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NO-glibenclamide derivatives: Prototypes of a new class of nitric oxide-releasing anti-diabetic drugs

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ABSTRACT

Endothelial dysfunction and consequent reduction of biosynthesis of endogenous nitric oxide (NO) play an important pathogenetic role in the cardiovascular complications associated with type II diabetes. In this work, the hybrid drugs **3a** and **3b**, nitrooxymethylbenzoate-derivatives of **1** (which is a hydroxylated active metabolite of glibenclamide **2**), are reported. The pharmacodynamic characterization of **3b** showed that its hypoglycaemic activity is enriched with additional NO-donor effects, conferring vasorelaxing and anti-platelet properties of potentially great usefulness for diabetes-related cardiovascular disorders.

1. Introduction

Type II diabetes mellitus is presently viewed as a multifactorial disease in which both metabolic and cardiovascular disorders co-exist and are somehow correlated, although the exact mechanisms of this possible interaction are not yet completely understood.[1] Really, the metabolic aspects of this complex disease are almost always associated with microvascular complications, leading to retinopathy, neuropathy and nephropathy. Also important macrovascular problems (myocardial ischemia, cerebrovascular accidents, hypertension, peripheral vasculopathy) accompain (and sometimes anticipate) type II diabetes[2–4] and really represent the main causes of mortality in diabetic patients.[5] To further remark the close correlation between cardiovascular and metabolic aspects in type II diabetes, it is widely observed that agents improving insulin sensitivity show also pressure lowering effects[6,7] and, conversely, antihypertensive drugs can lead to an improvement of insulin sensitivity in type II diabetic patients.[8]

The pathogenesis of diabetic cardiovascular complications seems to involve structural aspects, such as glycation of wall components of blood vessels, and functional aspects. The functional impairment, in particular, is substantially due to endothelial dysfunction, that is, the incapacity of vascular endothelium to play correctly its fundamental role of regulation of circulatory homeostasis, through biosynthesis and release of nitric oxide (NO) and other endothelial factors.[9] Such a reduced bioavailability of NO, with its pivotal properties (for example, the vasorelaxing and anti-platelet ones), synergizes with the metabolic alterations (dyslipidaemia, glycation end-products, oxidative stress), resulting in a dramatically increased incidence of atherosclerosis, blood hypercoagulability, vascular inflammation and remodeling, exacerbated vasospastic responses, hypertension, coronaropathy and stroke.[8,10]

Therefore, type II diabetes patients are frequently forced to follow multi-pharmacological therapeutic regimens composed by several drugs, targeted to glycaemic control on one hand, and to reduction of cardiovascular complications on the other hand. In particular, the usual anti-diabetics (biguanides, sulfonylureas, thiazolidinediones, etc.) are largely associated with drugs targeting hypertension (ACE-inhibitors, sartans, calcium channel blockers, etc.), dyslipidaemia (statins, fibrates, etc.) and/or platelet activation/aggregation (aspirin, clopidogrel, etc.).[8,11]

In the last years, the development of new pharmacodynamic hybrids or 'chimeras', that is, molecules showing two (or more) mechanisms of action, represented an encouraging field of research and furnished some interesting examples of drugs of potentially great usefulness, especially for complex cardiovascular diseases of multifactorial nature.[12] The addition of cleavable NO-releasing molecular portions to well-known 'old' drugs has been frequently

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used as a rational and versatile strategy, in order to enrich the fundamental pharmacological property of a given 'native' drug with the additional biological properties of NO.[13] This approach seemed to be particularly advantageous for pharmacological agents targeting type II diabetes. Therefore, in this work, we reported prototypical hybrid drugs, exhibiting the pharmacodynamic profiles of both hypoglycaemic agents and slow donors of exogenous NO. The sulfonylurea derivative 4-*trans*-hydroxyglibenclamide (**1**) was selected as 'native' molecule. Indeed, this hydroxy-derivative **1** is one of the two principal active metabolites of glibenclamide (**2**), a widely used insulin secretagogue sulfonylurea. Besides its biological effectiveness totally comparable with that of its precursor,[14,15] compound **1** exhibits a hydroxy group useful for bearing a cleavable ester moiety, obtained by condensation with suitable NO-releasing molecular portions (such as 3-nitrooxymethylbenzoic or 4-nitrooxymethylbenzoic acid). This paper reports the 3-nitrooxymethylbenzoate-derivative (**3a**) and 4-nitrooxymethylbenzoate-derivative (**3b**) of **1** (Fig. 1). Since preliminary studies showed that **3b** showed a vasorelaxing potency higher than **3a**, indicating a more satisfying NO-releasing effect, **3b** was selected for a first pharmacological characterization, aimed to identify the main pharmacodynamic features of the molecule.

2. Chemistry

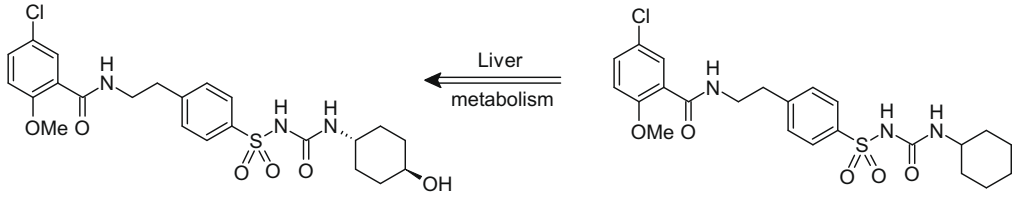
Compounds **3a** and **3b** were synthesized as indicated in Scheme 1, by condensation of the 4-*trans*-hydroxy-glibenclamide (**1**)[16] with the appropriate NO-donor linkers in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) and a catalytic amount of *N,N*-dimethylaminopyridine (DMAP). The nitrooxy esters were prepared as described in our previous work.[17]

3. Results and discussion

Since the fundamental issue for a pharmacodynamic hybrid is the retention of its main pharmacological profile, the first investigation was addressed to confirm the 'glibenclamide-like' hypoglycaemic activity and to compare it with the reference drugs, glibenclamide **2** and its metabolite **1**. As shown in Figure 2, the ip administration of **1** and **2** was followed by an evident reduction of non-fasting glycaemic levels in rats with streptozotocin-induced type II diabetes. In particular, the values of area under curve (AUC) of the glycaemic levels (expressed as mg/dl/2 h), relative to rats treated with vehicle, **1** and **2** were 45,318 ± 2308, 34,466 ± 912 and 33,113 ± 1156, respectively. The hypoglycaemic response recorded after ip administration of an equimolar dose of **3b** was almost completely equivalent to those evoked by the reference drugs (AUC = 36,683 ± 1208).

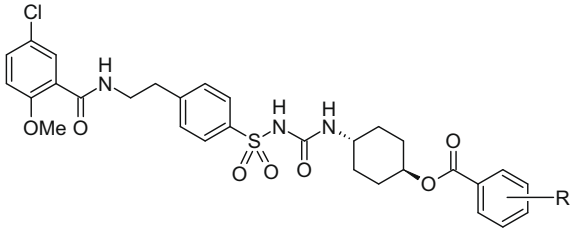
Furthermore, in order to confirm that the hypoglycaemic activity of **3b** was really due to the insulin secretagogue mechanism of action, typical of sulfonylureas, and to evaluate a potential usefulness of this pharmacological chimera for the human target, the investigation has been carried out also on human pancreatic islets hosting functional insulin-secreting beta-cells.[18,19] As shown in Figure 3, the challenge of pancreatic islets with a high glucose (20 mM) stimulus (i.e., the physiological secretagogue trigger) induced an increase of secretion of insulin, whose levels resulted about fivefold higher (insulin stimulation index SI = 4.9 ± 1.8) than those recorded in basal conditions (normal glucose, 3.3 mM). Consistent with its pharmacological role of insulin secretagogue, glibenclamide **2** (10 and 100 μM) caused a significant increase of insulin secretion in pancreatic islets exposed to normal glucose (SI = 4.3 ± 1.3 and 5.2 ± 1.9, respectively). Slightly improved responses were observed for **1**, which produced insulin SI of 6.0 ± 1.8 and 8.2 ± 2.2 (at 10 and 100 μM, respectively), further confirming that this is really a very active metabolite of glibenclamide (**2**). Compound **3b** exhibited an insulin secretagogue effectiveness which was completely comparable with that of **1** and slightly higher than that of **2**. The insulin SI parameters recorded in human pancreatic islets pre-treated with **3b** 10 and 100 μM were 6.3 ± 2.2 and 8.3 ± 3.6, respectively.

Once confirmed the retention of the above main pharmacological profile, the biological study was then addressed to evaluate and characterize the ancillary NO-releasing properties. The NO-releasing feature of **3b** were compared with that of glyceryltrinitrate (GTN). GTN was selected as reference NO-donor in this experimental protocol, because it is a well-known agent releasing NO with appreciable rate levels and mainly, because it is an organic ni-



1: 4-*trans*-hydroxy-glybenclamide

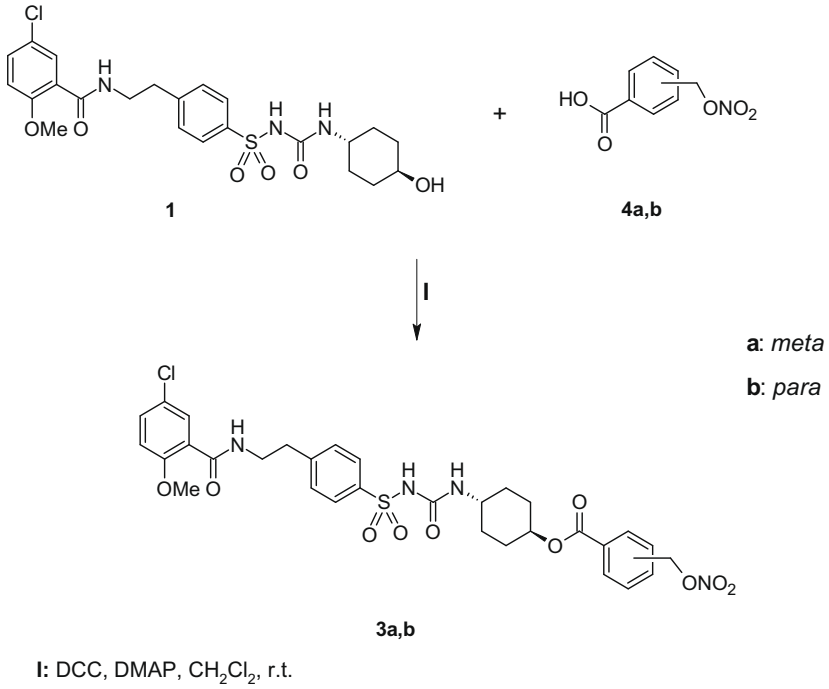
2: Glybenclamide



3a: R = 3-CH_2ONO_2

3b: R = 4-CH_2ONO_2

Figure 1. Chemical structures of glibenclamide (**2**), its hydroxylated active metabolite **1** and the two glibenclamide-related NO-releasing hybrids **3a** and **3b**.



Scheme 1. Synthetic route for the preparation of the two glibenclamide-related NO-releasing hybrids **3a** and **3b**.

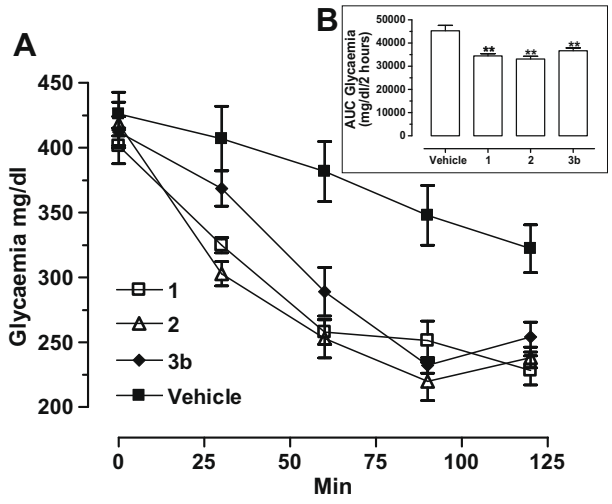


Figure 2. Hypoglycaemic effects of compounds **1**, **2** and **3b**. Panel A: Non-fasting glycaemic levels recorded in streptozotocin-induced diabetic rats after the intraperitoneal administration of vehicle, **1**, **2** or **3b**. Compound **2** and compound **1** were administered at a dose of 10 mg/kg, while **3b** was administered at the equimolar dose of 14 mg/kg. The corresponding levels of area under the curve (AUC) are also shown in the upper panel B.

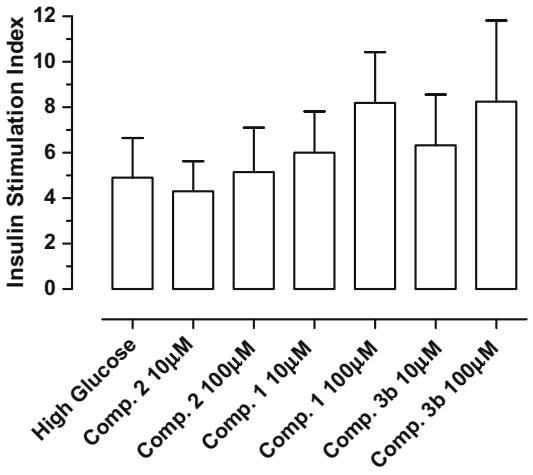


Figure 3. Insulin stimulation indexes recorded in human pancreatic islets after challenge with high glucose, **1**, **2** or **3b**.

trate (like **3b**), requiring bioactivation for the release of NO. The incubation of GTN in rat hepatic homogenate[20] led to the formation of NO (recorded by amperometric detection), indicating that these experimental conditions ensure an adequate environment for the bioactivation of organic nitrates. Also the incubation of **3b** in rat hepatic homogenate was followed by the formation of 'authentic' NO, which could be recorded by amperometric detection (Fig. 4). Since NO has a very short half-life (few seconds) and is rapidly metabolised with the formation of relatively stable end-products (such as nitrate and nitrite anions), it was not surprising that the 'steady state' concentrations of NO reached by GTN and **3b** (about 600 and 150 nM, respectively) were very lower

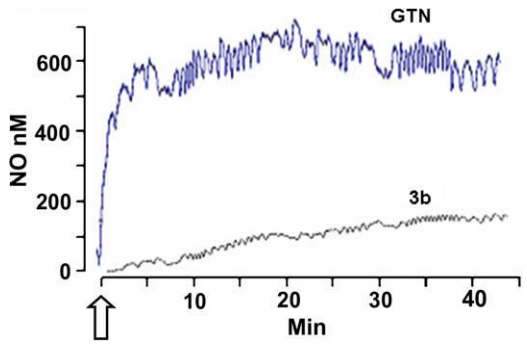


Figure 4. Representative amperometric tracing, showing the release of true NO from the reference drug glyceryltrinitrate (GTN) and from compound **3b**, both incubated in rat liver homogenate at the concentration 200 μM.

than the 'starting' concentrations. However, the comparison of the features of NO production from equimolar (200 μM) concentrations of **3b** or GTN allowed to observe that the formation of NO from the pharmacological chimera **3b** is a relatively slow process (preferable in this class of drugs[13]) and this remark is consistent with previous experimental observations on the same NO-releasing 'side chain'.[17,21,22] As expected, no release of NO was detected for **1** and **2**.

As concerns this complementary pharmacodynamic property, it is worthy to observe that such a modulated release of exogenous NO can induce a plethora of cardiovascular effects of potentially great usefulness in diabetes. Indeed, an enhancement of NO was linked to decreased endothelial dysfunction and vascular inflammation in diabetic rats; likewise, beneficial responses against diabetic endothelial dysfunction have been also associated with treatment with L-arginine (substrate of NO-synthase and precursor of NO).[23,24] In this study, in order to assess the real contribution of NO in the whole activity of these hybrids, the vasorelaxing and the anti-platelet effects were investigated, because of their direct and heavy implications in diabetic cardiovascular disorder.

Indeed, the impairment of endothelium-dependent vasodilator responses, mainly due to reduced levels of endogenous NO, is a well recognized characteristic of diabetic vascular disease.[25] Therefore, a possible NO-mediated vasorelaxing activity of **3b** was tested on isolated preparations of vascular smooth muscle. In particular, on endothelium-denuded rat aortic rings, this compound determined almost full vasorelaxing effects (E_{max} = 92 ± 5), with a pIC_{50} values of 6.75 ± 0.12. (Fig. 5). Full vasorelaxing effects were also induced by GTN (E_{max} = 98 ± 1), with higher levels of potency (pIC_{50} = 7.23 ± 0.07). As expected, sodium nitroprusside (SNP), selected as a rapid NO-donor, showed full vasorelaxing efficacy (E_{max} = 100 in all the experiments) and highest levels of potency (pIC_{50} = 8.73 ± 0.04). Compounds **1** and **2** were devoid of significant vasorelaxing effects. The vasorelaxing activity of **3a** was also assessed, showing lower levels of efficacy (E_{max} = 80 ± 2) and potency (pIC_{50} = 5.64 ± 0.05), reasonably related with a slower release of NO. This pharmacological behavior of the *meta* nitrooxymethyl derivative is in good agreement with data deriving from previous experimental investigations on other pharmacodynamic hybrids and on the NO-releasing characteristics of nitrooxymethyl benzoic acid-related side chains.[17,21,22]

In particular, the latter experimental work was specifically aimed to study a number of possible side chains, showing variable rates of NO-release. Among them, the two benzoic acid derivatives, bearing a nitrooxymethyl substituent in position *meta* or *para*, were identified as suitable side chains for the synthesis of NO-releasing hybrids. In fact, the two isomers were both endowed with modulated (but different) NO-releasing properties, but the *para* nitrooxymethyl derivative always showed a faster NO-donor rate.[17,21,22]

The implication of the typical NO-cGMP pathway in the vasodilator effects of **3b** was confirmed by observing that its responses were antagonised by ODQ (1*H*-[1,2,4] oxadiazolo [4,3-α] quinoxalin-1-one), inhibitor of guanilylcyclase (Fig. 5).

Finally, an impaired platelet function is typically recognised in diabetic patients, and increased platelet activation, aggregation and adhesion often precede (and, at least in part, contribute to) other cardiovascular complications.[26,27] Therefore, the anti-platelet effects potentially conferred by NO were tested turbidimetrically on human plasma. The strong aggregation induced by ADP in vehicle pre-treated platelets (73 ± 1%) was totally unaffected by **1** and **2** (76 ± 2% and 67 ± 3%, respectively), while it was significantly inhibited by **3b** and GTN (32 ± 4% and 28 ± 7%, respectively; Fig. 6).

4. Conclusions

In conclusion, this work reports the first examples of NO-anti-diabetic molecules, that is, hybrid drugs exhibiting both hypoglycaemic effects (due to the insulin secretagogue property, residing in the hydroxyglibenclamide portion) and further cardiovascular effects ensured by NO (released by the suitable side chains bearing the nitrooxy function). These NO-mediated cardiovascular properties can be viewed as a rational complementary pharmacodynamism and a precious enrichment for the anti-diabetic 'native' drug, since they might offer the possibility to correct both the glycaemic alteration and some of the most severe and dangerous cardiovascular complications of type II diabetes and, thus, to reduce the necessity of heterogeneous multi-pharmacological therapies. Therefore, these prototypical derivatives are useful templates for the potential development of the new pharmacological class of NO-releasing anti-diabetic drugs, obtained by chemical manipulation of the NO-donor portion and/or the native drug. Future pharmacological investigations will be also aimed to identify further probable beneficial effects due to NO, already observed for other classes of NO-releasing hybrids.[21,28] In particular, recovery of endothelial function and increased resistance of heart and other districts against ischaemia/reperfusion injury will be evaluated through appropriate experimental models.

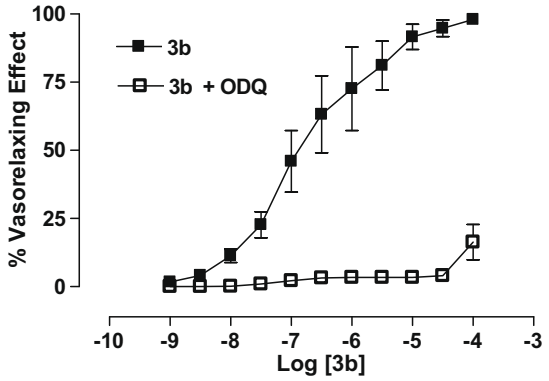


Figure 5. Concentration-vasorelaxing response curves for **3b** on vascular smooth muscle of rat aorta, observed in the absence or in the presence of ODQ (1 μM, inhibitor of guanilylcyclase).

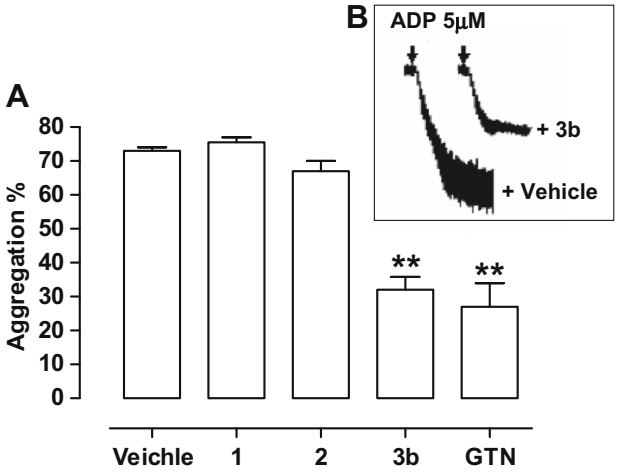


Figure 6. Panel A: The histograms represent the human platelet aggregating responses induced by ADP in the presence of vehicle, **1** (100 μM), **2** (100 μM), **3b** (100 μM) or GTN (100 μM). Panel B: representative turbidimetric tracing showing the aggregation induced by the addition of ADP 5 μM (arrows) in human platelet rich plasma pre-treated with vehicle or **3b** (100 μM).

5. Experimental

5.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. NMR spectra were obtained with a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references. Mass spectra were obtained on a Hewlett–Packard 5988 A spectrometer using a direct injection probe and an electron beam energy of 70 eV. The elemental compositions of the compounds agreed to within ±0.4% of the calculated value. Chromatographic separation was performed on silica gel columns by flash ((Kieselgel 40, 0.040–0.063 mm; Merck). Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum Silica Gel (60 F254) sheets that were visualized under a UV lamp. Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was always used as the drying agent. Commercially available chemicals were purchased from Sigma–Aldrich.

5.1.1. 3-[(Nitrooxy)methyl]benzoate of glibenclamide metabolite 3a

To a solution of glibenclamide metabolite **1**[16] (300 mg, 0.59 mmol) in CH_2Cl_2 (5 mL) and DMF (1 mL) was added 3-[(nitrooxy)methyl]benzoic acid **4a**[17] (116 mg, 0.59 mmol), DCC (146 mg, 0.71 mmol) and DMAP (6 mg). The resulting suspension was stirred at rt for 2 h, then was filtered and the solvent evaporated. The crude product was purified by column chromatographic eluting with $CHCl_3$/MeOH (95:5) to give **3a** (144 mg, 0.21 mmol, 35% yield) as a white solid: mp 70–72 °C. ^{1}H NMR ($CDCl_3$): δ 1.26–1.69 (m, 4H, cyclohexyl); 1.91–2.15 (m, 4H, cyclohexyl); 3.04 (t, 2H, J = 6.9 Hz, CH_2Ar); 3.65–3.78 (m, 3H, CH_2NH, CHNH); 3.82 (s, 3H, OMe); 4.90–5.00 (m, 1H, CHOCO); 5.47 (s, 2H, CH_2ONO_2); 6.46–6.34 (d, 1H, J = 7.7 Hz, NHCH); 6.89 (d, 1H, J = 8.8 Hz, Ar); 7.35–7.62 (m, 5H, Ar); 7.84–7.88 (m, 2H, Ar); 7.99–8.08 (m, 2H, Ar); 8.14–8.16 (m, 1H, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ 165.60; 164.21; 150.30; 147.04; 146.37; 138.07; 133.26; 133.10; 132.60; 132.15; 131.90; 130.81; 130.67; 130.02; 129.14; 127.36; 127.55; 122.70; 113.19; 74.14; 72.59; 56.55; 48.59; 40.78; 35.86; 30.40; 29.87. MS m/z: 688 (M^+ 88%). Anal. ($C_{31}H_{33}ClN_4SO_{10}$) C, H, N.

5.1.2. 4-[(Nitrooxy)methyl]benzoate of glibenclamide metabolite 3b

Compound **3b** was synthesized from glibenclamide metabolite **1**[16] (300 mg, 0.59 mmol) and 4-[(nitrooxy)methyl]benzoic acid **4b**[17] (116 mg, 0.59 mmol) following the same procedure described above for **3a** to give **3b** (131 mg, 0.19 mmol, 32% yield) as a white solid: mp 85–87 °C. ^{1}H NMR ($CDCl_3$): δ 1.10–2.04 (m, 8H, cyclohexyl); 3.02 (t, 2H, J = 7.6 Hz, CH_2Ar); 3.60–3.76 (m, 3H, CH_2NH, CHNH); 3.81 (s, 3H, OMe); 4.85–5.00 (m, 1H, CHOCO); 5.46 (s, 2H, CH_2ONO_2); 6.44 (d, 1H, J = 6.7 Hz, NHCH); 6.88 (d, 1H, J = 9.0 Hz, Ar); 7.35–7.46 (m, 5H, Ar); 7.87 (d, 2H, J = 8.2 Hz, Ar); 8.03 (d, 2H, J = 8.2 Hz, Ar); 8.13 (d, 1H, J = 2.7 Hz, Ar) ppm. ^{13}C NMR ($CDCl_3$): δ 169.18; 168.25; 165.34; 164.30; 156.05; 145.49; 137.32; 132.60; 131.87; 131.53; 130.14; 129.69; 128.52; 127.52; 126.88; 122.73; 113.10; 73.82; 72.59; 56.47; 48.26; 40.81; 35.75; 30.32; 29.83. MS m/z: 688 (M^+ 95%). Anal. ($C_{31}H_{33}ClN_4SO_{10}$) C, H, N.

5.1.3. 1-[4-[2-(5-Chloro-2-methoxybenzamido)ethyl]phenyl]-sulfonyl]-3-(*trans*-4-hydroxycyclohexyl) urea (glibenclamide metabolite) 1

Preparation of **1** and characterization data have been already reported.[16]

5.1.4. 3-[(Nitrooxy)methyl]benzoic acid 4a

Preparation of **4a** and characterization data have been already reported.[17]

5.1.5. 4-[(Nitrooxy)methyl]benzoic acid 4b

Preparation of **4b** and characterization data have been already reported.[17]

5.2. Pharmacology

Human samples were processed with the approval of our local Ethics Committee. All procedures on animals complied with the Guidelines of the European Community Council Directive 86/609, adopted by Italian law D.L. 116/92.

5.2.1. Diabetes induction and monitoring of the hypoglycaemic activity

Experiments were conducted on male Wistar rats (250–350 g) made diabetic (type 2 diabetes) using a single ip injection of Streptozotocin (STZ, Sigma–Aldrich) 50 mg/kg, dissolved in citrate buffer (pH 4.6). After five days, blood samples for measurement of glycaemia were taken from the tail vein and rats with blood glucose value <350 mg/dl were excluded from the study. Diabetics rats used for the study showed non-fasting glycaemia of 411 ± 9 mg/dl. Tested compounds and vehicle (DMSO, Riedel-de-Haën) were administered ip to diabetic rats. In particular, Glibenclamide (compound **2**, purchased from Sigma–Aldrich) and compound **1** were administered at a dose of 10 mg/kg, while **3b** was administered at the equimolar dose of 14 mg/kg. After the pharmacological treatments, blood samples were collected by puncture of tail vein and glycaemic levels were monitored for 2 h through the employ of commonly used sensors (New Glucocard G sensor and Glucocard Gmeter, Arkray, A. Menarini diagnostics).

5.2.2. Functional evaluation of on human pacreatic islets

Pancreata were obtained and processed with the approval of the local Ethics Committee, and islet isolation was performed by enzymatic digestion and density gradient purification. Handpicked islets were kept at 37 °C in a CO_2 incubator for 3–6 days before the experiments were performed, with the culture medium consisting of M199 medium, containing 5.5 mmol/l glucose, 10% (vol/vol) bovine serum and antibiotics. Insulin secretion studies were performed by the batch incubation technique, according to the procedure detailed elsewhere. Islet samples were kept at 37 °C for 45 min in Krebs-Ringer bicarbonate (KRB), 0.5% (vol/vol) albumin, pH 7.4, containing 3.3 mmol/l glucose (washing phase). Then, the medium was replaced with KRB containing 3.3 mmol/l glucose to assess basal insulin secretion during 45 min, after which the islets were challenged with 16.7 mmol/l glucose, or 3.3 mmol/l glucose plus 10 or 100 μmol/l of either glibenclamide (Compound **2**), its metabolite (Compound **1**) or the hybrid molecule **3b**. Insulin was quantified using an immunoradiometric assay (Pantec Forniture Biomediche, Turin, Italy). Insulin secretion results were expressed as stimulation index (SI, i.e., the release in response to a given stimulus over the basal release at 3.3 mmol/l glucose).

5.2.3. Nitric oxide amperometric detection

Adult male Wistar rats (250–300 g) were used to obtain the rat liver homogenate according some modifications of the procedures described by Kozlov et al.[20]

After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding, then the rat liver was removed to a beaker containing ice-cold sucrose buffer (K_2HPO_4 100 mM, EDTA 1 mM, KCl 1.15% p/v, Sucrose 0.25 M and EtOH 0.1%, pH 7,4 at rt), cut into small pieces and washed in order to remove the eventual blood residuals. After drying with paper, the weight of the li-

ver pieces was determined and the same buffer was added in a ratio of 1:6 liver/buffer (w/v). The liver was homogenized using Potter-Elvehjem homogenizer.

The obtained homogenate was filtrated through three layers of surgical gauze and maintained in ice during the time that precedes the experiment.

The experimental sample was composed by 2.5 ml of filtrated homogenate, 1 ml of re-suspension buffer (K_2HPO_4 100 mM and EDTA 0.1 mM, pH 7.4 at rt), 0.5 ml of glutathione 25 mM, 0.5 ml of NADPH 10 mM and NADH 10 mM. Glutathione, NADPH and NADH were dissolved in re-suspension buffer and maintained in ice before the experiment.

The experimental procedure was carried out at room temperature and under slow stirring.

After administration in rat hepatic homogenate, the release of NO from **3b** and Glyceryl Trinitrate (GTN, a rapid NO-donor used as the reference drug) 200 μM was monitored by amperometric detection using the NO-sensor tip (ISO-NOPF) and the APOLLO 4000 Free Radical Analyzer System as acquisition system.

5.2.4. Evaluation of the NO-mediated vasorelaxing properties

The effects of the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g). After a light ether anaesthesia, rats were sacrificed by cervical dislocation and bleeding.

The aortae were immediately excised, freed of extraneous tissues and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five mm wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with an unirecord microdynamometer (Buxco Electronics).

After an equilibration period of 60 min, the endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (30 mM)-precontracted vascular rings. A relaxation <10% of the KCl-induced contraction was considered representative of an acceptable lack of the endothelial layer, while the organs, showing a relaxation $\geqslant$ 10% (i.e., significant presence of the endothelium), were discarded. In the first series of experiments, we investigated the possible NO-releasing effect of the tested compounds.

From 30 to 40 min after the confirmation of the endothelium removal, the aortic preparations were contracted by a single concentration of KCl (30 mM) and when the contraction reached a stable *plateau*, threefold increasing concentrations of **3b** (1 nM–100 μM) were added.

Preliminary experiments showed that the KCl (30 mM)-induced contractions remained in a stable tonic state for at least 40 min.

The same experiments were carried out in the presence of a well-known guanylate-cyclase inhibitor: ODQ 1 μM which was incubated in aortic preparations after the endothelium removal confirmation.

5.2.4.1. Materials. Substances used in the experimental protocols were KCl (Carlo Erba) dissolved (3 M) in Tyrode solution, acetylcholine chloride (Sigma) dissolved (0.1 M) in EtOH 95% and further diluted in bidistillated water. ODQ (Sigma) was dissolved (1 mM) in EtOH 95% and further diluted in Tyrode solution. Compounds **3b, 2** and **1** were dissolved (10 mM) in DMSO and further diluted in Tyrode solution.

All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicles.

5.2.4.2. Data analysis. The vasorelaxing efficacy was evaluated as maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by KCl 30 mM. The parameter of potency was expressed as pIC_{50}, calculated as negative logarithm of the molar concentration of the tested compounds evoking a half reduction of the contractile tone induced by KCl 30 mM. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as mean ± standard error, for 5–10 experiments. Student *t*-test was selected as statistical analysis, $P < 0.05$ was considered representative of significant statistical differences. Experimental data were analyzed by a computer fitting procedure (software: GraphPad Prism 4.0).

5.2.5. In vitro anti-platelet activity

Human venous blood was obtained from healthy volunteers who had not taken any drug for at least two weeks. Volunteers were informed that blood samples were obtained for research purposes and that their privacy would be protected.

Platelet-rich plasma (PRP) was obtained by centrifugation at 80*g* for 40 min at room temperature. Then the top layer, PRP, was removed while the residual blood sample was centrifuged at 1550*g* for 25 min in order to obtain platelet-poor plasma (PPP). The PRP platelet number was adjusted at about 6×10^5 platelets/μL, through dilution with normal saline (NaCl 0.9%).

Platelets aggregation was determined, in diluted PRP, by an optical method, using a turbidimetric aggregometer (Elvi 840, Milan). In the aggregometer, contents of cuvettes (500 μL) were maintained at 37 °C and stirred constantly at 1000 rpm. Changes in light transmission through the diluted PRP and PPP were used for calibration, and represent minimum (0%, no aggregation) and maximum (100%, full aggregation) light transmission, respectively.

In preliminary experiments concentration–response curves to the aggregating agent (ADP) were obtained, and the 5 μM ADP concentration was selected. Aggregation % was recorded as increased light transmission after the addition of the aggregating stimulus and has been expressed as mean ± standard error, from groups of 10–12 samples (the samples of each group were collected from at least three different individuals). The inhibitory effects of compounds **1, 2, 3b**, GTN 100 μM) and vehicle on the ADP 5 μM-induced aggregation were evaluated. The tested compounds were added 2 min before the administration of ADP 5 μM. Control responses were obtained in the presence of drug vehicle only (DMSO). The final concentration of DMSO was 0.5%.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.06.049.

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