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Evaluation of the NO-releasing properties of NO-donor linkers

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Abstract

This work describes the synthesis of some benzoic (1–4) and alcoholic (5–7) nitrooxy derivatives, which are nitric oxide (NO) donors in themselves, and can also be seen as useful linkers that can be used in multi-target drugs capable of releasing NO. The NO-mediated vasorelaxing effects of the compounds were tested on endothelium-denuded isolated rat aortic rings pre-contracted with KCl. The pharmacological study of these compounds demonstrated that slight structural modification, such as the insertion of (a) methyl group(s) into the nitrooxymethyl chain or into the aromatic ring, and a change in the position of this nitrooxymethyl chain, could exert a marked (and potentially useful) influence on the NO releasing properties.

Introduction

The pharmacological treatment of many pathological states often requires the action of different and complementary pharmacodynamic mechanisms. In clinical practice, this is frequently achieved by the administration of 'cocktails' of drugs with different mechanisms of action. However, the benefits of this approach are often compromised by poor compliance, particularly in the treatment of asymptomatic chronic diseases such as hypertension (Eisen et al 1990).

In the last few years, the 'one drug–one target' paradigm has been shelved, and numerous multi-target drugs have been designed and synthesised. These compounds are designed to address a particular disease, with the goal of enhancing efficacy and/or improving safety compared with existing drugs. In this way, a single entity modulates multiple targets simultaneously by virtue of two (or more) pharmacodynamic properties resulting from the presence of overlapping or conjugated pharmacophores.

Compared with pharmacological cocktails, the use of a single multi-target drug presents certain advantages, such as easier prediction of pharmacokinetic/pharmacodynamic relationships, and improved compliance (Morphy & Rankovic 2006).

The release of nitric oxide (NO) is the mechanism of action underlying the pharmacological features and clinical applications of drugs such as the 'old' vasodilator nitrites and nitrates, reflecting the fundamental roles played by this small molecule in the cardiovascular system. A large number of important biological activities, principally connected with homeostasis of the cardiovascular system (Martelli et al 2006), are mediated by the production of endogenous NO, and could be mimicked by the administration of exogenous NO through NO-releasing drugs. Among these activities, the vasorelaxing effects are principally due to NO-induced activation of cytosolic guanylate cyclase in the vascular smooth muscle, with a consequent rise in the intracellular concentration of cGMP, and direct activation of potassium channels.

In addition to its vasorelaxing effects, NO controls platelet function (Furlong et al 1987; Radomski et al 1990). In particular, NO, which is produced by platelets as well as by endothelial cells, reduces platelet adhesion and aggregation. More recently, a possible role

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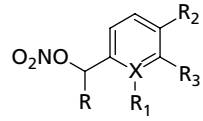
of NO in the process of ischaemic preconditioning has been debated extensively, and there is clear evidence that the administration of exogenous NO donors significantly reduces myocardial damage in ischaemia-injured hearts from different animal species (Nakano et al 2000; Qin et al 2004).

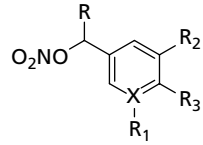
This type of NO-releasing ability has recently been identified as an interesting property to be added to molecules that already possess another fundamental pharmacodynamic effect. This strategy has led to the development of important multi-target drugs (Shaffer et al 1991; Minuz et al 1998; Villarroya et al 1999; Baraldi et al 2004; Breschi et al 2006) – interesting pharmacodynamic hybrids that combine a 'native' mechanism of action with NO donor activity, aiming to reduce possible side-effects (e.g. the gastric toxicity of aspirin) or to improve the therapeutic impact (e.g. to increase the antiplatelet activity of aspirin, or to increase the antihypertensive activity of the angiotensin receptor antagonists).

In order to obtain a multi-target drug possessing NO donor properties, it was important to conjugate the NO function to another drug with an easily cleavable group. The ester-based linker is a cleavable group which, as a result of the action of plasma esterases, is capable of releasing two individual drugs (NO and the native drug), which act independently.

In the last few years, many cleavable conjugates have been described (e.g. NO–aspirin, NO–sartans) (Shaffer et al 1991; Minuz et al 1998; Villarroya et al 1999; Baraldi et al 2004; Breschi et al 2006) containing an NO-releasing functionality linked via an ester group to a native drug. The chemical strategies usually employed to add NO donor properties to a known drug involved conjugation of the drug with different nitrogen-containing molecular portions. Among them, conjugation of a nitrooxy moiety, often via a molecular linker, probably represents the most convenient approach. In order to ensure appropriate conjugation between the NO-donor linker and a native drug, the nitrooxy group was inserted into different structures possessing carboxylic, phenolic or alcoholic functions suitable for forming a vulnerable bond such as the ester one. On this basis, and with the aim of studying the pharmacologic properties of these NO-donor structures and evaluating their use for building up new pharmacodynamic hybrids, the nitrooxymethyl-benzoic acids (**1**–**3**) and the 2-nitrooxymethyl-pyridinecarboxylic acid (**4**) have been synthesised; furthermore, as hydroxy-functionalised linkers the phenol derivative (**5**) and four nitrooxymethyl-benzylic alcohols (**6a**, **b** and **7a**,**b**) were also synthesised (Figure 1).



Compound	X	R	R_1	R_2	R_3
1a	C	H	H	H	COOH
2a	C	Me	H	H	COOH
3	C	H	Me	Me	COOH
4	N	H	–	H	COOH
5	C	H	H	H	OH
6a	C	H	H	H	CH_2OH
7a	C	Me	H	H	CH_2OH



Compound	X	R	R_1	R_2	R_3
1b	C	H	H	H	COOH
2b	C	Me	H	H	COOH
6b	C	H	H	H	CH_2OH
7b	C	Me	H	H	CH_2OH

Figure 1 NO-donor linkers with a carboxy (compounds **1a**, **b** and **4**), phenolic (compound **5**) or hydroxymethyl function (compounds **6a**, **6b**, **7a** and **7b**).

Materials and Methods

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. NMR spectra were obtained with a Varian Gemini 200 MHz spectrometer (Palo Alto, CA, USA). Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane and referenced from solvent references. Mass spectra were obtained on a Hewlett-Packard 5988 A spectrometer (Palo Alto, CA, USA) using a direct injection probe and an electron beam energy of 70 eV. The elemental compositions of the compounds agreed with the calculated values to within $\pm 0.4\%$. Chromatographic separation was performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck (NJ, USA) or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography on Merck aluminum silica gel (60 F254) sheets that were visualised under a UV lamp. Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was used as the drying agent.

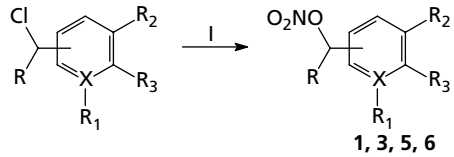
Synthesis

The synthesis of compounds **1**–**6** has been described previously (Breschi et al 2004, 2006). Figure 2 shows the general procedures used to obtain nitrooxy derivatives. Compounds **1**, **3**, **5** and **6** were obtained by reaction of the corresponding chloro-derivatives with silver nitrate in acetonitrile (method A, Figure 2). Compounds **2a**, **2b** and **4** were obtained by reaction with nitric acid and acetic anhydride at -10°C (method B, Figure 2). Compounds **7a** and **7b** were synthesised starting from the appropriate acetylbenzoic acids (**8a** and **8b**), which were reduced to the corresponding benzyl alcohols (**9a** and **9b**) with $LiAlH_4$ in THF (Scheme 1). Treatment of a solution of **9a** and **9b** in toluene with concentrated HCl afforded the monochloro derivatives **10a** and **10b**, which were submitted to reaction with $AgNO_3$ in CH_3CN to yield the corresponding nitro esters **7a** and **7b** (Figure 3).

*3-(2-Hydroxyethyl)benzyl alcohol (**9a**)*

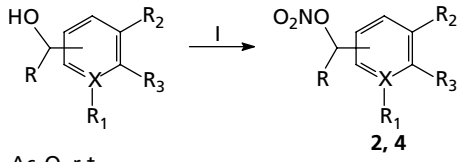
A solution of 3-acetylbenzoic acid (**8a**) (500 mg; 3.05 mmol) in THF (3 mL) was added to a solution of 1 M $LiAlH_4$ in THF

Method A



I: $AgNO_3$, CH_3CN, r.t.

Method B



I: HNO_3 conc., Ac_2O, r.t.

Figure 2 General methods for the synthesis of nitrooxymethyl derivatives starting from halogenated compounds (method A) or hydroxyalkyl compounds (method B).

(230 mg; 6.09 mmol) cooled to 0°C. The mixture was stirred at 0°C for 12 h, then water (1.7 mL) and 1 M NaOH (0.4 mL) were added, and the resulting suspension filtered. The solvent was evaporated to give **9a** as a yellow oil (432 mg, 2.84 mmol, 93% yield); ^{1}H NMR ($CDCl_3$) δ 1.46 (d, 3H, J = 6.5 Hz, CH_3); 4.63 (s, 2H, CH_2OH); 4.85 (q, 1H, J = 6.5 Hz, CH); 7.20–7.35 ppm (m, 4H, Ar). Anal. $C_9H_{12}O_2$ (C, H). Calcd: C, 71.03; H, 7.95. Found: C, 71.34; H, 7.64.

*4-(2-Hydroxyethyl)benzyl alcohol (**9b**)*
Compound **9b** was synthesised from **8b** (500 mg, 3.05 mmol) following the same procedure described above for the preparation of **9a**, to give **9b** as yellow oil (246 mg, 1.62 mmol, 53% yield); ^{1}H NMR ($CDCl_3$) δ 1.49 (d, 3H, J = 6.5 Hz, CH_3); 4.68 (s, 2H, CH_2OH); 4.90 (q, 1H, J = 6.5 Hz, CH); 7.32–7.40 ppm (m, 4H, Ar). Anal. $C_9H_{12}O_2$ (C, H). Calcd: C, 71.03; H, 7.95. Found: C, 71.05; H, 7.90.

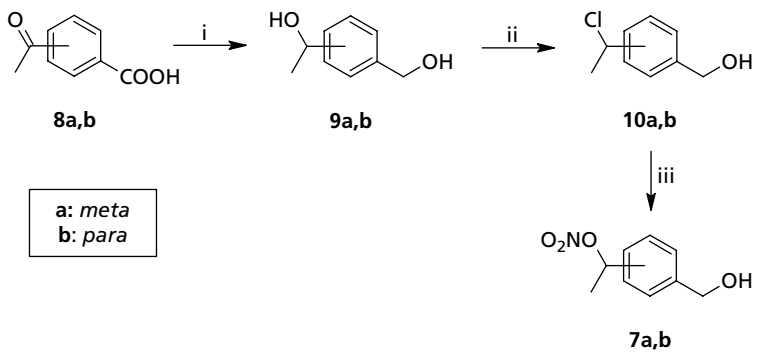
*3-(2-Chloroethyl)benzyl alcohol (**10a**)*
To a stirred suspension of compound **9a** (783 mg, 5.15 mmol) in toluene (26 mL), concentrated HCl (2.71 mL) was added at room temperature. The resulting solution was stirred for 12 h at room temperature. The solution was then washed with aqueous $NaHCO_3$ and water, the organic phase was separated and the aqueous layer extracted with CH_2Cl_2. The organic phase was dried and the solvent was evaporated to afford compound **10a** (583 mg, 3.42 mmol, 66% yield) as a colourless oil; ^{1}H NMR ($CDCl_3$) δ 1.85 (d, 3H, J = 6.9 Hz, CH_3); 4.72 (s, 2H, CH_2OH); 5.10 (q, 1H, J = 6.9 Hz, CH); 7.30–7.43 ppm (m, 4H, Ar). Anal. $C_9H_{11}OCl$ (C, H). Calcd: C, 63.35; H, 6.50. Found: C, 63.50; H, 6.24.

*4-(2-Chloroethyl)benzyl alcohol (**10b**)*
Compound **10b** was synthesised from compound **9b** (645 mg, 4.24 mmol) following the same procedure described above for the preparation of **10a**, to give **10b** (602 mg, 3.54 mmol, 83% yield) as a yellow oil; ^{1}H NMR ($CDCl_3$) δ1.86 (d, 3H, J = 6.7 Hz, CH_3); 4.71 (s, 2H, CH_2OH); 5.10 (q, 1H, J = 6.7 Hz, CH); 7.32–7.45 ppm (m, 4H, Ar). Anal. $C_9H_{11}OCl$ (C, H). Calcd: C, 63.35; H, 6.50. Found: C, 63.50; H, 6.24.

General procedure for preparation of nitro-esters

Method A
A solution of opportune chloride (3.42 mmol) in CH_3CN (9 mL) was added to a stirred solution of $AgNO_3$ (2.33 g, 13.69 mmol) in CH_3CN (5 mL). Stirring was continued over 4 h at room temperature in the dark. The precipitate (silver chloride) was then filtered off and the solvent evaporated. The crude product was triturated with $CHCl_3$ (20 mL) and filtered off to remove the unreacted silver nitrate and AgCl.

Method B
A solution of the opportune alcohol (2.0 mmol) in Ac_2O (2 mL) was added to a solution of HNO_3 (0.55 mL) and Ac_2O



i: $LiAlH_4$, THF, r.t.; **ii**: conc HCl, toluene, r.t., **iii**: $AgNO_3$, CH_3CN, r.t.

Figure 3 Process for the synthesis of compounds **7a**, **7b**, **8a**, **8b**, **9a**, **9b**, **10a** and **10b**.

(1.15 mL) cooled to −10°C. The mixture was stirred at −10°C for 2 h. The solvent was then evaporated to give a crude product (277 mg, 1.4 mmol, yield 69%) which was submitted to the subsequent reaction without any further purification.

*3-[(Nitrooxy)methyl]benzoic acid (**1a**)*
Compound **1a** was synthesised following the general procedure described in method A (Breschi et al 2004), to give compound **1a** (78% yield) as a white solid; mp 129–131°C; ^{1}H NMR ($CDCl_3$) δ 5.49 (s, 2H, CH_2ONO_2); 7.49–7.58 (m, 1H, Ar); 7.67 (d, 1H, J = 7.8 Hz, Ar); 8.14–8.17 (m, 2H, Ar); ^{13}C NMR ($CDCl_3$) δ 73.99 (CH_2ONO_2), {129.34, 130.18, 130.76, 131.29, 133.20, 134.19} (Ar–C), 171.08 (COOH). Anal. $C_8H_7NO_5$ (C, H, N): Calcd: C, 48.74; H, 3.58; N, 7.10. Found: C, 48.62; H, 3.73; N, 7.12.

*4-[(Nitrooxy)methyl]benzoic acid (**1b**)*
Compound **1b** was synthesised following the general procedure described in method A (Breschi et al 2006), to give compound **1b** (yield 54%) as a white solid; mp 144–146°C; ^{1}H NMR ($CDCl_3$) δ 5.50 (s, 2H, $CH4_2$); 7.50 (d, 2H, J = 8.2 Hz, Ar); 8.15 (d, 2H, J = 8.2 Hz, Ar); ^{13}C NMR ($CDCl_3\delta$ 73.72 (CH_2ONO_2), 128.65, 130.86, 138.27, 154.56 (Ar – C), 178.00 (COOH). MS (m/z) 197 (M^+, 3); 135 ($M^+ - ONO_2$, 46); Anal. $C_8H_7NO_5$ (C, H, N): Calcd: C, 48.74; H, 3.58; N, 7.10. Found: C, 48.45; H, 3.34; N, 7.42.

*2,6-Dimethyl-3-[(nitrooxy)methyl]benzoic acid (**3**)*
Compound **3** was synthesised following the general procedure described in method A (Breschi et al 2006), to give compound **3** (yield 90%) as a white solid; mp 123–125°C; ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 6H, CH_3) 5.47; (s, 2H, CH_2); 7.12 (d, 1H, J = 7.9 Hz, Ar); 7.33 (d, 1H, J = 7.8 Hz, Ar); ^{13}C NMR ($CDCl_3$) δ 16.40 (CH_3), 20.09 (CH_3), 73.19 (CH_2ONO_2), 128.23, 128.36, 132.20, 134.48, 134.88, 136.94 (Ar–C), 174.96 (COOH). Anal. $C_{10}H_{11}NO_5$ (C, H, N): Calcd: C, 53.33; H, 4.92; N, 6.22. Found: C, 50.04; H, 4.67; N, 6.06.

*3-(Hydroxymethyl)benzyl nitrate (**6a**)*
Compound **6a** was synthesised following the general procedure described in method A (Breschi et al 2006), and was obtained as a yellow oil (yield 91%); ^{1}H NMR ($CDCl_3$) δ 4.72 (s, 2H, CH_2OH); 5.43 (s, 2H, CH_2ONO_2); 7.32–7.44 (m, 4H, Ar); ^{13}C NMR ($CDCl_3$) δ 52.47 (CH_2OH), 73.12 (CH_2ONO_2), 129.13, 130.16, 130.64, 130.96, 132.82, 133.33 (Ar–C). Anal. $C_8H_9NO_4$ (C, H, N): Calcd: C, 52.46; H, 4.95; N, 7.65. Found: C, 52.37; H, 4.82; N, 7.72.

*4-(Hydroxymethyl)benzyl nitrate (**6b**)*
Compound **6b** was synthesised following the general procedure described in method A (Breschi et al 2006), giving compound **6b** (yield 74%) as a yellow oil; ^{1}H NMR ($CDCl_3$) δ 4.71 (s, 2H, CH_2OH); 5.42 (s, 2H, CH_2ONO_2); 7.35–7.44 (m, 4H, Ar); ^{13}C NMR ($CDCl_3$) δ 64.32 (CH_2OH), 73.19 (CH_2ONO_2), 126.79, 128.89, 131.00, 141.82 (Ar–C). Anal. $C_8H_9NO_4$ (C, H, N): Calcd: C, 52.46; H, 4.95; N, 7.65. Found: C, 52.52; H, 5.03; N, 7.28.

*1-[3-(Hydroxymethyl)phenyl]ethyl nitrate (**7a**)*
Compound **7a** was synthesised following the general procedure described in method A. The crude product was purified by column chromatography using hexane/AcOEt (7:3) as the eluent to give compound **7a** (227 mg, 1.15 mmol, yield 34%) as a yellow oil; ^{1}H NMR ($CDCl_3$) δ 1.64 (d, 3H, J = 6.6 Hz, CH_3); 4.73 (s, 2H, CH_2OH); 5.93 (q, 1H, J = 6.6 Hz, CH); 7.31–7.39 ppm (m, 4H, Ar). Anal. $C_9H_{11}NO_4$ (C, H, N): Calcd: C, 54.82; H, 5.62; N, 7.10. Found: C, 54.68; H, 5.73; N, 7.07.

*1-[4-(hydroxymethyl)phenyl]ethyl nitrate (**7b**)*
Compound **7b** was synthesised and purified as described for compound **7a**, giving compound **7b** (269 mg, 1.37 mmol, 39% yield) as a yellow oil; ^{1}H NMR ($CDCl_3$) δ 1.62 (d, 3H, J = 6.7 Hz, CH_3); 4.71 (s, 2H, CH_2OH); 5.93 (q, 1H, J = 6.7 Hz, CH); 7.31–7.42 ppm (m, 4H, Ar). Anal. $C_9H_{11}NO_4$ (C, H, N): Calcd: C, 54.82; H, 5.62; N, 7.10. Found: C, 54.74; H, 5.77; N, 7.35.

Pharmacological procedures

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609. The experimental protocol was approved by the Animal Care Committee of the University of Pisa.

In-vitro vascular protocols
The compounds were tested on isolated thoracic aortic rings from male normotensive Wistar rats (250–350 g). After a light ether anaesthesia, the rats were killed by cervical dislocation and exsanguination. The aortas were excised immediately and freed from extraneous tissues, and the endothelial layer was removed by gently rubbing the intimal surface with a hypodermic needle. Aortic rings (5 mm wide) were suspended under a preload of 2 g in 20 mL organ baths containing Tyrode solution (composition in mM: NaCl 136.8, KCl 2.95, $CaCl_2$ 1.80, $MgSO_4$ $7H_2O$ 1.05, NaH_2PO_4 0.41, $NaHCO_3$ 11.9, glucose 5.5), maintained at 37°C and gassed continuously with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded using an isometric transducer (Grass FTO3; West Warwick, RI, USA), connected to a preamplifier (Buxco Electronics; Wilmington, NC, USA) and to data acquisition software (MP 100, BIOPAC Systems Inc.; Goleta, CA, USA).

After an equilibration period of 60 min, endothelium removal was confirmed by the administration of acetylcholine (10 μM) to rings pre-contracted with 30 mM KCl. Relaxation that was less than 10% of the KCl-induced contraction was considered to be indicative of an acceptable lack of the endothelial layer; rings that showed relaxation of 10% or more were assumed to have significant amounts of endothelium remaining and were therefore discarded.

NO-mediated vasorelaxing effect: concentration–response curves
Aortic preparations were contracted with a single application of 30 mM KCl 30–40 min after confirmation of endothelium removal. When the contraction reached a stable plateau,

the test substance was added, in 3-fold increasing concentrations from 1 nM to 100 μM. Each aortic ring was used for only one concentration–response curve for a given test compound. Preliminary experiments showed that the KCl-induced contraction remained in a stable tonic state for at least 40 min.

The same experiments were carried out in the presence of the guanylate cyclase inhibitor, 1H-(1,2,4)-oxadiazolo-(4,3-a)-quinoxalin-1-one (ODQ; 1 μM), which was added to the aortic preparations after confirmation of endothelium removal.

Time course of vasorelaxing effect

Aortic preparations were contracted with a single application of 30mM KCl 30–40 min after confirmation of endothelium removal. Once a stable plateau contraction was reached, a single concentration (1μM, representing the mean of the pIC50 values (defined below) of all the test substances) of the compounds was added. The vasorelaxing effect of the added compounds was monitored for 50 min.

Data analysis

The vasorelaxing efficacy was expressed as the maximal vasorelaxing response as a percentage of the contractile tone induced by 30 mM KCl. The highest concentration of the test compounds that could be administered (100 μM) did not necessarily exert the maximal effect, in which case efficacy was expressed as the vasorelaxing response as a percentage of the contractile tone induced by 30 mM KCl evoked by this limit concentration. Potency was expressed as pIC50, calculated as the negative logarithm of the molar concentration of the test compound that evoked a 50% reduction of the contractile tone induced by 30 mM KCl. The pIC50 could not be calculated for compounds with efficacy below 50%. The above experimental data were obtained by a computer fitting procedure from the concentration–response curves using commercial software GraphPad Prism 4.0 (San Diego, CA, USA).

In order to describe the time course of the effects, the effect vs time curves were analysed using commercial software (GaphPad Prism 4.0) using the equation: $E = (EM \times T)/(Kt + T)$, where E is the vasorelaxing effect (expressed as a percentage of the contractile tone induced by 30 mM KCl) recorded at each min after administration of the test compounds (1 μM), EM is the maximum vasorelaxing effect (expressed as a percentage of the contractile tone induced by 30 mM KCl) induced by the test compounds (1 μM), T is the time (in min) correlated to a given E, Kt is the time (in min) required to reach $E = {}^1/_2EM$. This analysis allowed us to calculate the parameter of T25 (time, in min, required to reach an equi-effective level of vasorelaxing effect = 25% of the contractile tone induced by 30 mM KCl). This T25 parameter could not be calculated for compounds exhibiting an EM lower than or close to 25%.

The parameters of pIC50 and T25 are given as mean ± s.e. for 5–10 experiments, and were analysed statistically using one-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test. The concentration–response curves and the time–course curves were evaluated statistically using two-way ANOVA. *P* values below 0.05 were considered to be significant.

Results

Insertion of the nitrooxymethyl chain in the *meta* position (as in compounds **1a**, **2a**, **6a** and **7a**) or in the *para* position (as in compounds **1b**, **2b**, **6b** and **7b**) with respect to the carboxylic function (in the benzoic series) or to the hydroxymethyl function (in the benzylic series) was selected in order to investigate the potential influence played by steric and/or electronic factors on the NO-releasing properties of these compounds.

The nitrooxymethyl chain was also inserted into the aromatic ring (compound **3**) or into the carbon atom (compounds **2a** and **b** and **7a** and **b**) of one (or more) methyl group(s) in order to determine the potential influence of these groups on the NO-releasing kinetics of compounds **2**, **3** and **7**.

All the compounds synthesised were tested as vasodilator agents on rat aortic rings pre-contracted with a depolarising stimulus (30mM KCl). In this experimental model, all compounds exhibited full or almost full vasorelaxing activity. The potency (pIC50) and efficacy of the compounds tested and of reference drug sodium nitroprusside (SNP) are given in Table 1. The parameters describing the time-course profile of the vasorelaxing effect (T25 values), reflecting the NO release rates, are shown in Table 2.

In terms of potency, the pIC50 values were always lower than that of SNP (pIC50 = 8.73), and ranged between 4.83 (compound **7a**) and 7.21 (compound **6b**). The vasorelaxing effects of all the test compounds and of SNP were almost completely abolished by pre-incubation with ODQ (an

Table 1 The nitric oxide (NO)-mediated vasorelaxing efficacy of the synthesised compounds and sodium nitroprusside (SNP) was evaluated as the maximal vasorelaxing response, expressed as a percentage of the contractile tone induced by 30 mM KCl (% EM). Potency is given as the pIC50, calculated as the negative logarithm of the molar concentration of the test compounds evoking a 50% reduction in the contractile tone induced by 30 mM KCl. The pIC50 could not be calculated for compounds with efficacy below 50%. Values are given as mean ± s.e. for 5–10 experiments

Compound	pIC50	% EM
SNP	8.73 ± 0.04	100[a]
1a	5.80 ± 0.08	78 ± 9
1b	6.47 ± 0.02	100[a]
2a	5.64 ± 0.04	96 ± 2
2b	5.71 ± 0.02	100[a]
3	5.30 ± 0.03	98 ± 2
4	6.34 ± 0.02	100[a]
5	6.26 ± 0.05	100[a]
6a	7.03 ± 0.04	100[a]
6b	7.21 ± 0.03	100[a]
7a	4.83 ± 0.04	91 ± 1
7b	5.98 ± 0.02	100[a]

[a]These compounds produced 100% vasorelaxation in all the tests.

Table 2 Summary of the time-course profiles of the effects of the synthesised compounds and sodium nitroprusside. EM is the maximum vasorelaxing effect (expressed as a percentage of the contractile tone induced by 30 mM KCl) induced by the test compounds (1 μM). T25 is the time (in min) required to reach 25% of the vasorelaxation induced by 30 mM KCl. This parameter could not be calculated for compounds that had an EM value below 25%. Some compounds (indicated as ineffective) did not exhibit any significant vasorelaxing effect at 1 μM concentration. Values are expressed as means ± s.e. for 5–10 experiments

Compound	% EM	T25
SNP	98 ± 2	0.39 ± 0.08
1a	39 ± 8	8.25 ± 0.42
1b	65 ± 10	3.84 ± 0.48
2a	42 ± 2	8.83 ± 0.58
2b	50 ± 7	6.25 ± 0.21
3	Ineffective	–
4	49 ± 3	4.68 ± 0.33
5	73 ± 1	2.00 ± 0.27
6a	90 ± 1	0.48 ± 0.02
6b	90 ± 4	0.45 ± 0.03
7a	Ineffective	–
7b	26 ± 1	Not calculable

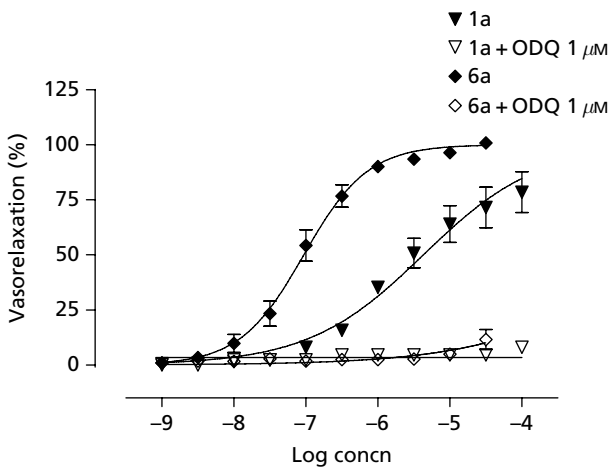


Figure 4 Representative examples of the effect of the guanylate cyclase inhibitor ODQ (1 μM) on the vasorelaxing effects of compounds **1a** and **6a**. The vasorelaxing efficacy is expressed as the maximal vasorelaxing response as a percentage of the contractile tone induced by 30 mM KCl.

inhibitor of guanylate cyclase and thus of the NO–cGMP pathway), indicating a clear involvement of NO release in their mechanism of action (Figure 4). Although the pIC50 value was clearly influenced by the rate of NO release, analysis that focused more closely on evaluating the development of the vasorelaxing effect over time (through the time-course protocol and the T25 parameter) seemed to be a more appropriate approach to describe this aspect. Indeed, the T25 parameter reflects the time needed to achieve a particular level of vasorelaxation (25% of the pre-contraction by KCl) induced by 1 μM of the compounds tested. Thus, the T25 parameter could be viewed as an indirect indicator of the rate of release of NO from the drug. As expected, SNP (a well-known rapid NO releasing agent) exhibited a very low T25 value (0.39 min) and two compounds (**6a** and **6b**) showed similar T25 values (0.48 min and 0.45 min, respectively). Compound **5** exhibited a slightly higher T25 value (2.00 min) than that of SNP, but the two values did not differ significantly. All the other compounds proved to be NO donor agents possessing a significantly lower rate of NO release, with T25 values ranging between 3.84 min (compound **1b**) and 8.83 min (compound **2a**); Compound **7b** gave a modest vasorelaxing effect (EM = 26%), which did not allow calculation of T25; compounds **3** and **7a** did not evoke any significant vasorelaxing effect, indicating that these two compounds were the slowest NO donors of the series.

Discussion

Although this preliminary study has focused on a limited number of lead compounds, some aspects of the structure–activity relationships can be hypothesised in terms of the influence of slight structural variations on the NO releasing properties of the nitrooxy group.

Considering the carboxylic derivatives, a comparison of compounds **1a** vs **1b** and **2a** vs **2b** showed that the *para* position ensured more effective NO release. In particular, compound **1b** was more potent than compound **1a**; also the T25 value of **1b** was significantly lower than that of **1a**, indicating a more rapid release of NO. Although **2a** and **2b** analogues showed almost equal potency and efficacy, release of NO was more rapid from **2b**, indicated by the significantly lower T25 value of compound **2a**. Insertion of a methyl group into the nitrooxymethyl chain did not seem to have any significant influence in the *meta*-substituted analogues **1a** and **2a**, and seemed to have a negative impact for the *para*-substituted analogues **1b** and **2b**: compound **2b** was less potent than compound **1b**, and, consistent with this, NO release was slower with this compound. Insertion of two methyl groups into the aromatic ring (compound **3**) led to a decrease in potency compared with compound **1a**, and, consistent with this, compound **3** (1 μM) did not possess any significant vasorelaxing effect in the time-course protocol. Replacement of the benzene ring of compound **1a** with a pyridine system (compound **4**), and replacement of its carboxy function with a hydroxy group (compound **5**), caused improvements in both the vasorelaxing potency and the rate of NO release. The benzylic derivatives **6a** and **6b** were the most potent of the compounds tested, and their NO release rates similar to that with SNP. In the benzylic series, the *para*-substituted compound **7b** was a more potent NO donor than the *meta*-substituted compound **7a**. However, the positive effect due to this structural feature was not evident for the analogues **6a** and **6b**, which had almost equal pIC50 and T25 values. The negative influence due to the insertion of the methyl group into the nitrooxymethyl chain observed in the benzoic series was even more evident in the benzylic compounds (compare compound **7a** vs **6a**, and **7b** vs **6b**). In particular, compound **7a** was the least potent of all the compounds tested, and did not exhibit any vasorelaxing effect in the time-course

protocol, while compound **7b** exhibited a poor vasorelaxing effect which did not allow evaluation of T25.

This study aimed to develop some representative NO-donor structures that might be useful as linkers to be added to native drugs, in order to obtain pharmacodynamic hybrids. The key aim with such hybrids is the presence of the two mechanisms of action; however, correct balancing of the two pharmacodynamic properties is another fundamental aspect. In particular, depending on the characteristics of the native drug (mechanism of action, posology, therapeutic indication), the release of NO should be correctly modulated in order to obtain well-calibrated levels of additive biological effects of NO itself. The availability of different NO-donor linkers possessing different NO release rates thus represent a necessary tool for this purpose.

The main findings of this work indicate that the shift of the nitrooxymethyl chain from the *meta* to the *para* position, in both benzoic and in benzylic derivatives, significantly influences the NO donor properties and achieves more effective release. Furthermore, the insertion of a methyl group into the nitrooxymethyl chain, as well as the presence of two methyl groups on the aromatic system, reduced the rate of NO release. It is reasonable to hypothesise that in a pharmacodynamic hybrid, the NO releasing rate of a given NO-donor linker might be influenced by the presence of other molecular portions (i.e. the native drug). However, this work indicates that variations in the structure of the NO-donor linkers described above ensure almost the same trend of NO-releasing properties as that shown by the hybrid compounds (Breschi et al 2004, 2006). This hypothesis seems to be confirmed by previous results concerning NO-releasing derivatives, in which losartan and its active metabolite EXP3174 were conjugated with some of the linkers described in this work (Breschi et al 2004, 2006), the NO-releasing properties of these hybrids were, in some cases, different from those exhibited by the corresponding NO-donor linkers alone.

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