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A new class of furoxan derivatives as NO donors: mechanism of action and biological activity

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1 The mechanism of action and biological activity of a series of R-substituted and di-R-substituted phenylfuroxans is reported.

2 Maximal potency as vasodilators on rabbit aortic rings, precontracted with noradrenaline (1 μM), was shown by phenyl-cyano isomers and by the 3,4-dicyanofuroxan, characterized by a potency ratio 3-10 fold higher than glyceryl trinitrate (GTN). This effect was reduced upon coincubation with methylene blue or oxyhaemoglobin (10 μM).

3 The furoxan derivatives showing maximal potency as vasodilators were also able to inhibit collagen-induced platelet aggregation, with IC_{50} values in the sub-micromolar range.

4 The furoxan derivatives were able to stimulate partially purified, rat lung soluble guanylate cyclase; among the most active compounds, the 3-R-substituted isomers displayed a higher level of stimulatory effect than the 4-R analogues.

5 Solutions (0.1 mM) of all the tested furoxans, prepared using 50 mM phosphate buffer, pH 7.4, (diluting 10 mM DMSO stock solutions) did not release nitric oxide (NO) spontaneously; however in presence of 5 mM L-cysteine, a significant NO-releasing capacity was observed, which correlated significantly with their stimulation of the guanylate cyclase activity.

Keywords: Furoxans; nitric oxide; vasodilatation; haemoglobin; guanylate cyclase; platelet aggregation

Introduction

Since the early observations on the existence of endothelium-dependent relaxation and endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), this field of research has become an important area of biological interest (Ignarro, 1990). In 1987, two groups, independently, forwarded the hypothesis that EDRF could be closely related to nitric oxide (NO) radical (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). These observations were extended in a multiplicity of investigations (Moncada *et al.*, 1991), which led to the present concept of the role of NO as a transduction mechanism and as an effector molecule.

The possibility that there may be more than one type of EDRF cannot be excluded (Rubanyi & Vanhoutte, 1987), but certainly NO provides a comprehensive explanation for the majority of the pharmacological findings on EDRF: NO plays an important role in the vasodilator process (Holtz *et al.*, 1984), is a potent inhibitor of platelet aggregation (Radomski *et al.*, 1987), and is able to kill bacterial cells when released by macrophages (Hibbs *et al.*, 1987).

NO is synthesized from the amino acid L-arginine by the action of an enzyme, NO synthase (Bredt & Snyder, 1990; Bredt *et al.*, 1991), in addition, extension of the original hypothesis by Needleman *et al.* (1973) on the requirement of tissue thiols for the vasodilator action of glyceryl trinitrate (GTN), led to the identification of S-nitrosocysteine, a labile but potent vasorelaxant that works through the action of liberated NO (Ignarro *et al.*, 1981). Several reports (Seth & Fung, 1993) have suggested that a cytochrome P450 related enzyme could be responsible for the production of NO from organic nitrates, in cells not derived from the vascular smooth muscle. More recently a membrane-bound enzyme responsible for generating NO from GTN in these latter cells had been characterized (Seth & Fung, 1993). The enzyme activity was enhanced by several thiols and was suppressed by several reagents known to bind free sulphydryl groups.

These findings have prompted the development of compounds acting as NO-donating pro-drugs, devoid of nitrate tolerance (Needleman & Johnson, 1973) and with a longer duration of action than classic organic nitrate esters (Yamada *et al.*, 1991; Ånggård, 1991).

Recently, we described a new class of compounds, with a furoxan structure, which have been shown to possess NO-mimetic pharmacological activities (Calvino *et al.*, 1992; Ghigo *et al.*, 1992). The mechanism of action of furoxancarboxamides at the molecular level has been investigated by Feelisch *et al.* (1992) who found that these compounds react with sulphydryl groups of low molecular weight thiols and proteins. Furoxans are thus pro-drugs, which increase the level of guanosine 3':5'-cyclic monophosphate (cyclic GMP) via liberation of NO, as found for classic nitrovasodilators.

In structure-activity relationship studies, we have also investigated the influence of different substitutions on the furoxan ring, i.e. bis-(phenylsulphonyl) and 3- or 4-(1,1-dinitroethyl), in relation to their vasodilator and platelet antiaggregatory profile (Ferioli *et al.*, 1993; Gasco *et al.*, 1993a,b).

In the present paper, the *in vitro* biological activity of twenty R-substituted phenylfuroxans (derivatives **2-11 a, b**), as well as of three di-R-substituted furoxans (derivatives **1, 12, 13**), is described; their proposed mechanism of action as NO donors makes them interesting models in the design of new drugs.

Methods

Synthesis of the compounds

All the derivatives were synthetized according to the methods previously reported (Gasco & Boulton, 1981; Gasco *et al.*,

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1993a,b). The purity (>98%) of the samples used for the pharmacological tests was checked by h.p.l.c. (RP-C18 column, 5 μm, 250 × 4.6 mm; eluent; methanol/water).

Study of the vasodilator effects

Male New Zealand white rabbits weighing 1.8–2.3 kg were anaesthetized with sodium pentobarbitone (40–50 mg kg^{-1}) via an ear vein, the carotid artery was cannulated and the animals bled. The thorax was rapidly opened and the aorta was removed carefully, to protect the endothelial lining, cleared of fat and connective tissue and cut in 2–3 mm wide transverse rings. Four rings were joined together by surgical silk to form a chain and placed in a glass organ bath containing Krebs Henseleit bicarbonate solution at 37°C, constantly oxygenated with a mixture of 95% O_2/5% CO_2. After an equilibration period of 1 h under a basal tension of 2 g, changes in isometric contraction were monitored by a force transducer (Basile, mod. 7004) connected to a Gemini 7070 Basile pen recorder.

Responses to vasodilator agents were studied following the protocol described previously (Ferioli *et al.*, 1993). Glyceryl trinitrate (GTN, 0.01-3.0 μM) was used as a reference standard.

The composition (mM) of the Krebs buffer was: NaCl 118.90, KCl 4.66, KH_2PO_4 1.18, $MgSO_4$ 1.10, $CaCl_2$ 2.52, glucose 5.55, $NaHCO_3$ 25 (Merck; Darmstadt, Germany); pH was 7.4.

The following compounds were used: acetylcholine HCl (Sigma Chemical Company; St. Louis, Missouri, U.S.A.), glyceryl trinitrate (Perlinganit Lösung; Schwarz Pharma; Monheim, Germany), dimethylsulphoxide (Sigma), noradrenaline bitartrate (Sigma), ascorbic acid (Merck), methylene blue (Sigma).

Preparation of haemoglobin

Bovine haemoglobin Type 1 (H-2500 Sigma Chemical Co.) contains a mixture of oxyhaemoglobin and the oxidized derivative methaemoglobin. Pure oxyhaemoglobin was prepared, protected from the light, by adding to a solution of commercially available haemoglobin in phosphate saline buffer, pH 7.4, a 10 fold molar excess of the reducing agent sodium dithionite ($Na_2S_2O_4$), at 4°C. Within a few minutes, the mixture was centrifuged at 1000 *g* for 10 min, at 4°C, and the supernatant loaded onto a chromatographic column (Sephadex G-25 coarse, Pharmacia, Uppsala, Sweden) and eluted with phosphate buffer.

The purity of the oxyhaemoglobin solution was determined spectrophotometrically (λ_{max} = 576 nm, 541 nm; ε = 14600, 13800) and the solutions were frozen in aliquots at −20°C and stored for 15 days.

Platelet aggregation studies

Human platelet aggregation was determined by the Born turbidimetric technique (Born, 1962), according to the procedure previously described (Ferioli *et al.*, 1993). The activity of each compound was tested 5 times with platelet rich plasma (PRP) obtained from different donors. IC_{50} values were generated from regression analysis of concentration-response curves.

Preparation of guanylate cyclase from rat lungs

Male, Sprague Dawley rats (Charles River, Calco (CO), Italy), weighing 200–220 g were anaesthetized with urethane (1 g kg^{-1}, i.p.) and killed by decapitation. The procedure was carried out at +4°C; pieces (100 g) of rat lung were chopped, suspended in 300 ml buffer [(Tris/HCl 10 mM, containing sucrose (0.25 M), ethylendiamino-tetraacetic acid (EDTA, 1 mM) and dithiothreitol (DTT, 2 mM), pH 7.4] and homogenized in a Polytron homogenizer. The homogenate was centrifuged at 500 *g* for 10 min, the supernatant collected and further centrifuged at 105,000 *g* for 1 h. The high speed supernatant (cytosol fraction) was applied to a column (4 × 15 cm) of DEAE cellulose (DE-52, Whatman) which was pre-washed (2.0 l) with buffer A [Tris/HCl (10 mM) containing DTT (2 mM), at pH 7.4]. The column was further washed with buffer A (1.0 l) followed by washing (1.0 l) with buffer B [Tris/HCl (10 mM), DTT (2 mM), NaCl (0.1 mM), at pH 7.4]. For the elution step, the column was finally washed with buffer C [Tris/HCl (10 mM), DTT (2 mM), NaCl (0.5 mM), at pH 7.4] and 10 ml fractions were collected. Fractions containing enzymic activity were collected and dialysed overnight against buffer A. The dialysate was applied to a column (1.5 × 19 cm) of Blue Sepharose CL-6B (Pharmacia) previously equilibrated with 1.0 l of buffer B. The enzyme was eluted with buffer C and 3 ml fractions were collected. The main active fractions (absorbance at 280 nm above 0.1) were pooled and dialysed overnight against buffer A. This procedure removed most of the haemoglobin from the eluate of the DE-52 column. The dialysate was frozen in aliquots at −70°C, thawed before use and not refrozen. Using SDS-polyacrylamide gel electrophoresis, about 50% of the protein migrated at 72 kD, the molecular weight reported for soluble GC subunit of bovine lung (Nakane *et al.*, 1988; 1990).

Assay of guanylate cyclase activity

The guanylate cyclase activity was measured by preincubating the enzyme (10 μg) for 5 min at 37°C in 50 mM Tris/HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 10 mM ethylenglycol-*bis*-(α-aminoethyl ether)-*N,N,N,N*, tetraacetic acid (EGTA), 5 mM L-cysteine and 0.5 mM of all the tested furoxans. The reaction was started by addition of 0.2 mM guanosine-5'-triphosphate (GTP) in a final volume of 300 μl and terminated after 20 min at 37°C, by heating for 3 min at 90°C. Cyclic GMP was measured by a radioimmunoassay kit using [^{3}H]-cyclic GMP (Amersham). The generation of cyclic GMP was linear with time (10–30 min) and cyclic GMP-phosphodiesterase inhibitors did not affect enzymatic activity, so they were not added to reaction mixture. The maximal enzymatic activity (E) was expressed in pmol cyclic GMP min^{-1} mg^{-1} protein. Values are given as means ± s.e.mean (n = 6–11). It refers to the activity displayed by each furoxan compound at 0.5 mM.

Furoxan compounds did not show cross-reactivity in the cyclic GMP radioimmunoassay. Protein was calculated according to Lowry *et al.* (1951).

Measurement of NO_2^-

Solutions (0.1 mM) of all the tested furoxans were prepared at 37°C, using 50 mM phosphate buffer, pH 7.4, (diluting 10 mM DMSO stock solutions) in presence or absence of 5 mM L-cysteine; after 1 h under gentle stirring, 1 ml aliquots of drug solutions were mixed with 0.25 ml aliquots of Griess reagent (4% sulphanilamide; 0.4% N-naphtylethylendiamine dihydrochloride; 10% H_3PO_4, w/v) and incubated at room temperature for 10 min to form an azo-dye. Absorbance was monitored at 550 nm using a UV-1204 Shimadzu spectrophotometer and NO_2^- was quantified with $NaNO_2$ as a standard (linearity of the standard curve was from 0.01 to 0.1 mM). The capacity to release NO was expressed as NO_2^-%, i.e. the ratio between the number of NO_2^- mol detected and the number of furoxan mol, multiplied by 100.

Statistical analysis

EC_{50} values represent the concentration required to produce 50% relaxation in a noradrenaline-contracted strip (n = 4–6). R_M% represents the peak relaxation observed at the maximal drug concentration employed.

Analysis of concentration-response curves was performed

using ALLFIT computer programme; comparisons were made by Student's t test. A probability of 0.05 or less was considered significant.

Linear regression analyses were performed according to Coburn (1987) on a IBM AT personal computer. The figures in parentheses for the regression lines are 95% confidence intervals; r = correlation coefficient ($r^2 \times 100$: variance in the data 'explained' by the regression equation); s = standard deviation from regression; P = level of significance; n = number of data.

Results

Vasodilator effects

The vasodilator activity of the compounds under investigation, expressed as their EC_{50} values ± 95% confidence limits (CL), as well as percentage of maximal relaxation (R_M% values), is reported in Table 1. Compounds **2a**, **2b**, **3a** and **4a** were inactive, while the EC_{50} values of the other derivatives ranged between 0.005–100 μM. Maximal potency was shown by compounds **11b**, **11a** and **13**, characterized by a potency ratio 3–10 fold higher than GTN. The majority of the furoxan derivatives (with the exclusion of compounds **1**, **3a**, **3b**, **4a**, **5b**), caused complete relaxation of the vascular tone, induced by noradrenaline. Furoxans showing the highest potency as vasodilators were also tested on aortic rings that had been pretreated (15 min) with methylene blue or oxyhaemoglobin (10 μM) (Figure 1). The relative EC_{50} values (see legend to Figure 1) reflect the expected rightward shift.

Effects on platelet aggregation

Compounds **7a**, **7b**, **11a**, **11b**, **12** and **13** were also tested for their ability to inhibit collagen-induced platelet aggregation. They showed IC_{50} values in the sub-micromolar range (Table 2).

Activation of soluble guanylate cyclase

The ability of the furoxan derivatives to activate the partially purified, soluble guanylate cyclase, obtained from rat lung, expressed as maximal enzymatic activity (E), is shown in Table 1.

Diphenylfuroxan **1** is characterized by low activity; substitution of the CH_3, NH_2, C_6H_5S, $COOCH_3$ groups for 3-phenyl or 4-phenyl moiety affords derivatives with similar (**2a**, **2b**, **3b**, **4a**) or slightly enhanced (**3a**, **4b**, **5a**, **5b**) biological activity, when compared to **1**.

A different situation occurs with the remaining pair of isomers (**6a**, **6b**; **7a**, **7b**: **8a**, **8b**; **9a**, **9b**; **10a**, **10b**; **11a**, **11b**), in which the 3-R-substituted isomers always display a level of stimulatory effect higher than that displayed by the 4-R analogues. The 3-R derivatives of this group can be listed in increasing order of efficacy as follows: **11b>10b>9b>**sodium nitroprusside (SNP)$\geq$**8b>7b>6b**.

Finally the high degree of efficacy as activators of guanylate cyclase of compounds **12** and **13** is noteworthy.

Formation of nitrite from furoxans

The capacity of furoxans to release NO (measured as NO_2^-) upon coincubation with 5 mM L-cysteine, is reported in Table 1. There was no spontaneous formation of NO; NO releasing-capacity followed the series: **10b = 10a$\leq$9b>12>**

Table 1 Chemical structure and biological activity of NO-releasing furoxans

Compound	*R*	*R'*	*Vasodilator activity* EC_{50} (μM) ± 95% CL	*Vasodilator activity* R_M %	*Stimulation of guanylate cyclase activity* E	*Release of NO* NO_2^- %
GTN			0.06 ± 0.03	111 ± 3	Not tested	45 ± 1
SNP			Not tested	Not tested	2182 ± 106	58 ± 6
1	Ph	Ph	100 ± 3	50 ± 1	179 ± 5	3 ± 2
2a	CH_3	Ph	Inactive	Inactive	309 ± 40	7 ± 3
2b	Ph	CH_3	Inactive	Inactive	270 ± 13	1 ± 1
3a	NH_2	Ph	–	46 ± 2	391 ± 46	2 ± 2
3b	Ph	NH_2	70.5 ± 0.3	71 ± 7	104 ± 15	0 ± 0
4a	Ph-S	Ph	–	48 ± 4	175 ± 46	8 ± 1
4b	Ph	Ph-S	3.5 ± 0.3	102 ± 2	464 ± 102	3 ± 2
5a	$COOCH_3$	Ph	3.6 ± 1.2	116 ± 10	482 ± 36	5 ± 3
5b	Ph	$COOCH_3$	67.6 ± 1.2	66 ± 9	348 ± 75	2 ± 2
6a	Cl	Ph	0.5 ± 0.1	116 ± 4	230 ± 20	2 ± 2
6b	Ph	Cl	1.7 ± 0.3	123 ± 2	714 ± 26	14 ± 1
7a	Ph-SO_2	Ph	1.7 ± 0.3	98 ± 5	575 ± 38	5 ± 2
7b	Ph	Ph-SO_2	0.63 ± 0.06	98 ± 5	1008 ± 68	22 ± 1
8a	$CONH_2$	Ph	6.1 ± 6.4	128 ± 4	517 ± 113	2 ± 0
8b	Ph	$CONH_2$	6.0 ± 1.2	127 ± 6	2062 ± 130	7 ± 4
9a	OCH_3	Ph	5.1 ± 2.6	128 ± 4	484 ± 36	5 ± 2
9b	Ph	OCH_3	0.5 ± 0.3	124 ± 6	2656 ± 55	76 ± 2
10a	NO_2	Ph	7.2 ± 2.9	98 ± 2	374 ± 27	76 ± 3
10b	Ph	NO_2	1.0 ± 0.6	110 ± 4	2847 ± 180	80 ± 1
11a	CN	Ph	0.007 ± 0.002	122 ± 3	1212 ± 126	6 ± 2
11b	Ph	CN	0.005 ± 0.008	124 ± 4	3744 ± 192	40 ± 2
12	Ph-SO_2	Ph-SO_2	0.049 ± 0.015	125 ± 4	2615 ± 73	59 ± 4
13	CN	CN	0.016 ± 0.006	113 ± 6	2087 ± 417	38 ± 7

R_M %: percentage of the maximum vascular relaxation. E: maximal enzymatic activity. NO_2^-%: ratio between the number of NO_2^- mol detected and the number of furoxan mol, multiplied by 100. The EC_{50} value has not been reported when R_M % was less than 50%. Basal enzymatic activity was 0.077 ± 0.004 nmol min^{-1} mg^{-1} prot. Values are given as means ± s.e.mean for 6 to 11 replicates.

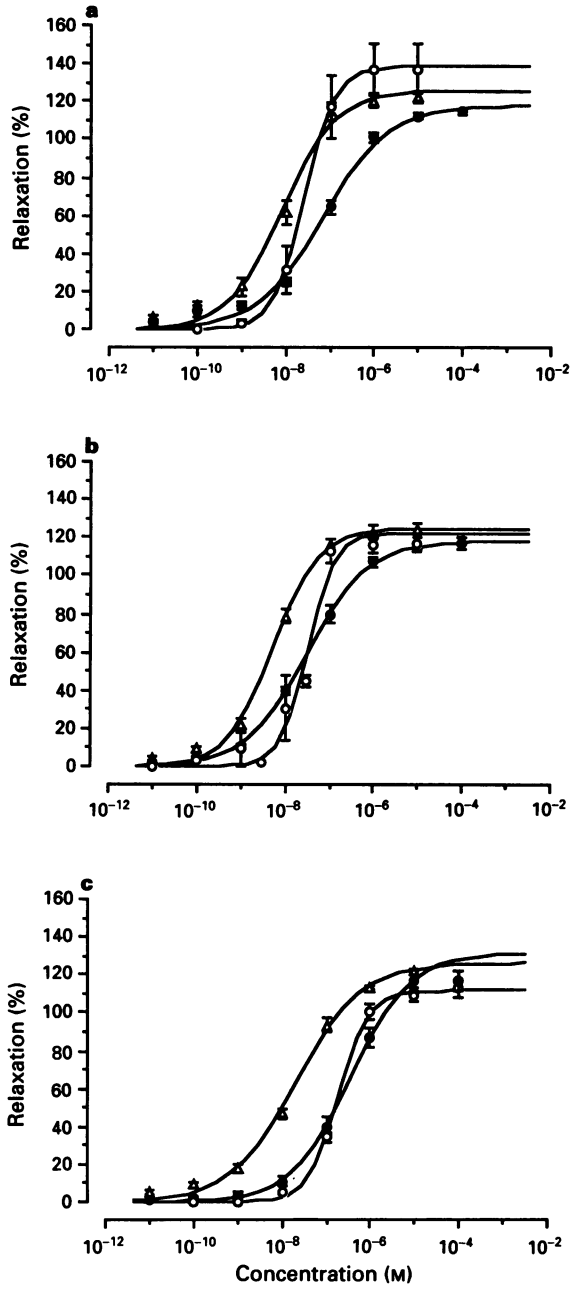

Figure 1 Concentration-response curves of the vasodilator activity of selected furoxan compounds, in the absence (Δ) or presence of methylene blue (10 μM) (●) or oxyhaemoglobin (10 μM) (○). EC_{50} (μM) ± 95% CL: (a) compound **11a**, control 0.007 ± 0.002, +MB 0.07 ± 0.02 ($P<0.05$), +HbO_2 0.030 ± 0.005 ($P<0.05$); (b) compound **11b**, control 0.005 ± 0.001, + MB 0.031 ± 0.009 ($P<0.05$), +HbO_2 0.032 ± 0.005 ($P<0.05$); (c) compound **13**, control 0.016 ± 0.004, +MB 0.29 ± 0.05 ($P<0.05$), +HbO_2 0.32 ± 0.02 ($P<0.05$). Each value is the mean ± 95% CL of 4–6 different experiments.

Table 2 Inhibitory effect of selected furoxan compounds on collagen-induced, human platelet aggregation

Compound	*IC_{50} ± 95% confidence limits* (μM)
SNP	2.2 ± 0.5
7a	3.4 ± 1.4
7b	0.38 ± 0.02
12	0.31 ± 0.05
11a	0.84 ± 0.12
11b	0.20 ± 0.02
13	0.24 ± 0.02

11b = 13>7b>6b>11a and remaining compounds. No release of NO was observed in the absence of L-cysteine.

Comparison of the data reported above with those of stimulation efficacy of the guanylate cyclase activity (E), shows a statistically significant degree of correlation, able to explain only 46.3% of variance (Equation 1; Coburn, 1987).

Equation 1: E = 26.7 (±6.3) %NO_2^- + 500.9 (±208.7)
$r = 0.68$ $s = 797.9$ $P<0.01$ $n = 23$.

The furoxan derivatives **11b**, **10a** and **8b** show 'residuals' (observed *minus* estimate) larger than twice the standard deviation; if these compounds are removed, the 'cleaned' *equation 2* is obtained.

Equation 2: E = 34.1 (±2.5) %NO_2^- + 305.9 (±73.8)
$r = 0.96$ $s = 272.5$ $P<0.01$ $n = 20$

This equation is very satisfactory from a statistical point of view and explains 91.4% of variance. Only derivative **11a** is now an outlier and its removal affords Equation 3.

Equation 3: E = 34.8 (±1.9) %NO_2^- + 253.8 (±59.1)
$r = 0.98$ $s = 211.5$ $P<0.01$ $n = 19$

This equation is highly satisfactory and explains 95.1% of variance.

Discussion

The results described in the present study indicate that the furoxan compounds represent interesting prototypes of NO-donating pro-drugs characterized by significant biological activity.

The majority of the furoxan compounds studied possess vasodilator properties, with adequate efficacy to cause complete functional antagonism of the noradrenaline-induced vascular tone. Their vasodilator action cannot be ascribed to antagonism of the α_1-adrenoceptor, since a similar vasodilator profile was also evident in aortic ring depolarized by K^+ (data not shown). In addition, in a limited number of experiments, the vasodilator effect of furoxans was tested in vascular preparations in which the endothelium had been completely removed through rubbing of the intima, and found to be fully independent of endothelial integrity, as already reported for organic nitrates.

The mechanism of action of the furoxan compounds seems to be due to a thiol-dependent formation of NO, since no or minimal spontaneous formation of NO was observed after 1 h in absence of cysteine. Moreover, a high, statistically significant degree of correlation has been observed between the stimulation efficacy of the guanylate cyclase activity (E) and ability to release NO, for nineteen derivatives of the series. This is in line with what has been reported for the widely used nitrovasodilators, glyceryl trinitrate and sodium nitroprusside, which release nitric oxide on redox activation (Ignarro *et al.*, 1981; Kruszyna *et al.*, 1987). The high degree of statistical significance observed, might have been facilitated by the experimental model employed, based on the simple interaction between a substrate (NO) and a purified enzyme (guanylate cyclase). At the moment we have no evidence to explain the behaviour of four 'outliers' (**11a**, **11b**, **10a**, **8b**). A convincing argument to explain the behaviour of 4-nitro-3-phenylfuroxan **10a**, which displays a negative residue, is that this compound quickly undergoes nucleophilic substitution by action of a thiol group affording nitrites and a derivative similar to **4a**, both weak activators at the concentration used in the experiment. In effect we found that **10a** easily reacts with thiol derivatives giving 4-SR substituted products (Gasco *et al.*, unpublished results). No clear correlation was found when $1/EC_{50}$ values of the vasodilator activity of the different furoxans were plotted vs E or %NO_2^-. The considerable shift of the EC_{50} values for the vasodilator activity of the furoxan compounds, observed in the presence of methylene blue or oxyhaemoglobin, provides further sup-

port in favour of a NO-mediated, cyclic GMP-dependent mechanism of action.

Some selected furoxan compounds showing the highest potency as vasodilators, also inhibit collagen-induced, human platelet aggregation. This is consistent with the hypothesis that furoxan compounds, by releasing NO, share a common action mechanism both as antiaggregating agents and as vasodilators (Feelish *et al.*, 1992; Ghigo *et al.*, 1992).

Generally speaking as far as the nitric oxide release and the ability to activate the guanylate cyclase of the investigated furoxans are concerned, in the most active compounds ($\%NO_2^- > 10\%$, E pmol cyclic GMP min^{-1} mg^{-1} protein > 500) the 4-R substitution is more efficient than the corresponding 3-R substitution. Important substituents are CN, NO_2, OCH_3, $PhSO_2$, Cl, $CONH_2$ (in part) groups. Worthy of note is the high activity level of both the di-cyano and di-phenylsulphonyl substituted furoxans which suggest that furoxans, disubstituted with electron withdrawing moieties, should be very active compounds.

As pointed out before, as far as the vasodilator properties of the series are concerned, the structure-activity relationships are complex. The results we recently obtained in the study of NO-release by **11b** and other furoxan derivatives, under the action of thiophenol, suggest that this process is a complex pathway, strongly dependent on the nature of the substituent at the heterocyclic ring (work in progress).

Taken together our results indicate that furoxans represent a new class of compounds with an interesting potential as vasodilators and antiaggregating agents. The extensive structure-activity and -reactivity studies now in progress will hopefully clarify whether furoxans may provide a means to tailor NO lifetime and transport properties, and to elicit specific biological responses.

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