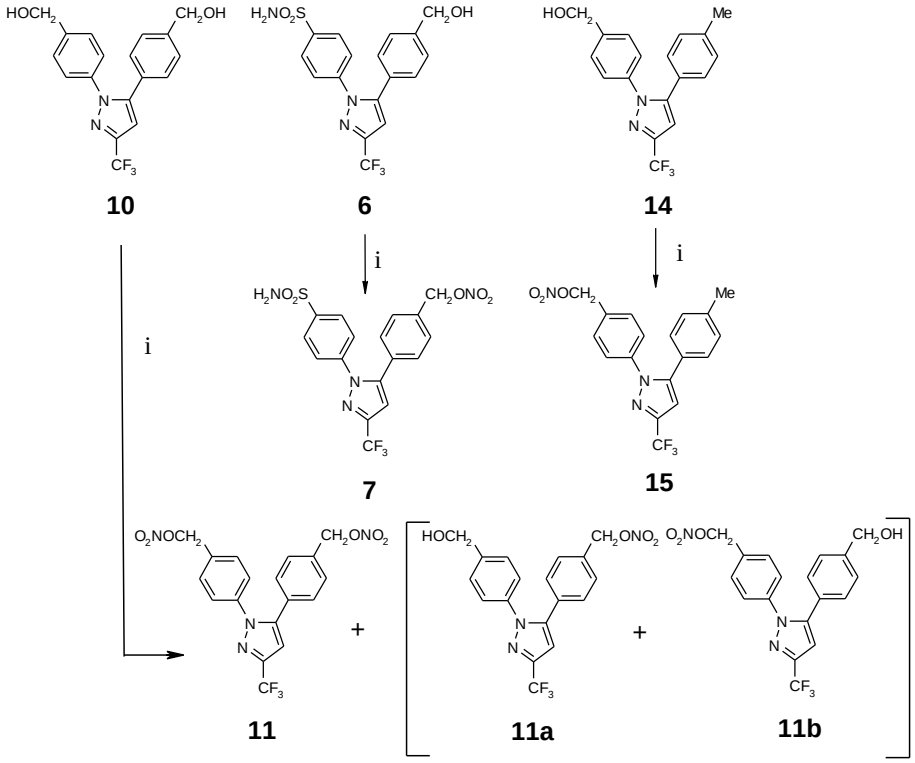
Celecoxib
1

Scheme 1.



Scheme 1: i) MeONa, CF_3COOEt, MeOH, refluxing; ii) refluxing in MeOH, (EtOH for **6**); iii) $LiAlH_4$, THF.

Scheme 2.



Scheme 2: i) Ph_3P, $AgNO_3$, NBS, - 15° → r. t.