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Synthesis and preliminary biological profile of new NO-donor tolbutamide analogues

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ABSTRACT

We describe a new class of NO-donor hypoglycemic products obtained by joining tolbutamide, a typical hypoglycemic sulfonylurea, with a NO-donor moiety through a hard link. As NO-donors we chose either furoxan (1,2,5-oxadiazole 2-oxide) derivatives or the classical nitrooxy function. A preliminary biological characterization of these compounds, including stimulation of insulin release from cultured rat pancreatic β-cells and in vitro vasodilator and anti-aggregatory activities, is reported.

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia, with disorders of the carbohydrate, fat, and protein metabolism. It causes long-term complications in target organs, including retina, kidney, peripheral nerves, and the cardiovascular system. From the etiological standpoint, diabetes mellitus is caused by defects in the secretion and/or action of insulin. Two main types of diabetes are recognized: type 1 (T1DM), which is primarily due to pancreatic islet beta-cell autoimmune destruction, and typically causes ketoacidosis, and type 2 (T2DM), the commoner form of diabetes, which results from defects of insulin secretion and is almost always associated with the body cells' inability to adequately respond to insulin (insulin resistance).[1] Diabetes is now one of the most significant public health problems, due to a worldwide increase in this disease.[2] This increase foreshadows a rapid increase in chronic diabetic complications, including blindness, renal failure, peripheral neuropathy, and multiple athero-thrombotic lesions, involving coronary, cerebral, and peripheral arteries. There is general agreement that arterial damage is the most important factor involved in the high incidence of morbidity and reduced life-expectancy in diabetic patients. T2DM, in particular, is characterized by a complex profile of cardiovascular risk, due to the presence of hyperglycemia, dyslipidemia, arterial hypertension, endothelial dysfunction, and multiple hemostatic alterations.[3–5] It is recognized that impaired formation and/or availability of endothelium-derived nitric oxide (NO, EDRF) may be a crucial factor in the development of cardiovascular events in diabetes mellitus.[6] NO is an endogenous messenger, believed to play a significant role in the maintenance of micro- and macro-vascular homeostasis. It inhibits platelet adherence and aggregation, decreases leukocyte chemotaxis, promotes endothelial regeneration and angiogenesis, reduces vascular smooth-muscle cell (VSMC) constriction, migration and proliferation, and enhances VSMC apoptosis.[7] NO triggers a complex cascade of events in target cells, by activating soluble guanylate cyclase (sGC), which induces synthesis of 3,5-cyclic guanosine monophosphate (cGMP) leading to activation of the cGMP-dependent protein kinase PKG (NO/cGMP/PKG pathway).[8]

In T2DM, therapeutic guidelines recommend an aggressive multiple intervention policy, including hypoglycemic, hypotensive and anti-platelet aggregation drugs, in the setting of cardiovascular prevention. Thus also in diabetes, as for other complex diseases requiring the administration of multiple drugs, there is today great interest in the availability of hybrid drugs, known also as polyvalent or multifunctional drugs, which simultaneously modulate more than one target.[9,10] In particular, compounds deriving from the hybridization of a hypoglycemic drug with NO-donor moieties would appear to be of great interest for the treatment of T2DM. Compounds of this type have recently been characterized, namely NO-donor pro-drugs of the hydroxylated active metabolite of glibenclamide, a well-known hypoglycemic agent.[11] As a further

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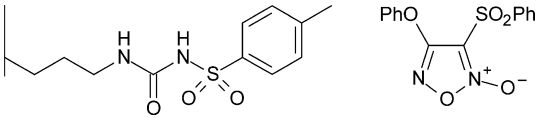
development of our work on NO-donor hybrid compounds, we here describe a new class of NO-donor hypoglycemic products, which are not co-drugs, obtained by joining tolbutamide **1** (Chart 1), a typical hypoglycemic sulfonylurea, with a number of NO-donor moieties (NO-donor tolbutamides), through a hard link. As NO-donors, we chose either furoxan (1,2,5-oxadiazole 2-oxide) derivatives, in view of their remarkable ability to induce anti-aggregatory effects[12] and vasodilation without inducing tolerance,[13] or the classical nitrooxy function (ONO_2). It is generally accepted that furoxans release NO in the presence of thiol co-factors,[14] while nitrooxy derivatives require enzymatic bioactivation to do so.[15] In order to modulate the amount of exogenous NO supplied by the constructs, we used either differently-substituted furoxan derivatives, or moieties containing one or two nitrate groups. The synthesis of these products is reported and discussed, together with a preliminary biological characterization, including the stimulation of insulin release from cultured rat pancreatic β-cells (INS-1E line) and in vitro vasodilator and anti-aggregatory activities. Since both inhibitory and stimulatory effects, on glucose-induced insulin secretion by INS-1E cells, have been ascribed to NO, the simple NO-donor substructures (see Schemes 4 and 5) present in these products were also tested, as controls.[16]

Synthesis of the target products and of the intermediates used for their preparation is summarized in Schemes 1–3. Briefly, the intermediate mononitrate **4** was obtained by action of the 4-hydroxybenzenesulfonamide (**2**) on the previously-described tosylate of 3-hydroxypropyl nitrate **3**, in the presence of NaOH (Scheme 1). The final mononitrate **5** was prepared by treating **4** with CuCl and butyl isocyanate in DMF. The dinitrate target compound **8** was synthesized in a similar manner, starting from dinitrate **7**, in turn obtained by action of iodine and $AgNO_3$ on **6**. The sulfonamide **6** is the product of the nucleophilic attack of the sodium salt of **2** on allyl bromide, in ethanol.

Nucleophilic attack of **2** on 3,4-bisphenylsulfonylfuroxan (**9**), in the presence of Na_2CO_3 in DMF, afforded the 3-phenylsulfonyl substituted furoxan **10** in a regioselective manner (Scheme 2).



1 30

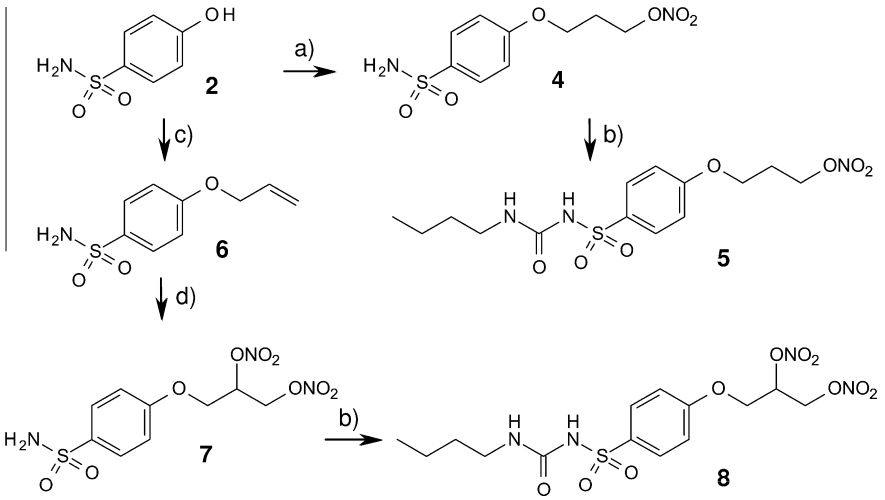
Chart 1. Structure of Tolbutamide (**1**) and of compound **30**.

The final furoxan **11** was obtained starting from **10**, following the procedure used to prepare the nitrates **5** and **8**. The 4-bromomethyl-3-phenylfuroxan **13** was obtained by action of $SOBr_2$ on the corresponding hydroxyderivative **12**. The 4-bromomethylfuroxans, appropriately substituted at the 3-position, were subjected to reaction with **2** in the presence of NaOH or Na_2CO_3, to give the intermediate solfonamides **16**–**18**. These products were transformed into the corresponding target compounds (**19**, **20**, **21**) via the usual reaction with CuCl and butyl isocyanate. The general procedure to prepare the target compounds **5**, **8**, **11**, **19**, **20**, **21** and their spectral characterization are reported in reference.[17]

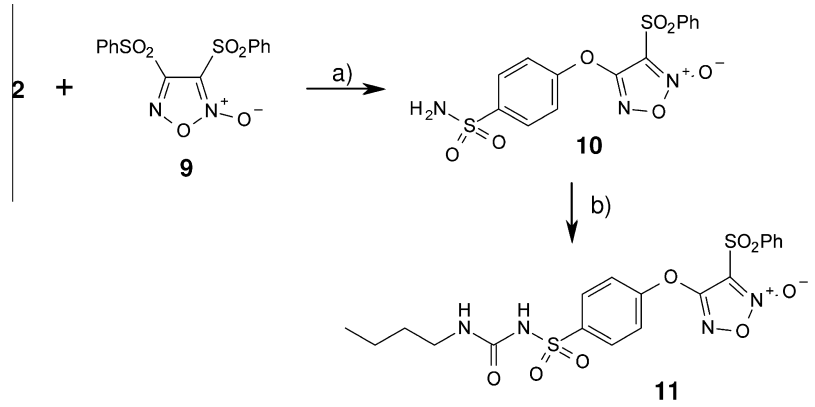
The simple substructures containing NO-donor nitrooxy functions **24**, **26**, used for control purposes, were obtained via the pathways outlined in Scheme 4. The mononitrate **24** was prepared under Mitsunobu conditions,[18] namely by treating the adduct of Ph_3P and diisopropylazodicarboxylate (DIAD) in THF solution with phenol **22**, followed by addition of the alcohol **23**. The dinitrate **26** was synthesized by treating a mixture of silver nitrate and (allyloxy)benzene (**25**) with iodine in acetonitrile at room temperature, followed by reflux. The simple NO-donor furoxans **27**–**29** were prepared by procedures reported in Scheme 5. Furoxans **27**, **28** were obtained from the related (bromomethyl)furoxans **13**, **14** through nucleophilic displacement of bromine by sodium salt of phenol. Finally, the cyano substituted furoxan **29** was prepared by trifluoroacetic anhydride dehydration of the parent amide **28** in THF solution, in the presence of pyridine. The 3-phenylsulfonyl substituted furoxan **30** (Chart 1) was synthesized as reported in the literature.[19]

The ability of the NO-donor hybrids to release NO was assessed by detecting nitrite, the principal final product of NO oxidative metabolism, using the Griess reaction. After incubation in rat liver homogenate, nitrates **5**, **8** generated nitrite (**8** > **5**). The furoxan-tolbutamides (**11**, **20**, **21**) produce nitrite when incubated with an excess of L-cysteine in buffer solution (pH = 7.4). Under these conditions, the NO release from compound **19** was undetectable. Parallel experiments, on INS-1E cells exposed to the NO-donor hybrids, showed a significant production of nitrite for compounds **8**, **11**, **19**, and **21** (see Supplementary data).

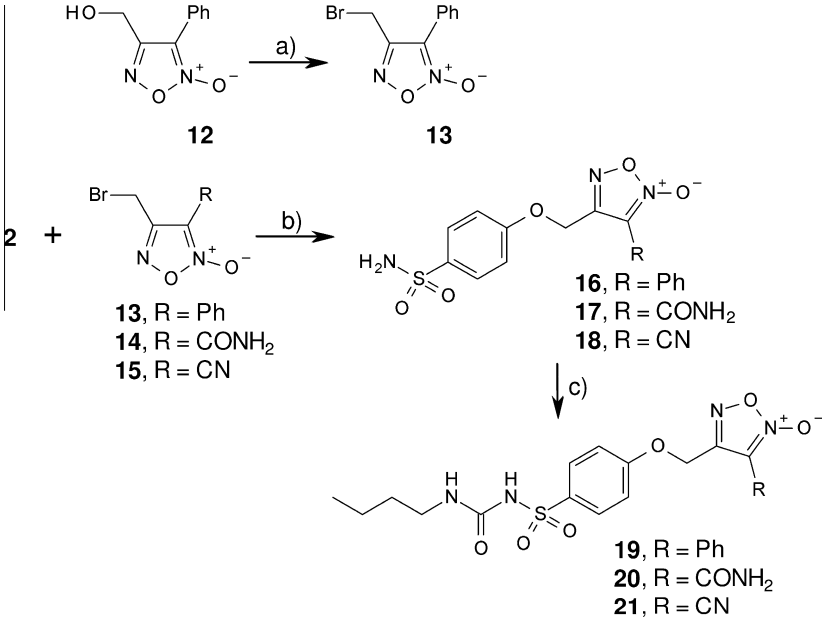
The ability of the synthesized products to supply exogenous NO to blood vessels was evaluated by assessing their capacity to induce relaxation of rat aorta strips pre-contracted with phenylephrine, following a procedure described elsewhere.[20] Both nitrate and furoxan derivatives of tolbutamide were found to determine relaxation of the contracted strips in a concentration dependent



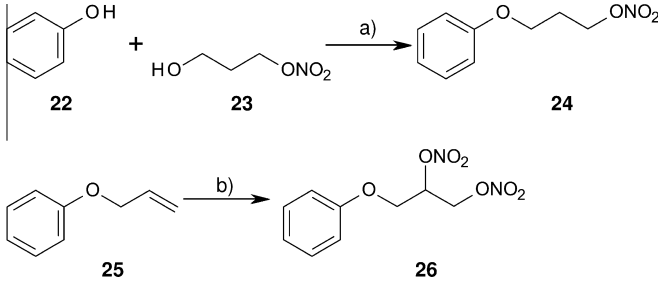
Scheme 1. (a) Tosylpropylnitrate (**3**), NaOH, EtOH; (b) CuCl, butylisocyanate, DMF; (c) allylbromide, NaOH, EtOH; (d) I_2, $AgNO_3$, CH_3CN, rt then reflux.



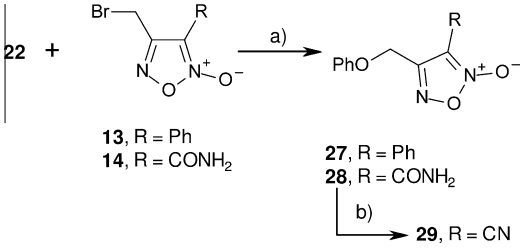
Scheme 2. (a) Na_2CO_3, DMF; (b) CuCl, butylisocyanate, DMF.



Scheme 3. (a) $SOBr_2$, DMF; (b) NaOH, EtOH for R = Ph, $CONH_2$; Na_2CO_3, DMF for R = CN; (c) CuCl, butylisocyanate, DMF.



Scheme 4. (a) Ph_3P, DIAD, THF; (b) I_2, $AgNO_3$, CH_3CN, rt then reflux.



Scheme 5. (a) Na, THF, 0 °C to rt; (b) $(CF_3CO)_2O$, Py, THF, 0 °C to rt.

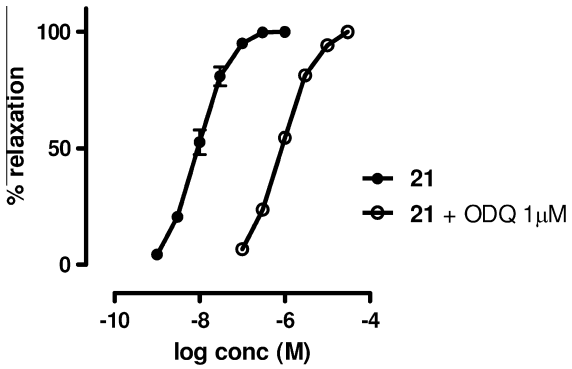


Figure 1. Concentration response curves for vasodilator activity of compound **21** in the absence and in the presence of ODQ.

manner, as reported for the example in Figure 1. The vasodilator potencies of the products, expressed as EC_{50}, are given in Table 1. When the experiments were repeated in the presence of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a well-known inhibitor of sGC, a significant reduction in the vasodilator effect of the products occurred (Table 1). This strongly supports the

Table 1
Anti-aggregatory and vasodilator activity of compounds **1**, **5**, **8**, **11**, **19**, **20**, **21**

Compound	Anti-aggregatory activity		Vasodilator activity
	IC_{50} (μM)(CL 95%) [+50 μM ODQ]	% inhibition ± SEM at 300 μM[a]	EC_{50} (μM) ± SEM [+1 μM ODQ]
1		6.4 ± 4.3	Inactive
5		1.1 ± 1.1	1.6 ± 0.2 [b]
8		5.6 ± 4.5	0.90 ± 0.10 [61 ± 16]
11	10 (8.9–12) [34 (32–37)]		0.0024 ± 0.0001 [0.22 ± 0.04]
19		13 ± 4	2.4 ± 0.2 [b]
20		5.2 ± 1.0	0.74 ± 0.09 [57 ± 1]
21	26 (22–30) [209 (188–232)]		0.010 ± 0.001 [0.90 ± 0.06]

[a] Due to the low activity of the compound, IC_{50} could not be calculated. In this case the percent of inhibition is reported at 300 μM.
[b] In the presence of 1 μM ODQ, EC_{50} values were >100 μM.

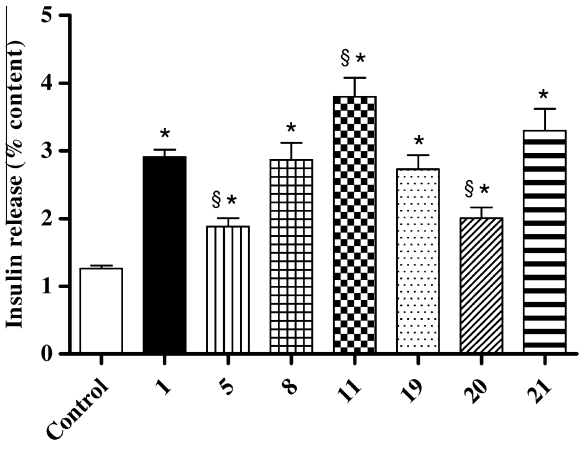


Figure 2. Insulin release from INS-1E cell line exposed to **1**, **5**, **8**, **11**, **19**, **20**, and **21** at 200 μM concentration in the presence of 2 mM glucose (control). Data are mean values ± SEM of 8–12 determinations for tolbutamide derivatives and 20–24 determinations for control and **1**. *p <0.05 at least versus control; §p<0.05 at least versus 1.

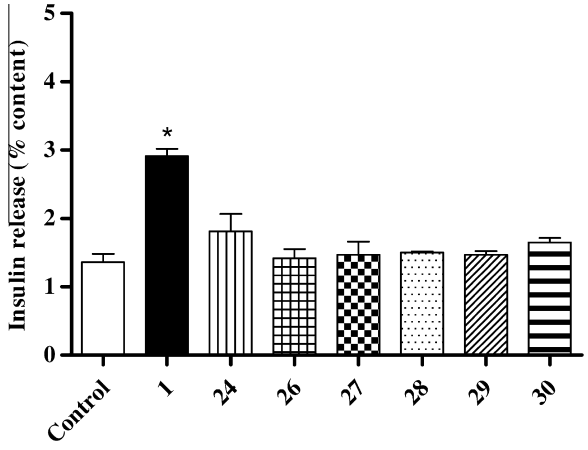


Figure 3. Insulin release from INS-1E cell line exposed to **1**, **24**, **26**, **27**, **28**, **29**, and **30** at 200 μM concentration in the presence of 2 mM glucose (control). Data are mean values ± SEM of 8–12 determinations for NO-donor derivatives and 20–24 determinations for (control) and **1**. *p <0.05 at least versus (control).

involvement of NO in the vasodilator action. Analysis of the data shows that the most potent vasodilator is the 3-phenylsulfonylfuroxan derivative **11**, which is active in the nM range, followed in order by the 3-CN substituted compound **21** and the 3-carbamoyl analogue **20**. The 3-phenylfuroxan derivative is the least potent vasodilator of the furoxan series, having an EC_{50} (2.4) in the low μM range. The potency ranking parallels the electron-withdrawing properties of substituents at the 3-position of the ring. This is in keeping with the hypothesis that NO release by the furoxan system is triggered by nucleophilic attack of a thiol cofactor at the 3-position of the ring.[14,21] The nitrates also display potencies in the low μM range, the di-ester **8** being somewhat more potent than the mono-ester **5**.

The anti-aggregatory effects of the compounds were evaluated by measuring collagen-induced platelet aggregation of human platelet-rich plasma (PRP), following a procedure reported elsewhere.[22] The results, expressed as either IC_{50} or, when IC_{50} could not be calculated, as the percentage of inhibition at the maximal concentration tested (300 μM), are in Table 1. Analysis of the data shows that the potent vasodilator hybrid furoxans **11** and **21** are also potent anti-aggregatory agents. This finding is in agreement with the hypothesis that platelets possess the appropriate machinery for inducing NO release from furoxans.[12] The involvement of NO in the anti-aggregatory effect is demonstrated by the substantial loss of this property when the experiments are carried out in the presence of ODQ. The low activity of the remaining two hybrid furoxans, **19** and **20**, is presumably due to their lower capacity to release NO, and in the case of **20** also to a hydrophilic–lipophilic balance that is not suitable to penetrate the platelets. The lack of antiaggregatory action displayed by hybrid nitric acid esters **5** and **8** is not surprising, in view of the established poor capacity of platelets to produce NO from nitrates.[23]

Tolbutamide is a well-known hypoglycemic drug belonging to the class of sulfonylureas, which act by stimulating insulin release from pancreatic β cells, through a specific interaction with the SUR-1 subunit of the ATP-dependent K^+ channel, leading to the channel's closure, beta-cell depolarization, opening of voltage-dependent Ca^{2+} channels, increased Ca^{2+} fluxes into beta cells,

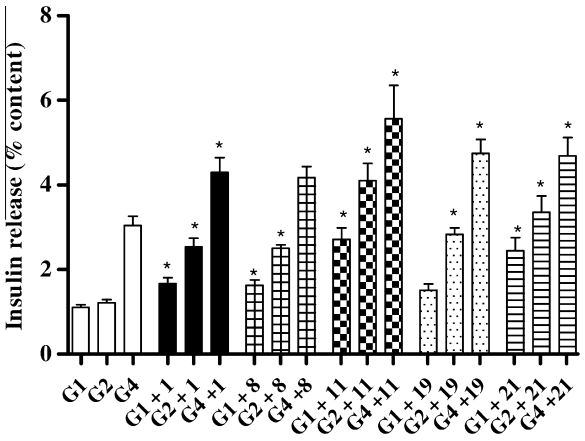


Figure 4. Insulin release from INS-1E cell line exposed to **1**, **8**, **11**, **19**, **21** at 200 μM concentration in the presence of different glucose concentrations (**G1**, **G2**, **G4**). Data are mean values ± SEM of at least 5 determinations for tolbutamide derivatives and 15–20 determinations for glucose alone and for **1**. $^{*}p$ <0.05 at least versus corresponding glucose concentrations.

and ultimately insulin secretion.[24] In order to verify whether the NO-donor tolbutamide analogues described here retain the insulin secretagogue properties of the native drug, we studied all the products, as well as the corresponding simple NO-donor substructures (i.e., the two nitric esters **24**, **26** and the four furoxans **27**–**30**), for their ability to stimulate insulin secretion from the well-differentiated rat pancreatic β cell line INS-1E, which releases insulin in a glucose-regulated manner.[25,26] INS-1E cells were incubated in KRB/Hepes buffer in the presence of various glucose concentrations and 200 μM of product; the results are in Figures 2, 3 and 4. Tolbutamide, which was tested as a reference compound, enhanced insulin release by approximately 2.5 times versus control cells (Fig. 2). Under the same conditions, all synthesized hybrid products likewise significantly increased insulin release versus 2 mM glucose, although to different extents. Some products increased insulin secretion as much as tolbutamide (namely compounds **8**, **19**, **21**) or to a greater extent (compound **11**), whereas others (compounds **5** and **20**), were less effective than tolbutamide. In parallel experiments we also tested some compounds at a concentration of 100 μM which, in most cases significantly increased insulin secretion with respect to the control, but to a lesser extent than the higher concentration (see Supplementary data).

As the simple NO-donor moieties (**24**, **26**–**30**), tested at 200 μM under the same experimental conditions, did not significantly increase insulin release (Fig. 3), it is reasonable to assume that the role of NO, if any, on glucose-induced insulin secretion from INS-1E cells by the NO-tolbutamides, must be negligible.

The hybrid NO-donor compounds showing insulin-secretory effects similar to or higher than that of tolbutamide, at 2 mM glucose, were also challenged at various glucose concentrations. As shown in Figure 4, the results substantially confirm that all NO-releasing compounds (i.e., **8**, **11**, **19**, **21**) exhibit patterns of insulin secretion similar to that of tolbutamide, characterized by an increasing effect depending on glucose concentrations up to 4 mM. It should be pointed out that when INS-1E cells are exposed to glucose concentrations higher than 4 mM (e.g., 5.6 or 11 mM), tolbutamide as well as its NO-donor derivatives are unable to further increase glucose-stimulated insulin secretion (see Supplementary data).

Thus, in most cases, the structural modifications induced, with the goal of obtaining tolbutamide analogues endowed with NO-releasing properties, do not hamper the compounds' effectiveness in terms of insulin secretion, although there may be some change with respect to tolbutamide.

One problem with hybrid drugs, in which the stoichiometry between the two pharmacophores present is necessarily 1:1, is that these pharmacophores must display their biological activities in the same concentration range. This is a difficult goal to achieve, and success can only be evaluated through in vivo experiments. The results of this in vitro study indicate that the most interesting products for in vivo testing are compounds **5**, **19**, **20** in which the tolbutamide pharmacophore is combined with substructures endowed with low/moderate NO dependent activity. Compounds **11** and **21** are too potent NO-donors to give rise to a balanced hybrid drug in vivo; however, a dedicated study will be necessary to confirm this, in view of the probable different pharmacokinetic properties of the products.

In conclusion, we successfully obtained new hybrid compounds, which are not co-drugs, that combine the insulin secretagogue properties of tolbutamide with the ability to restore endothelial function, due to their ability to activate the NO/cGMP/PKG pathway. The products were obtained by combining tolbutamide with NO-donor nitrooxy and furoxan moieties endowed with different NO-releasing capabilities. Compounds **5**, **19**, and **20**, in which the tolbutamide pharmacophore is combined with substructures that display low or moderate NO-dependent vasodilator activity, emerged as models for which additional in vivo studies would be worthwhile, as they are potentially useful as drugs to treat diabetes.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.103.

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17. To a solution of the appropriate benzensulfonamide derivative (1.06 mmol) in DMF (10 mL), stirred under inert atmosphere, butylisocyanate (0.24 mL, 2.17 mmol) and CuCl (0.01 mmol) were added. After 24 h the reaction mixture was poured into ice water (50 mL) under vigorous stirring and 2N HCl was added until the precipitation of a white solid that was filtered. Compound **5**: yield: 91%. M.p. = 101 °C (from EtOH/H_2O 1/1 v/v). ^{1}H-NMR (DMSO-d_6) δ 0.86 (3H, *t*, -$CH_2CH_2CH_2CH_3$), 1.10–1.34 (4H, *m*, -$CH_2CH_2CH_2CH_3$), 2.17 (2H, *t*, -$CH_2CH_2ONO_2$), 2.93 (2H, *q*, -$CH_2CH_2CH_2CH_3$), 4.16 (2H, *t*, -OCH_2-), 4.69 (2H, *t*, -CH_2ONO_2), 6.39–6.43 (1H, *m*,-$NHCH_2$-), 7.11 (2H, *d*, Arom), 7.82 (2H, *d*, Arom), 10.39 (1H, *s*, -SO_2NH-); ^{13}C NMR (DMSO-d_6) δ 13.4, 19.2, 26.0, 31.2, 38.7, 64.5, 70.7, 114.4, 129.4, 131.9, 151.2, 161.6; MS (CI) m/z 376 $(M+1)^+$. Compound **8**: yield: 89%. M.p. = 135 °C (from EtOH). ^{1}H NMR (DMSO-d_6) δ 0.81

(3H, *t*, -$CH_2CH_2CH_2CH_3$), 1.13–1.32 (4H, *m*, -$CH_2CH_2CH_2CH_3$), 2.93 (2H, *q*, -$CH_2CH_2CH_2CH_3$), 4.41–4.56 (2H, *m*, -OCH_2-), 4.91–5.10 (2H, *m*, -CH_2ONO_2), 5.81–5.85 (1H, *m*, -$CHONO_2$), 6.42 (1H, *t*, -$NHCH_2$-), 7.15 (2H, *d*, Arom), 7.86 (2H, *d*, Arom), 10.42 (1H, *s*, -SO_2NH-); ^{13}C NMR (DMSO-d_6) δ 14.0, 19.8, 31.7, 39.1, 65.9, 70.3, 78.2, 115.1, 130.0, 133.2, 151.8, 166.7; MS (CI) m/z 437 $(M+1)^+$. Compound **11**: yield: 15%. M.p. = 160 °C (from EtOH). ^{1}H NMR (DMSO-d_6) δ 0.80 (3H, *t*, -$CH_2CH_2CH_2CH_3$), 1.10–1.33 (4H, *m*, -$CH_2CH_2CH_2CH_3$), 2.95 (2H, *q*, -$CH_2CH_2CH_2CH_3$), 6.52–6.56 (1H, *m*, -$NHCH_2$-), 7.67 (2H, *d*, Arom), 7.76 (2H, *t*, Arom), 7.89–8.02 (5H, *m*, Arom), 10.67 (1H, *s*, -SO_2NH-); ^{13}C-NMR (DMSO-d_6) δ 13.8, 19.6, 31.5, 39.1, 111.7, 119.9, 128.9, 130.10, 130.2, 136.6, 137.0, 138.2, 151.5, 156.2, 157.6; MS (CI) m/z 497 $(M+1)^+$. Compound **19**: yield: 89%. M.p. = 156.5 °C (from EtOH/H_2O 1/1 v/v). ^{1}H NMR (DMSO-d_6) δ 0.81 (3H, *t*, -$CH_2CH_2CH_2CH_3$), 1.10–1.34 (4H, *m*, -$CH_2CH_2CH_2CH_3$), 2.93 (2H, *q*, -$CH_2CH_2CH_2CH_3$), 5.57 (2H, *s*, -OCH_2-), 6.44 (1H, *t*, -$NHCH_2$-), 7.23 (2H, *d*, Arom), 7.57–7.62 (3H, *m*, Arom), 7.83–7.86 (4H, *m*, Arom), 10.47 (1H, *s*, -SO_2NH-); ^{13}C NMR (DMSO-d_6) δ 13.4, 19.2, 31.2, 38.7, 61.3, 114.3, 115.0, 121.9, 127.6, 129.0, 129.4, 130.7, 133.2, 151.0, 153.7, 160.4; MS (CI) m/z 447 $(M+1)^+$. Compound **20**: yield: 27%. M.p. = 182 °C (from EtOH). ^{1}H NMR (DMSO-d_6) δ 0.90 (3H, *t*, -$CH_2CH_2CH_2CH_3$), 1.11–1.35 (4H, *m*, -$CH_2CH_2CH_2CH_3$), 2.94 (2H, *q*, -$CH_2CH_2CH_2CH_3$), 5.53 (2H, *s*, -OCH_2-), 6.43 (1H, *t*, -$NHCH_2$-), 7.24 (2H, *d*, Arom), 7.78 (1H, *s*, -SO_2NH-), 7.80 (2H, *d*, Arom), 8.50 (1H, *s br*, -$CONH_2$), 10.44 (1H, *s br*, -$CONH_2$); ^{13}C NMR (DMSO-d_6) δ 13.6, 19.3, 31.3, 38.7, 61.5, 110.5, 114.9, 129.5, 133.0, 151.4, 154.9, 155.7, 160.9; MS (CI) m/z 414 $(M+1)^+$. Compund **21**: yield: 86%. M.p. = 160 °C (from EtOH/H_2O 1/1 v/v). ^{1}H NMR (DMSO-d_6) δ 0.92 (3H, *t*, -$CH_2CH_2CH_2CH_3$), 1.11–1.34 (4H, *m*, -$CH_2CH_2CH_2CH_3$), 2.94 (2H, *q*, -$CH_2CH_2CH_2CH_3$), 5.62 (2H, *s*, -OCH_2-), 6.44 (1H, *t*, -$NHCH_2$-), 7.32 (2H, *d*, Arom), 7.92 (2H, *d*, Arom), 10.47 (1H, *s*, -SO_2NH-); ^{13}C NMR (DMSO-d_6) δ 13.4, 19.2, 31.2, 38.7, 60.8, 98.2, 106.2, 114. 9, 129.5, 133.4, 151.2, 154.4, 160.2; MS (CI) m/z 396 $(M+1)^+$.

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20. Thoracic aortas were isolated from male Wistar rats weighing 180–200 g (Harlan Italy Laboratories, S. Pietro al Natisone, Italy). 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; the vessels were helically cut and four to six strips were obtained from each aorta. The tissues were mounted under 1.0 g of tension in organ baths containing 30 mL of Krebs-bicarbonate buffer with the following composition: 111.2 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.0 mM KH_2PO_4, 12.0 mM $NaHCO_3$, and 11.1 mM glucose, maintained at 37 °C and gassed with 95:5% O_2/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} ± SEM (μM). The effects of 1 μM ODQ upon relaxation were evaluated in a separate series of experiments, in which ODQ was added to the organ bath 5 min before the contraction. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab. The addition of the drug vehicle (DMSO) had no appreciable effect on the contraction level. At least five experiments for each compound were perfomed.
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22. Venous blood samples were obtained from healthy volunteers who had not taken any drugs for at least 2 weeks. Volunteers, who were treated according to the Helsinki protocol for biomedical experimentation, gave their informed consent to the use of blood samples for research purposes. PRP was prepared by centrifugation of citrated blood at 210g 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 min after the addition of the stimulus. Collagen at submaximal concentrations (0.8–1.5 μg/mL) was used as the platelet activator in PRP. Compounds under study were preincubated with PRP 10 min before the 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 ODQ (50 μM). At least four experiments for each compound were performed. The anti-aggregatory activity of tested compounds is evaluated as percent inhibition of platelet aggregation compared to control samples. For most active compounds, IC_{50} values could be calculated by nonlinear regression analysis; otherwise, percent inhibition at the maximal concentration tested (300 μM) is reported.
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26. *Cell culture*: INS-1E cells were cultured in a humidified atmosphere containing 5% CO_2 in complete medium composed of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 50 μM 2 mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. The maintenance culture was passaged once a week by gentle trypsinisation.
Insulin secretion: The secretory response to glucose and other secretagogues was tested in INS-1E cells between passages 54 and 65. Cells were seeded in 24-well plates at a density of 4×10^4 cells/cm^2 in RPMI medium. After 48 h, medium was removed and cells were pre-incubated for 1 h at 37 °C in Krebs-Ringer bicarbonate buffer with HEPES (KRBH) containing 0.5% bovine serum albumin (BSA, fraction V, Sigma) and basal glucose concentrations (1 or 2 mM, according to the protocol). At the end of the pre-incubation time, cells were washed and then incubated for 1 h at 37 °C in 1 mL fresh KRBH buffer containing 0.5% BSA and suitable glucose concentrations, in the presence or absence of tolbutamide and its derivatives, used at concentrations of 100 or 200 μM. At the end of the incubation, the buffer was collected for insulin determination. Finally, 1 mL of cold acidified ethanol (150:47:3, v/v, absolute ethanol/H_2O/concentrated HCl) was added to the cells and left for 24 h in order to extract their insulin content.
Insulin was measured in the buffer and in the acid-ethanol extract by radioimmunoassay according to Herbert et al. (Herbert V.; Lau K. S.; Gottlied C.W.; Bleicher S. J. Coated charcoal immunoassay of insulin. *J. Clin. Endocrinol.* 1965, *25*, 1375), using rat insulin as a standard. The sensitivity and the coefficient of variation of the radioimmunoassay were as follows: detection limit 0.13 ng/mL, intra-assay variation 3.3%, inter-assay variation 10.5%.