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Searching for new NO-donor aspirin-like molecules: Furoxanylacyl derivatives of salicylic acid and related furazans

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ABSTRACT

A new group of derivatives of salicylic acid containing NO-donor furoxans, and the related des-NO-furazans, were synthesized and evaluated as new aspirin-like molecules. Their stability was assessed in acid (pH 1) and physiological solutions (pH 7.4), and in human serum. No compound exhibited COX-inhibitory activity against COX-1 and COX-2 isoforms, when tested up to 100 μM, respectively, on isolated platelets and on monocytes. Phenylsulfonyl- and cyano-substituted furoxans inhibited platelet aggregation induced by collagen in human platelet-rich plasma, through a cGMP dependent mechanism. Furoxan derivatives displayed cGMP-dependent vasodilator activities, tested on rat aorta strips precontracted with phenylephrine. All products showed anti-inflammatory activity similar to that of ASA, tested on rats by the carrageenan-induced paw edema assay. Unlike ASA, all products showed markedly reduced gastrotoxicity in a rat lesion model.

1. Introduction

The major limitation on the prolonged use of aspirin (ASA) **1** (Fig. 1) is its gastrotoxicity, responsible for gastric ulceration, exacerbation of peptic ulcer symptoms, gastrointestinal hemorrhage, erosive gastritis, delay in ulcer healing and, in some cases, death.[1–3]

This is a prominent problem, in view of the widespread use of ASA to treat headache, rheumatic pain and inflammation, as well as for its effective antithrombotic activity.[1,4,5] A number of new therapeutic perspectives are emerging for this enigmatic and intriguing drug, including its ability to reduce the risk of colorectal adenoma or cancer, and that of the recurrence of colorectal adenoma in high risk patients.[6–8] The beneficial pharmacological actions of ASA are predominantly dependent on its ability to inhibit both isoforms of the COX-enzyme, with a preference for the COX-1 isoform, with consequent inhibition of prostanoid production.[9] Unlike the other nonsteroidal anti-inflammatory drugs (NSAIDs), ASA irreversibly inhibits both COX-1 and COX-2, by forming a covalent bond with the serine residue (Ser^{530}) positioned in the arachidonic acid-binding channel of the enzymes. COX-1 is prevalently a constitutive enzyme, present in many tissues and cells, including platelets, and it is thought to be largely responsible for the antithrombotic effect of ASA. By contrast, inhibition of the COX-2 enzyme, prevalently an inducible isoform expressed by inflammatory stimuli in many tissues, is largely responsible for the drug's anti-inflammatory, analgesic, and antiproliferative actions.[9] ASA's gastrotoxicity involves local and systemic mechanisms.[1,10,11] Local mechanisms include interaction with phospholipids, weakening of the hydrophobic surface barrier in membranes, and the diffusion and subsequent entrapment of the drug into the mucosal cell, with consequent trapping of hydrogen ions, or both. These events principally depend on the product's pK_a and lipophilicity. The systemic mechanisms are mainly related to the drug's ability to inhibit the COX-1 enzyme present in gastric epithelial cells, and consequently to block the production of prostaglandins, known to be crucial in defending the gastric mucosa. In view of the gastrosparing and anti-inflammatory actions exerted by nitric oxide (NO),[12,13] one of the strategies proposed to overcome the problem of ASA's gastrotoxicity is that of linking the drug with an appropriate NO-donor moiety. Most such products are obtained by linking the NO-donor moieties to the carboxylic site of aspirin through an ester linkage (Fig. 1A).[14–17] Recently, we proposed a new class of NO-donor aspirin-like molecules obtained by substituting the acetyl group of aspirin with acyl groups, containing nitrooxy NO-donor moieties (Fig. 1B).[18] In order to explore the influence exerted by the nature of the NO-donor moiety on the pharmacological profile of this new type of products, we here describe the synthesis and pharmacological activity of a new class

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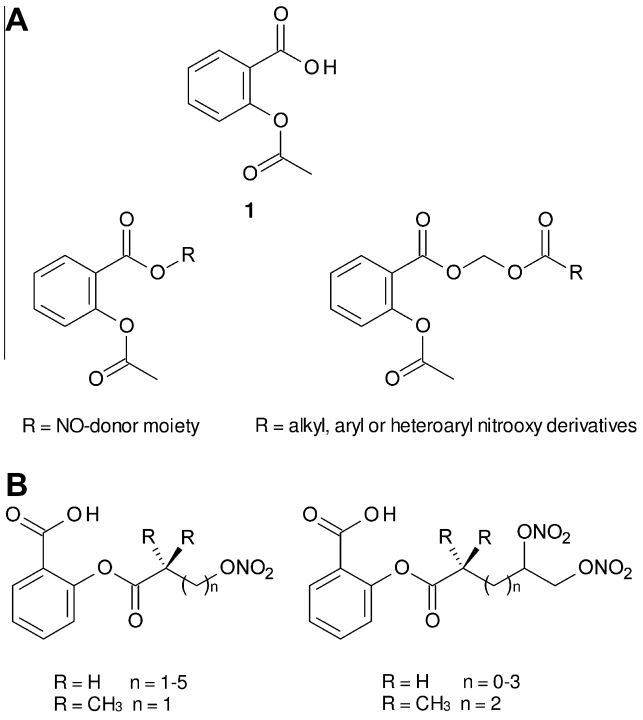


Figure 1. (a) Structure of aspirin and general structures of NO-donor aspirin co-drugs; (b) general structure of NO-donor salicylic acid co-drugs.

of compounds, obtained by substituting acyl groups containing NO-donor furoxan moieties for acetyl group of ASA (compounds **13**, **14**, **17** and **18**, Fig. 2). Furoxans are NO-donors able to release NO through a mechanism different from that occurring for the organic esters of nitric acid. It is generally accepted that they can produce NO under the action of thiol cofactors,[19] while nitrooxy derivatives require principally enzymatic metabolism.[20] This new class of products might be expected to display a pharmacological profile different from that observed for the nitrooxy analogues,[21] in the light of both the different structure and the different NO-release mechanism of the corresponding NO-donor moieties. In this study, the related furazan derivatives **13a**, **14a**, **17a** and **18a** (Fig. 2), devoid of NO-releasing (des-NO) properties, are also considered, for comparison purposes.

2. Results and discussion

2.1. Chemistry

The synthesis of the end products required the preliminary preparation of the carboxylic acid **4**, **4a**, **5**, **5a**, **8**, **8a**, **10** and **10a** (Scheme 1). Phenyl- and phenylsulfonyl-substituted compounds **4**, **4a**, **5** and **5a**, were obtained by oxidation of the corresponding alcohols **2**, **2a**, **3** and **3a** with Jones reagent in acetone solution. To prepare carbamoyl- and cyano-substituted compounds **8**, **8a**, **10** and **10a**, 4-bromomethylfuroxan-3-carboxamide (**6**) was treated with methyl thioglycolate in acetonitrile solution in the presence of Et_3N to give the ester **7**. Ester **7,** containing the furoxan ring, was reduced with trimethyl phosphite ($P(OCH_3)_3$) to give the resulting furazan ester **7a**. Dehydration of **7** and **7a** with trifluoroacetic anhydride in THF, in the presence of pyridine (Py), afforded **9** and **9a**. Hydrolysis of **7**, **7a**, **9** and **9a** with 6 M hydrochloric acid in dioxane yielded the expected acids. The preparation of the final products **13**, **13a**, **14**, **14a**, **17**, **17a**, **18** and **18a** is depicted in Scheme 2. The appropriate acids were transformed into the corresponding chlorides **11**, **11a**, **12**, **12a**, **15**, **15a**, **16** and **16a**. These products were immediately coupled with salicylic acid in THF or CH_2Cl_2 solution in the presence of pyridine to give the target compounds.

2.2. Stability in aqueous buffered solutions and human serum

The stability of all end products was studied in aqueous solutions at pH 1 and 7.4, as well as in human serum. The progress of the transformation was evaluated by high-performance liquid chromatography (HPLC). The results are shown in Table 1. All products remained largely unchanged after 3 h of incubation in acidic medium. At physiological pH, both furoxans and furazan analogues were transformed into salicylic acid, accompanied prevalently by the native heterocyclic acids. The transformation strictly followed first-order kinetics. The observed pseudo-first-order rate constants (k_{obs}) and the half-lives ($t_{1/2}$, Table 1) were determined by fitting the remaining ester against time with one-phase exponential decay equation (Graph Pad, Prism software vers. 5).

Analysis of Table 1 shows that the products containing a sulfur atom α-positioned to the ester function are less stable than the others, and that the stability of pairs of related furazan and furoxan esters is similar. Different results were obtained working in human serum, where the compounds can interact with serum esterases and other serum proteins, in particular with albumin. After 2 h, stoichiometric amounts of salicylic acid were recovered from carbamoyl- and cyano-substituted compounds containing sulfur, **17**, **17a**, **18** and **18a**. Conversely, the native acid components were only partly recovered because of their instability in this medium. In the case of the remaining products, the formation of salicylic acid was not stoichiometric, but was accompanied by a number of other metabolites. In all cases, the disappearance of the initial products followed a pseudo-first-order kinetics, with half-lives markedly lower than those measured at physiological pH, and falling within a narrower range.

2.3. COX inhibitory activity

Compounds were assayed for COX-1 and COX-2 inhibition. COX-1 inhibitory activity was evaluated on isolated human

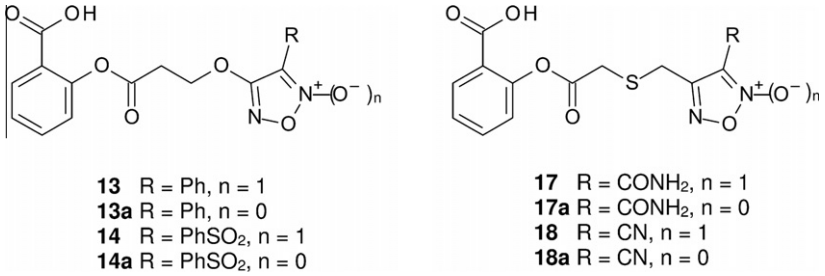
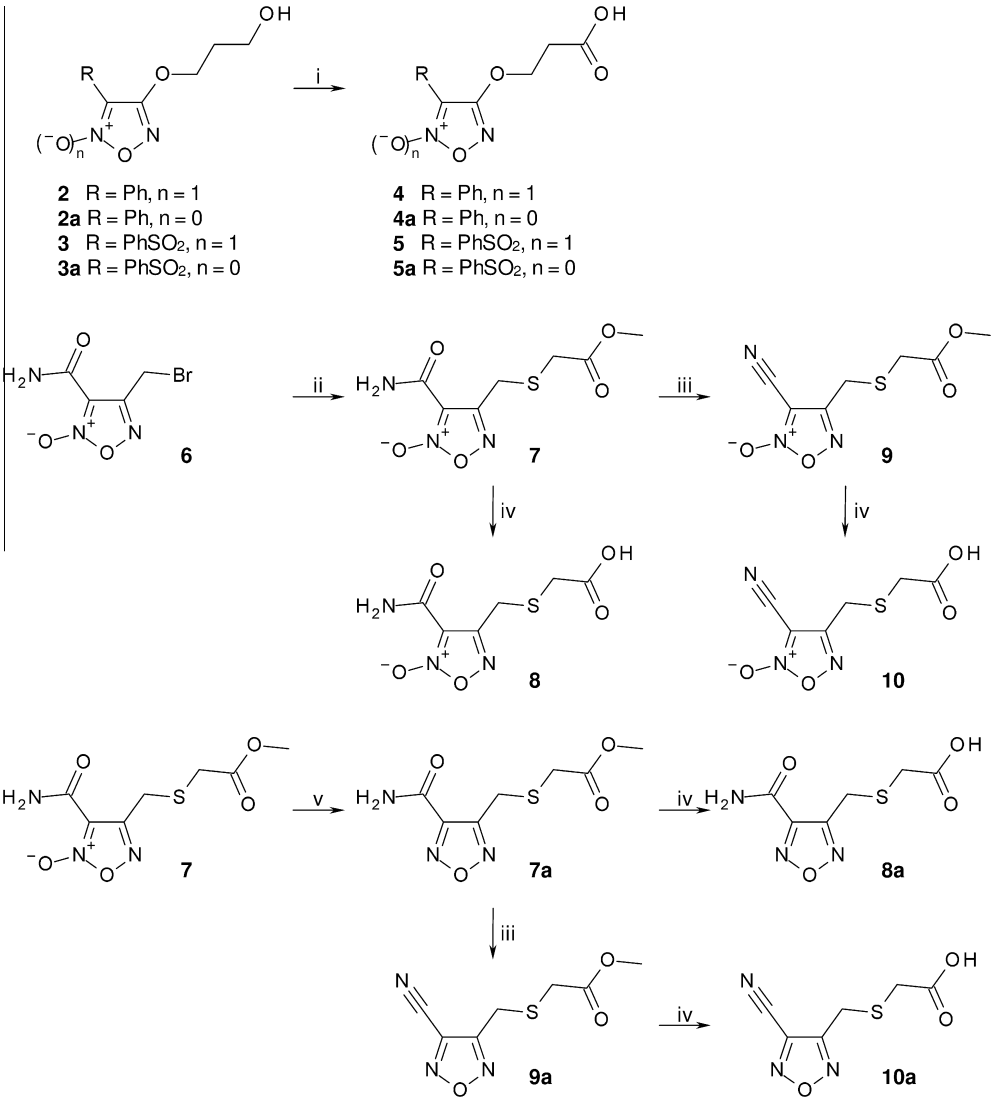


Figure 2. Structure of the new compounds.



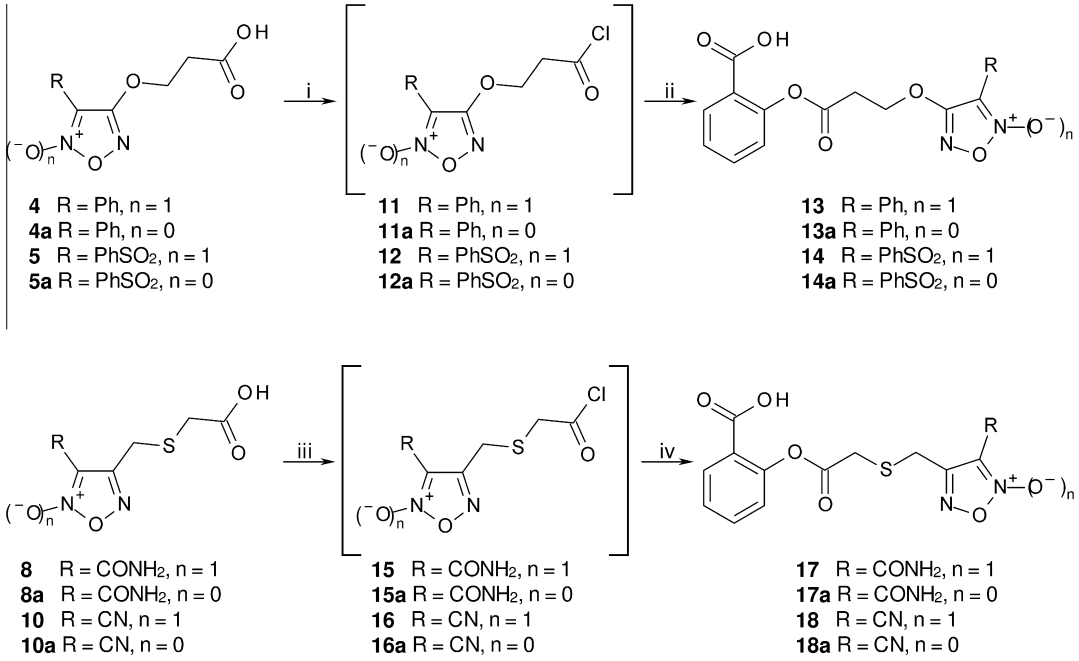
Scheme 1. Reagents and conditions: (i) Jones reagent, acetone, 0 °C; (ii) $HSCH_2COOCH_3$, Et_3N, CH_3CN; (iii) $(CF_3CO)_2O$, Py, THF, 0 °C; (iv) 6 M HCl, dioxane, 70 °C; (v) $P(OCH_3)_3$, reflux.

platelets. In this assay, washed platelets were treated with different concentrations of the products for 30 min, before stimulation of thromboxane B_2 (TXB_2) production with the calcium ionophore A23187. After a fixed time, the cells were lysed and the TXB_2 produced was quantitated by EIA.

COX-2 inhibitory activity was evaluated on isolated human monocytes, in which COX-2 protein expression had been induced by overnight lipopolysaccharide (LPS) treatment. After addition of arachidonic acid, PGE_2 production was assessed by EIA. Neither the NO-donor furoxan derivatives nor the des-NO furazan analogues were able to inhibit either COX isoform, when tested up to 100 μM. This is probably due to the steric bulk of the heterocyclic acyl moieties, which prevent binding of the products to the active cleft of the enzyme, and then its acylation. Conversely a number of nitrooxyacyl derivatives of salicylic acid, previously described by our group, were shown to be potent irreversible inhibitors of both enzymes.[21]

2.4. Platelet anti-aggregatory activity

Both the furoxan and the furazan series were studied for their action on collagen-induced platelet aggregation of human platelet-rich plasma. The results, expressed as IC_{50}, or as percentage of inhibition at the highest concentration tested when IC_{50} could not be calculated, are reported in Table 2. The data show that the furazan derivatives display either no activity or very weak activity. The only exception is the amide compound **17a**, which displays low antiaggregatory action. Compounds in the furoxan series display very weak antiaggregatory action, while the phenylsulfonyl- and the cyano-substituted members **14** and **18** behave as potent antiaggregatory agents. The potency of these two furoxan derivatives decreased if the tests were carried out in the presence of ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), a well-known inhibitor of soluble guanylate cyclase (sGC), an enzyme that mediates the antiaggregatory effects of NO. This is thus in keeping with the involvement of NO in the antiaggregatory effects of furoxans. The NO-donor aspirin obtained by linking the 3-cyanofuroxan-4-yl-methyl substructure present in **18** to the carboxylic group of the lead, through an ester bridge, possessed potent antiaggregatory activity. This activity was shown to be due to abundant NO-release in the platelets, following the synergistic effect of high intracellular concentrations of glutathione (GSH) and ascorbate in these kind of cells.[22] The furoxan amide compound **17** acts as a rather good antiaggregatory agent; however, its antiplatelet activity was found not



Scheme 2. Reagents and conditions: (i) $SOCl_2$, dry THF; (ii) Py, salicylic acid, dry THF; (iii) $SOCl_2$, dry CH_2Cl_2; (iv) Py, salicylic acid, dry CH_2Cl_2.

Table 1
Stability in aqueous buffered solutions and human serum of aspirin (**1**) and of compounds **13**, **13a**, **14**, **14a**, **17**, **17a**, **18** and **18a** incubated at 37 °C

Compound	Stability		
	Buffered solutions		Human serum $t_{1/2}$ (min)[c]
	pH 1% unchanged at 3 h[a]	pH 7.4 $t_{1/2}$ (min)	
1	90	–[b]	63
13	98	288	35
13a	98	318	40
14	96	360	30
14a	97	370	35
17	97	45	27
17a	97	50	27
18	96	33	13
18a	96	38	14

[a] SEM ⩽ 1%.
[b] %Unchanged at 3 h = 90; SEM ⩽ 1%.
[c] SEM ⩽ 0.2.

to be influenced by the presence of ODQ, suggesting that NO-dependent sGC activation plays little or no role in this action. As in the case of the related furazancarboxyamide **17a**, an antiaggregatory mechanism that is neither COX- nor NO-dependent must be involved. Indeed, preliminary results obtained with these two products indicate that they behave as antagonists at the thromboxane receptor (IC_{50} (CL 95%) μM: 38 (37–38) and 39 (37–42) for compound **17** and **17a**, respectively). The above picture indicates that the potential value of this new series of furoxanylacyl derivatives of salicylic acid as anti-thrombotic agents is limited, since none of these compounds act as irreversible inhibitors of the COX-enzymes, unlike aspirin and some its nitrooxyacyl analogues.[21]

2.5. Vasodilator activity

The vasodilatory effects of the furoxan derivatives were evaluated on endothelium-denuded rat aorta strips, pre-contracted with phenylephrine. All the furoxan products were capable of relaxing the contracted tissue in a concentration-dependent manner. The vasodilator potencies, expressed as EC_{50}, are shown in Table 2. Their order of potency was **14** > **18** > **13** ⩾ **17**. When the experiments were repeated in the presence of 1 μM ODQ, the potencies decreased, in keeping with NO-induced activation of the sGC being the mechanism underlying this effect. The most potent products, namely **14** and **18**, also displayed the most potent NO-dependent antiaggregatory activity. Two properties of this new class of vasodilators that differ from those of their nitrooxy analogues are of interest: their greater potency and the probable lack of tolerance development. This latter aspect was studied in vivo for the simple 4-hydroxymethylfuroxan-3-carboxyamide (CAS 1609),[23] whose substructure is present in **17**, and for the simple 4-ethoxy-3-phenylsulfonylfuroxan (CHF 2363),[24] whose substructure is present in **14**. Both these two products were found to be devoid of tolerance development.

2.6. Anti-inflammatory activity

All products in the furoxan and furazan series were tested on carrageenan-induced paw edema in conscious rats, together with aspirin (ASA) and salicylic acid. Injection of carrageenan into the rat's hind paw produced immediate swelling, which peaked at 4–5 h. Aspirin, administered by the intragastric route at 120 mg/kg just prior to carrageenan injection, significantly reduced (43.0 ± 3.3%) paw edema at 3 h, compared to vehicle-treated animals (Table 2). Salicylic acid acted similarly, reducing paw edema by 37%. The furoxan products significantly inhibited paw edema, from 37% to 55%, when administered intragastrically at doses equimolar with aspirin 120 mg/kg (Table 2). The most efficacious inhibitors were the phenylsulfonyl- and cyano-substituted furoxans **14** and **18**, for which inhibition reached 50% and 55%, respectively. Either the NO released by the products,[25] and/or the salicylic acid deriving from their metabolic transformation, could be involved in this activity.[26] The finding that the des-NO furazan analogues also displayed potent anti-inflammatory action (about 40% inhibition) appears to minimize the role played by NO in the observed effects, suggesting conversely that salicylic acid plays a paramount role. Indeed, both salicylic acid and the present series of

Table 2
Antiaggregatory, vasodilator, anti-inflammatory and ulcerogenic activity of ASA, salicylic acid and compounds **13**, **13a**, **14**, **14a**, **17**, **17a**, **18** and **18a**

Compound	Antiaggregatory activity		Vasodilator activity	Anti-inflammatory activity	Ulcerogenic activity
	IC_{50} (CL 95%) μM [+50 μM ODQ]	%Inhibition ± SEM at 300 μM	EC_{50} (μM) ± SEM [+1 μM ODQ]	%Inhibition of paw volume increase[c]	Lesion index (mm)[d]
ASA (**1**)	54 (49–60)		///	43.0 ± 3.3**	50.0 ± 3.8
Salicylic acid				36.8 ± 4.2*	1.1 ± 0.6***
13	[a]	17.0 ± 0.2	11 ± 2[b]	37.6 ± 4.8*	0.3 ± 0.2***
13a	[a]	12 ± 1	///	38.6 ± 5.4**	1.2 ± 0.5***
14	7.8 (5.5–11)		0.014 ± 0.003	49.6 ± 9.6**	0.4 ± 0.4***
	[152 (121–190)]		[0.97 ± 0.03]		
14a	[a]	2.6 ± 2.6	///	38.8 ± 10.1**	0.3 ± 0.2***
17	66 (57–76)		23 ± 3	39.4 ± 4.8**	0.0 ± 0.0***
	[59 (46–74)]				
17a	144 (110–190)		[b]	39.6 ± 9.4**	0.3 ± 0.2***
18	7.3 (5.1–10)		0.040 ± 0.010	54.9 ± 8.1**	1.2 ± 1.2***
	[88 (80–97)]		[2.4 ± 0.4]		
18a	[a]	36 ± 2	///	38.9 ± 3.8**	0.2 ± 0.2***

[a] Due to the low activity of the compound, IC_{50} could not be calculated. In this case the percentage inhibition is reported at 300 μM.
[b] In the presence of 1 μM ODQ, EC_{50} values were >100 μM.
[c] Determined in the carrageenan–induced paw edema model at 3 h in rats. Compounds were administered by intragastric route at doses equimolar with ASA 120 mg/kg. Edema volume in vehicle–treated group was taken as 100%; * $P < 0.05$; ** $P < 0.01$ versus vehicle (Dunnett's test).
[d] Determined in rats after intragastric administration of the compounds at doses equimolar with ASA 120 mg/kg. Gastric lesions were determined 3 h later; *** $P < 0.001$ vs. ASA (Newman–Keuls test).

compounds are ineffective as inhibitors of prostanoid synthesis under in vitro conditions, while being as effective as aspirin in inhibiting the rat paw edema. As for the mechanism involved, it has been proposed that the inhibition of COX-2 enzyme induction (via inhibition of NFkB signaling) may represent the major mode of action of salicylates in vivo.[26] Conversely, in some of the previously-described nitrooxy analogues, COX-acylation might also contribute to their in vivo anti-inflammatory activity.

2.7. Acute gastric mucosal damage

All compounds, including aspirin and salicylic acid as reference standards, were assessed for their ulcerogenic properties in conscious rats. The development of gastric lesions was assessed 3 h after intragastric administration of the compounds, and lesions were quantified by determining the 'lesion index' on the basis of their greatest length in millimeters (Table 2). In the present study aspirin, administered at 120 mg/kg, produced macroscopically-detectable gastric damage, characterized by mucosal necrosis and hemorrhage (lesion index = 50 ± 3.8). By contrast salicylic acid, tested under the same conditions, showed much lower gastrotoxicity, in keeping with findings that it lacks this side effect in experimental animals.[26] All the NO-donor products, as well as their des-NO furazan analogues, also displayed greatly reduced gastrotoxicity when administered at doses equimolar with aspirin. In the light of the fact that the products are not COX-inhibitors, and of the finding that the des-NO furazan analogues are devoid of gastrotoxicity (Table 2), a possible explanation for this finding is that they behave in vivo essentially as pro-drugs of salicylic acid. By contrast, in the COX-inhibitor nitrooxyacyl analogues, a role of NO in the reduced gastrotoxicity must be considered.

3. Conclusions

A new series of salicylic acid derivatives, containing NO-donor furoxan moieties linked to the hydroxyl group through an ester bridge, were designed together with the corresponding des-NO furazan analogues. The furoxan derivatives display antiaggregatory, vasodilator and anti-inflammatory properties, and show very limited gastrotoxicity when tested in conscious rats. The mechanisms responsible for these properties are somewhat different from those of the nitrooxy analogues previously described, due to both the different structure and the different NO-release mode of the respective NO-donor moieties. In particular in this new series of compounds, the antiaggregatory activity is dependent on the thiol-induced NO-release by furoxan moieties in platelets and the products are unable to inhibit COX-enzyme. The comparison with the corresponding des-NO furazans indicates that salicylic acid formed following hydrolysis under the action of serum esterases, is the main responsible of the anti-inflammatory activity of the series. The designed products are an emblematic example of how the appropriate selection of the NO-releasing group is a critical aspect in designing NO-hybrid compounds.

4. Experimental section

4.1. Chemistry

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 at 300 and 75 MHz, respectively, using TMS as internal standard. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700. Melting points were determined with a capillary apparatus (Büchi 540). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM); PE stands for 40–60 petroleum ether. Progress of the reactions was monitored by thin layer chromatography (TLC) on 5 × 20 cm plates with a layer thickness of 0.25 mm. Anhydrous magnesium sulfate was used as drying agent for the organic phases. Organic solvents were removed under vacuum at 30 °C. Preparative HPLC was performed on a Lichrospher® C_{18} column (250 × 25 mm, 10 μm) (Merck Darmstadt, Germany) with a Varian ProStar mod-210 with Varian UV detector mod-325. Elemental analyses (C, H, N) were performed and the results are within ±0.3% of the theoretical values, for compound **17a** the results are within ±0.5% of the theoretical values. Compounds **2**,[22] **2a**,[22] **3**,[27] **3a**,[28] **5**[29] and **6**,[30] were synthesized as indicated in the literature. CAS1609 was a gift from Sanofi-Aventis Deutschland GmbH.

4.1.1. General procedure for the preparation of 4, 4a and 5a

A solution of Jones reagent 2.5 M (19 mL, 46.62 mmol) was added to a stirred solution of the appropriate alcohol (18.65 mmol) in acetone (150 mL), cooled at 0 °C. The mixture was allowed to

reach rt and stirred for 4 h. *i*PrOH (10 mL) was added and the mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc (150 mL) and extracted with a saturated solution of $NaHCO_3$ (3 × 20 mL). The aqueous layers were acidified with 6 M HCl and extracted twice with EtOAc (50 mL). The combined organic layers were dried and concentrated under reduced pressure to give the desired compound.

4.1.1.1. 3-(3-Phenylfuroxan-4-yloxy)propanoic acid (4). 59% yield; white solid; mp = 136.5-137 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.01 (t, J = 6.0 Hz, 2H, –CH_2COOH), 4.78 (t, J = 6.0 Hz, 2H, –OCH_2–), 7.41–7.50 (m, 3H, C_6H_5), 8.05–8.07 (m, 2H, C_6H_5), 11.41 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 33.3, 66.7, 107.3, 121.9, 125.7, 128.8, 129.3, 161.9, 171.6 ppm; MS m/z 251 $(M+H)^+$.

4.1.1.2. 3-(4-Phenylfurazan-3-yloxy)propanoic acid (4a). 71% yield; white solid; mp = 105.5–108 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.00 (t, J = 6.0 Hz, 2H, –CH_2COOH), 4.74 (t, J = 6.0 Hz, 2H, –OCH_2–), 7.43–7.47 (m, 3H, C_6H_5), 7.92–7.96 (m, 2H, C_6H_5), 11.41 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 34.4, 68.0, 125.4, 127.9, 129.6, 131.3, 145.6, 163.7, 177.0 ppm; MS m/z 235 $(M+H)^+$.

4.1.1.3. 3-[(4-Phenylsulfonyl)furazan-3-yloxy]propanoic acid (5a). 68% yield; mp = 99.5–103.5 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 2.80 (t, J = 5.8 Hz, 2H, –CH_2COOH), 4.55 (t, J = 5.8 Hz, 2H, –OCH_2–), 7.76 (t, 2H, C_6H_5), 7.92 (t, 1H, C_6H_5), 8.07 (d, 2H, C_6H_5), 12.62 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 33.3, 69.8, 128.5, 130.1, 136.1, 136.7, 148.6, 160.7, 171.3 ppm; MS m/z 299 $(M+H)^+$.

4.1.2. Methyl 2-[(3-aminocarbonylfuroxan-4-yl)methyl]thioacetic acid (7)

To a solution of 4-bromomethylfuroxan-3-carboxyamide (3.1 g, 14 mmol) in CH_3CN (100 mL) methyl thioglycolate (1.3 mL, 14 mmol) and Et_3N (1.95 mL, 14 mmol) were added. After 1 h the mixture was diluted with CH_2Cl_2 (100 mL), washed with H_2O (100 mL), 1 M NaOH (50 mL), brine (50 mL), dried and concentrated under reduced pressure to give the title compound as a white solid, 72% yield; mp = 92.5–93.5 °C (from iPr$_2$O). ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 3.33 (s, 2H, –CH_2COOCH$_3$), 3.75 (s, 3H, -CH_3) 4.16 (s, 2H, –CH_2S–), 6.27 (br s, 1H, CONH$_2$), 7.58 ppm (br s, 1H, CONH$_2$); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 26.7, 33.1, 52.7, 110.1, 156.1, 156.2, 170.3 ppm; MS m/z 248 $(M+H)^+$.

4.1.3. Methyl 2-[(4-aminocarbonylfurazan-3-yl)methyl]thioacetic acid (7a)

A solution of **7** (3.1 g, 12.54 mmol) in P(OMe)$_3$ (20 mL) was heated under reflux for 17 h. The mixture was then poured into iced 6 M HCl (200 mL) and the white solid thus obtained was filtered. The crude product was purified by flash chromatography (PE/EtOAc 70:30 v/v) to give the title compound as a white solid, 90% yield; mp = 83–84 °C (from iPr$_2$O). ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 3.29 (s, 2H, –CH_2COOCH$_3$), 3.73 (s, 3H, CH_3), 4.24 (s, 2H, –CH_2S–), 6.29 (br s, 1H, CONH$_2$), 6.83 ppm (br s, 1H, CONH$_2$); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 23.8, 33.1, 52.6, 146.9, 152.9, 158.7, 170.3 ppm; MS m/z 232 $(M+H)^+$.

4.1.4. General procedure for the preparation of 9 and 9a

To a solution of the appropriate amide derivative (0.3 g, 1.21 mmol) in dry THF (10 mL) stirred under N_2 at 0 °C, dry pyridine (200 μL, 2.42 mmol) and, dropwise, $(CF_3CO)_2O$ (320 μL, 2.3 mmol) were added. After 20 min the reaction is complete; the mixture was diluted with Et_2O (15 mL), washed with H_2O (10 mL) and twice with 0.5 M HCl (10 mL). The organic layer was dried and concentrated under reduced pressure. The crude product was purified by flash chromatography (PE/EtOAc 90:10 v/v) to give the desired compound.

4.1.4.1. Methyl 2-[(3-cyanofuroxan-4-yl)methyl]thioacetic acid (9). 89% yield; colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 3.30 (s, 2H, –CH_2COOCH$_3$), 3.74 (s, 3H, CH_3), 3.93 ppm (s, 2H, –CH_2S–); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 26.1, 32.9, 52.9, 96.6, 105.0, 154.4, 169.6 ppm; MS m/z 230 $(M+H)^+$.

4.1.4.2. Methyl 2-[(4-cyanofurazan-3-yl)methyl]thioacetic acid (9a). 84% yield; colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 3.24 (s, 2H, –CH_2COOCH$_3$), 3.74 (s, 3H, CH_3), 4.10 ppm (s, 2H, –CH_2S–); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 23.3, 32.8, 52.8, 106.9, 132.7, 153.9, 169.6 ppm; MS m/z 214 $(M+H)^+$.

4.1.5. General procedure for the preparation of 8, 8a, 10 and 10a

To a solution of the appropriate methyl ester (6.3 mmol) in dioxane (30 mL), 6 M HCl (30 mL) was added and the mixture heated at 70 °C for 4 h. After concentration under reduced pressure, the residue was dissolved in EtOAc (30 mL) and the solution washed with H_2O (50 mL), and extracted twice with a saturated solution of $NaHCO_3$ (30 mL). The aqueous layers were acidified and extracted twice with EtOAc (30 mL). The organic layers were washed with brine (50 mL), dried and concentrated under reduced pressure to give the desired product.

4.1.5.1. 2-[(3-Aminocarbonylfuroxan-4-yl)methyl]thioacetic acid (8). 83% yield; white solid; mp = 110–112 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.36 (s, 2H, –CH_2COOH), 4.05 (s, 2H, –CH_2S–), 7.79 (br s, 1H, CONH$_2$), 8.45 (br s, 1H, CONH$_2$), 12.69 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 26.3, 33.3, 110.3, 155.8, 156.6, 171.0 ppm; MS m/z 234 $(M+H)^+$.

4.1.5.2. 2-[(4-Aminocarbonylfurazan-3-yl)methyl]thioacetic acid (8a). 67% yield; white solid; mp = 151–157 °C (toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.29 (s, 2H, –CH_2COOH), 4.14 (s, 2H, –CH_2S–), 8.18 (br s, 1H, CONH$_2$), 8.54 (br s, 1H, CONH$_2$), 12.75 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 23.3, 33.4, 148.3, 153.8, 158.4, 170.9 ppm; MS m/z 218 $(M+H)^+$.

4.1.5.3. 2-[(3-Cyanofuroxan-4-yl)methyl]thioacetic acid (10). 97% yield; white solid; mp = 87–88 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.35 (s, 2H, –CH_2COOH), 4.05 (s, 2H, –CH_2S–), 12.64 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 25.9, 33.3, 97.8, 105.7, 155.4, 170.7 ppm; MS m/z 216 $(M+H)^+$.

4.1.5.4. 2-[(4-Cyanofurazan-3-yl)methyl]thioacetic acid (10a). 92% yield; white solid; mp = 34–37 °C (from toluene). ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 3.32 (s, 2H, –CH_2COOH), 4.16 (s, 2H, –CH_2S–), 10.87 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 23.6, 33.1, 107.2, 133.0, 154.1, 175.7 ppm; MS m/z 200 $(M+H)^+$.

4.1.6. General procedure for the preparation of 13, 13a, 14 and 14a

$SOCl_2$ (334 μL, 4.58 mmol) and a few drops of dry DMF were added to a solution of the appropriate carboxylic acid (3.82 mmol) in dry THF (20 mL), stirred under N_2 at rt. Stirring was continued for 7 h at rt. The solution of the acyl chloride thus obtained was slowly added to a stirred solution of salicylic acid (0.53 g, 3.82 mmol) and dry Py (463 μL, 5.73 mmol) in dry THF (20 mL) kept under N_2 at 0 °C. The mixture was allowed to reach rt and then stirred overnight. The mixture was diluted with Et_2O (50 mL) and washed twice with 2 M HCl (50 mL). The combined organic layers were dried and concentrated under reduced

pressure. The crude product was purified by preparative HPLC (Lichrospher 250–25 C_{18}, CH_3CN/H_2O/TFA 50:50:0.1, flow 39 mL/min, λ224 nm, injection 4 mL, solution 51 mg/mL) to give the desired compound.

4.1.6.1. 2-({3-[(3-Phenylfuroxan-4-yl)oxy]propanoyl}oxy)benzoic acid (13). 61% yield; white solid; mp = 152–154 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.27 (t, J = 6.0 Hz, 2H, –CH_2COO–), 4.80 (t, J = 6.0 Hz, 2H, –CH_2O–), 7.14–8.07 (m, 9H, C_6H_4 + C_6H_5), 13.16 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 33.9, 66.5, 107.9, 122.3, 124.1, 124.2, 126.6, 126.7, 129.3, 131.1, 131.9, 134.3, 150.3, 162.4, 165.8, 169.4 ppm; MS m/z 371 (M+H)$^+$. Anal. Calcd for $C_{18}H_{14}N_2O_7$: C, 58.38; H, 3.81; N, 7.56. Found: C, 58.55; H, 3.91; N, 7.34.

4.1.6.2. 2-({3-[(4-Phenylfurazan-3-yl)oxy]propanoyl}oxy)benzoic acid (13a). 37% yield; white solid; mp = 141–146 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.25 (t, J = 6.0 Hz, 2H, –CH_2COO–), 4.78 (t, J = 6.0 Hz, 2H, –CH_2O–), 7.18–7.98 (m, 9H, C_6H_4 + C_6H_5), 13.16 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 33.8, 68.2, 123.9, 124.0, 124.4, 126.5, 127.4, 129.4, 131.3, 131.7, 134.1, 145.4, 150.1, 163.3, 165.6, 169.2 ppm; MS m/z 355 (M+H)$^+$. Anal. Calcd for $C_{18}H_{14}N_2O_6$: C, 61.02; H, 3.98; N, 7.91. Found: C, 61.13; H, 4.02; N, 7.74.

4.1.6.3. 2-[(3-{[(3-Phenylsulfonyl)furoxan-4-yl]oxy}propanoyl)oxy]benzoic acid (14). 32% yield; white solid; mp = 169.5–170 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.16 (t, J = 6.0 Hz, 2H, –CH_2COO–), 4.71 (t, J = 6.0 Hz, 2H, –CH_2O–), 7.21 (d, 1H, C_6H_4), 7.43 (t, 1H, C_6H_4), 7.63–7.70 (m, 3H, C_6H_4 + C_6H_5), 7.86 (t, 1H, C_6H_4), 7.98 (d, 1H, C_6H_4), 8.08 (d, 2H, C_6H_5), 13.16 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 33.3, 66.6, 110.4, 123.6, 123.7, 126.3, 128.2, 129.8, 131.5, 133.9, 136.0, 137.1, 149.8, 158.6, 165.4, 168.6 ppm; MS m/z 435 (M+H)$^+$. Anal. Calcd for $C_{18}H_{14}N_2O_9S$: C, 49.77; H, 3.25; N, 6.45. Found: C, 49.67; H, 3.28; N, 6.53.

4.1.6.4. 2-[(3-{[(4-Phenylsulfonyl)furazan-3-yl]oxy}propanoyl)oxy]benzoic acid (14a). 38% yield; white solid; mp = 139.5–140 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.16 (t, J = 5.8 Hz, 2H, –CH_2COO–), 4.71 (t, J = 5.8 Hz, 2H, –CH_2O–), 7.21 (d, 1H, C_6H_4), 7.43 (t, 1H, C_6H_4), 7.63–7.70 (m, 3H, C_6H_4 + C_6H_5), 7.86 (t, 1H, C_6H_5), 7.98 (d, 1H, C_6H_4), 8.08 (d, 2H, C_6H_5),13.16 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 33.4, 69.0, 123.6, 123.7, 126.3, 128.6, 130.0, 131.4, 133.9, 136.0, 136.6, 148.6, 149.8, 160.7, 165.3, 168.6 ppm; MS m/z 419 (M+H)$^+$. Anal. Calcd for $C_{18}H_{14}N_2O_8S$ 0.25 H_2O: C, 51.12; H, 3.46; N, 6.62. Found: C, 51.00; H, 3.30; N, 6.54.

4.1.7. General procedure for 17, 17a, 18 and 18a

$SOCl_2$ (190 μL, 2.57 mmol) and dry DMF (1 mL) were added to a solution of the appropriate carboxylic acid (2.15 mmol) in dry CH_2Cl_2 (10 mL), stirred under N_2 at rt. Stirring was continued for 1 h at rt. The solution of the acyl chloride thus obtained was slowly added to a stirred solution of salicylic acid (0.24 g, 1.72 mmol) and dry Py (260 μL, 3.22 mmol) in dry CH_2Cl_2 (20 mL) kept under N_2 at 0 °C. The mixture was allowed to reach rt and then stirred overnight. The mixture was washed twice with 2 M HCl (20 mL). The combined organic layers were dried and concentrated under reduced pressure. The crude product was purified as follows.

4.1.7.1. 2-{[(3-Aminocarbonylfuroxan-4-yl)methyl]thioacetoxy}benzoic acid (17). Purified by flash chromatography (eluent PE/EtOAc/HCOOH 70:30:0.1 v/v/v) to give the title compound in 69% yield as white solid; mp = 143.5–144.5 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.74 (s, 2H, –CH_2COO–), 4.15 (s, 2H, –CH_2S–), 7.21 (d, 1H, C_6H_4), 7.41 (t, 1H, C_6H_4), 7.67 (t, 1H, C_6H_4), 7.82 (br s, 1H, CONH_2), 7.95 (d, 1H, C_6H_4), 8.48 ppm (br s, 1H, CONH_2); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 26.2, 33.1, 110.0, 119.1, 123.4, 126.3, 131.4, 133.9, 149.8, 155.6, 156.3, 165.4, 168.4 ppm; MS m/z 354 (M+H)$^+$. Anal. Calcd for $C_{13}H_{11}N_3O_7S$: C, 44.19; H, 3.14; N, 11.89. Found: C, 43.80; H, 3.18; N, 12.00.

4.1.7.2. 2-{[(4-Aminocarbonylfurazan-3-yl)methyl]thioacetoxy}benzoic acid (17a). Purified by flash chromatography (eluent CH_2Cl_2/MeOH/HCOOH 98:2:0.1 v/v/v) to give the title compound in 69% yield as white solid; mp = 154–157 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.70 (s, 2H, –CH_2COO–), 4.25 (s, 2H, –CH_2S–), 7.21 (d, 1H, C_6H_4), 7.39 (t, 1H, C_6H_4), 7.68 (t, 1H, C_6H_4), 7.95 (d, 1H, C_6H_4), 8.20 (br s, 1H, CONH_2), 8.57 (br s, 1H, CONH_2), 13.20 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 23.4, 33.3, 123.7, 126.3, 131.4, 133.9, 148.1, 149.8, 153.2, 158.2, 165.4, 168.3, 170.7 ppm; MS m/z 338 (M+H)$^+$. Anal. Calcd for $C_{13}H_{11}N_3O_6S$ 0.5 H_2O: C, 45.09; H, 3.49; N, 12.13. Found: C, 45.27; H, 3.36; N, 11.65.

4.1.7.3. 2-{[(3-Cyanofuroxan-4-yl)methyl]thioacetoxy}benzoic acid (18). Purified by reverse phase flash chromatography (eluent H_2O/CH_3CN/TFA 60:40:0.1 v/v/v) to give the title compound in 38% yield as white solid; mp = 121.5–124.5 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.82 (s, 2H, –CH_2COO–), 4.20 (s, 2H, –CH_2S–), 7.19 (d, 1H, C_6H_4), 7.42 (t, 1H, C_6H_4), 7.67 (t, 1H, C_6H_4), 7.96 (d, 1H, C_6H_4), 13.18 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 25.4, 32.8, 98.7, 106.2, 123.4, 123.7, 126.4, 131.4, 133.9, 149.8, 155.8, 165.3, 168.2 ppm; MS m/z 336 (M+H)$^+$. Anal. Calc. for $C_{13}H_9N_3O_6S$: C, 46.57; H, 2.70; N, 12.53. Found: C, 46.27; H, 2.76; N, 12.27.

4.1.7.4. 2-{[(4-Cyanofuroxan-3-yl)methyl]thioacetoxy}benzoic acid (18a). Purified by preparative HPLC (Lichrospher 250–25 C_{18}, CH_3CN/H_2O/TFA 45:55:0.1, flow 39 mL/min, λ 224 nm, injection 3 mL, solution 51 mg/mL) to give the title compound in 46% yield as a white solid; mp = 114–115.5 °C (from toluene). ^{1}H NMR (300 MHz, DMSO-d_6, TMS): δ = 3.76 (s, 2H, –CH_2COO–), 4.35 (s, 2H, –CH_2S–), 7.16 (d, 1H, C_6H_4), 7.41 (t, 1H, C_6H_4), 7.68 (t, 1H, C_6H_4), 7.95 (d, 1H, C_6H_4), 13.19 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6, TMS): δ = 23.1, 33.3, 107.7, 123.3, 123.6, 126.4, 131.4, 133.7, 133.9, 149.8, 155.1, 165.3, 168.3 ppm; MS m/z 320 (M+H)$^+$. Anal. Calcd for $C_{13}H_9N_3O_5S$ 1.5 H_2O: C, 45.09; H, 3.49; N, 12.13. Found: C, 45.18; H, 3.17; N, 12.47.

4.2. Stability studies

4.2.1. Evaluation of stability in aqueous buffered solutions

A solution of each compound (10 mM) in acetonitrile was added to HCl 0.1 M or to phosphate buffer pH 7.4 (50 mM) preheated at 37 °C; the final concentration of the compound was 100 μM. The resulting solution was maintained at 37 ± 0.5 °C and, at appropriate time intervals, a 20 μL aliquot of reaction solution was analyzed by RP-HPLC, as described below.

4.2.2. Stability in human serum

A solution of each compound (10 mM) in acetonitrile was added to human serum (Sigma) preheated to 37 °C; the final concentration of the compound was 200 μM. The resulting solution was incubated at 37 ± 0.5 °C and, at appropriate time intervals, 300 μL of reaction mixture were withdrawn and added to 300 μL of acetonitrile containing 0.1% trifluoroacetic acid, in order to deproteinize the serum. The sample was sonicated, vortexed and then centrifuged for 10 min at 2150 g, the clear supernatant was filtered through 0.45 μm PTFE filters (Alltech) and analyzed by RP-HPLC.

The reverse-phase HPLC procedure separated and quantitated the remaining compound and salicylic acid. HPLC analyses were performed with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A), and a diode-array detector (DAD) (model G1315B) integrated in the HP1100 system. Data analysis was done by the HP ChemStation system (Agilent Technologies). The analytical column was a Nucleosil 100-5C18 Nautilus (250 × 4.6 mm, 5 μm particle size) (Macherey-Nagel). The mobile phase consisting of acetonitrile/water (55:45) 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 (for compounds) and 240 nm (for salicylic acid) referenced against a 600 nm wavelength. Quantitation was done using calibration curves of compounds and relative metabolites chromatographed under the same conditions; the linearity of the calibration curves was determined in a concentration range of 1–200 μM (r^2 >0.99).

4.3. Anti-inflammatory activity

Male Wistar rats, weighing 180–200 g (Harlan, S. Pietro al Natisone, Italy) were individually housed in hanging stainless-steel cages with grid floors, at constant room temperature (25 ± 1 °C) and humidity (60 ± 5%), with an artificial 12:12 h light/dark cycle. Edema was induced in conscious rats by intraplantar injection into the right hindpaw of 0.1 mL of 1% carrageenan, suspended in 1% carboxymethylcellulose (CMC). Immediately after carrageenan injection, compounds or vehicle (CMC, 1%) were administered intragastrically to different groups of rats in a volume of 10 mL kg^{-1}. Salicylic acid and NO-aspirin derivatives were administered at a dose equimolar with aspirin 120 mg kg^{-1}. Groups of 6–8 animals were used. Paw volume was measured with a water plethysmometer (Basile, Comerio, Italy) immediately before carrageenan injection and 3 h afterwards. The edema reduction in treated animals was expressed as percentage inhibition of the edema observed in vehicle-treated animals, considered as 100. The results obtained are presented as mean ± SEM. Statistical analysis was performed with ANOVA followed by Dunnett test.

4.4. Gastrotoxicity

Male Wistar rats, weighing 180–200 g (Harlan, S. Pietro al Natisone, Italy) were individually housed in hanging stainless-steel cages with grid floors, at constant room temperature (25 ± 1 °C) and humidity (60 ± 5%), with an artificial 12:12 h light/dark cycle. They were deprived of food but not of water 24 h before the experiments. Groups of rats (n = 8–10) were given aspirin 120 mg kg^{-1} by intragastric route or equimolar doses of the compounds under study (vehicle CMC 1%). Rats were sacrificed 3 h after the administration of the compounds. Immediately after the sacrifice, the stomachs were removed, opened along the lesser curvature and examined for the assessment of mucosal lesions. The stomachs were laid on a flat surface under a stereomicroscope. The glandular mucosa was examined and each individual hemorrhagic lesion was measured along its greatest length (<1 mm: rating = 1; 1–2 mm: rating = 2; >2 mm: rating according to their greatest length). The lengths of the lesions were summed to give an overall total, designated as the lesion index, for each stomach. The results obtained are presented as mean ± SEM. Statistical analysis was performed with ANOVA followed by Newman–Keuls test.

4.5. Inhibition of human platelet aggregation in vitro

Venous blood samples were obtained from healthy volunteers who had not taken any drug for at least two weeks. Volunteers, who were treated according to Helsinki protocol for biomedical experimentation, gave their informed consent to the use of blood samples for research purposes. Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 200g for 20 min. 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 °C for 10 minutes after addition of the stimulus. Collagen at submaximal concentration (0.8–1.5 μg mL^{-1}) was used as platelet activator in PRP. Compounds under study were preincubated with PRP 10 min before addition of the stimulus (collagen). Vehicle alone (0.5% DMSO) added to PRP did not affect platelet function in control samples. The role of NO and sGC in the inhibitory effect was investigated using the sGC inhibitor, ODQ (50 μM). At least five experiments for each compound were performed.

To study the antagonism at the thromboxane receptor human blood was anticoagulated with citrate solution and treated with 1 mM acetylsalicylic acid; PRP was prepared as described above. PRP samples were incubated with compounds or vehicle (0.5% DMSO) and challenged with the agonist U-46619 (0.5–2.5 μM). Incubation and registration times were the same as described previously. At least four experiments for each compound were performed.

The antiaggregatory activity of tested compounds is evaluated as% inhibition of platelet aggregation compared to control samples. For most active compounds IC_{50} values could be calculated by nonlinear regression analysis, otherwise% inhibition at maximal concentration tested (300 μM) is reported.

4.6. Vasodilator activity

Thoracic aortas were isolated from male Wistar rats weighing 180–200 g. As few animals as possible were used. The purposes and the protocols of our studies have been approved by Ministero della Salute, Rome, Italy. The endothelium was removed and the vessels were helically cut: three strips were obtained from each aorta. The tissues were mounted under 1.0 g tension in organ baths containing 30 mL of Krebs-bicarbonate buffer with the following composition (mM): NaCl 111.2, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.0, $NaHCO_3$ 12.0, glucose 11.1, maintained at 37 °C and 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 L-phenylephrine. When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. Results are expressed as EC_{50} ± SE (μM). The effects of 1 μM ODQ on relaxation were evaluated in separate series of experiments in which it was added to the organ bath 5 min before the contraction. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab. Addition of the drug vehicle (DMSO) had no appreciable effect on contraction level.

4.7. COX-1 inhibition

In order to evaluate COX-1 inhibitory activity, washed platelets from individual donors were prepared as described elsewhere.[18] Aliquots of platelets (200 μL at 10^8 cells mL^{-1}) were incubated with inhibitors at different concentrations (diluted from 400-fold concentrated solutions in DMSO) for 30 min at 37 °C, before addition of calcium ionophore (A23187, 2 μM). After 10 min at 37 °C, the reactions were terminated with 100 μL of methanol. TXB_2 in the supernatant was determined by enzyme immunoassay (Cayman Chemical). Percent inhibition of TXB_2 production in compound-treated samples was calculated by comparison with untreated control samples. Background (no ionophore) TXB_2 production was subtracted from each value obtained in the presence of ionophore.

4.8. COX-2 inhibition

In order to evaluate COX-2 inhibitory activity, human monocytes were isolated from buffy coats obtained from the SIMT (Banca del sangue, AOU San Giovanni Battista Hospital, Turin, Italy). Mononuclear cells were isolated as described elsewhere.[18] The monocytes (10^6 cells mL^{-1}) were incubated with LPS (10 μg mL^{-1}) for 18 h at 37 °C in a 5% CO_2 atmosphere. Aliquots of LPS-stimulated monocytes were centrifuged (300*g*, 8 min) and resuspended in fresh FCS-free RPMI-1640 medium. Each compound (diluted from 200-fold concentrated solutions in DMSO) was added at different concentrations to the monocyte suspensions; the aliquots of monocytes were then incubated at 37 °C in a 5% CO_2 atmosphere for 30 min, prior to stimulation with arachidonic acid (100 μM) for 15 min. Cell suspensions were centrifuged and cell-free supernatants were stored at −20 °C until eicosanoid determination was completed. PGE_2 in the supernatant was determined by EIA (Cayman Chemical). Percent inhibition of PGE_2 production in compound-treated samples was calculated versus untreated control samples. Background (no arachidonic acid) PGE_2 production was subtracted from each value obtained in the presence of arachidonic acid.

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