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Original article

Structural and biological characterization of new hybrid drugs joining an HDAC inhibitor to different NO-donors.

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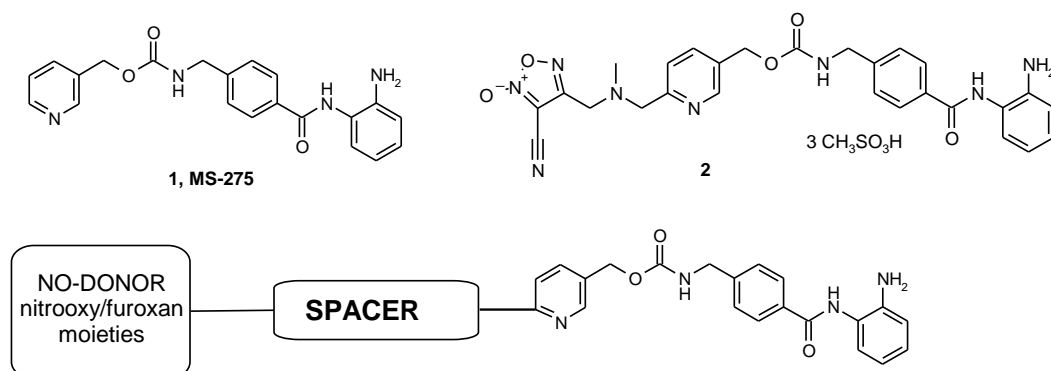
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Graphical abstract



Structure of MS-275 (1) and block structure of NO-donor MS-275 analogues.

Highlights

- Hybrid molecules bearing the selective class I HDAC inhibitor MS-275 and NO donors display a broad therapeutic potential.
- Dinitroxy compounds or furoxan derivatives are the most promising moieties to design this new class of hybrid drugs.
- The MS-275 coupled with the dinitroxy compound **31** showed exciting functional features during muscle differentiation.

Abstract

HDAC inhibitors and NO donors have already revealed independently their broad therapeutic potential in pathologic contexts. Here we further investigated the power of their combination in a single hybrid molecule. Nitrooxy groups or substituted furoxan derivatives were joined to the α -position of the pyridine ring of the selective class I HDAC inhibitor MS-275. Biochemical analysis showed that the association with the dinitrooxy compound **31** or the furoxan derivative **16** gives hybrid compounds the ability to preserve the single moiety activities. The two new hybrid molecules were then tested in a muscle differentiation assay. The hybrid compound bearing the moiety **31** promoted the formation of large myotubes characterized by highly multinucleated fibers, possibly due to a stimulation of myoblast fusion, as implicated by the strong induction of myomaker expression. Thanks to their unique biological features, these compounds may represent new therapeutic tools for cardiovascular, neuromuscular and inflammatory diseases.

Keywords: Histone deacetylases; nitric oxide; multitarget drugs; muscle differentiation; vasodilatation.

Introduction

Histone deacetylases (HDACs) are 18 enzymes grouped in four classes (HDAC I, II, III, IV), deputed to remove acetyl groups from lysine residues of histones and non-histone proteins. This epigenetic modification introduces positive charges in target proteins and prevents the introduction of other post-translation modifications (PTMs) including methylation, neddylation and ubiquitination.[1] In nucleosomes the presence of acetylated histones enables chromatin opening, DNA unwinding and consequent transcriptional activation. The removal of this PTM by HDACs leads to chromatin condensation and transcriptional silencing.[2,3] HDACs class I, II, IV are zinc-dependent metallo-proteins, whereas those belonging to class III, also named “sirtuins”, are NAD⁺-dependent enzymes. As HDACs induce structural changes into the chromatin that significantly alter cellular transcriptome, several pharmacologic tools have been generated so far to control their activity. Specifically, HDAC inhibitors (HDACi) have been developed for their ability to induce *in vitro* growth arrest, differentiation and/or apoptosis[4,5] as antitumor drugs.[6] In 2006, Vorinostat (suberoylanilide hydroxamic acid, SAHA), structurally similar to Trichostatin A (TSA), the first natural hydroxamic acid able to inhibit HDACs[7], has been approved by the food and drug administration (FDA) as HDAC inhibitor for the treatment of refractory cutaneous T-cell lymphoma.[8] Other more class-specific HDACi, are currently under phase II/III clinical trials as antitumor drugs.[6,9] The most recent drugs have been designed based on a pharmacophore model, where the crystal structure of the HDAC-like protein (HDLP), a homologue of mammalian class I/II HDACs, was complexed to SAHA and (*R*)- TSA.[10,11] This pharmacophore model, used to reveal the structure-activity relationships (SAR) of various inhibitor classes, contains a substructure able to bind the Zn²⁺ ion, present in the active site of the enzyme, a spacer, localized in the access channel to the active site, and a hydrophobic surface recognition group, which interacts with residues on the rim of the active site.[12] Very recent results show potential application of HDACi also in a number of non-cancer pathologies including inflammatory (eg. chronic obstructive pulmonary disease (COPD)), immunological (eg. asthma, rheumatoid arthritis),[13] neurological (eg. Alzheimer’s and Parkinson’s diseases), cardiovascular (eg. heart ischemia, heart failure, arrhythmia, cardiac hypertrophy)[14] and neuromuscular (eg. Duchenne and Becher’s muscular dystrophies)[15–18] diseases. Specifically, class I HDACs are strongly activated and highly expressed in muscular dystrophies and their inactivation by HDACi or specific siRNAs is able to slow the disease progression.[15–18] Specifically, HDACi induce bigger myofiber size and counteract the continuous activation of satellite cells typical of dystrophic muscles by activation of the follistatin. The outcome of the HDACi treatment is resistance to muscle function degeneration and reduction of inflammatory infiltrate in dystrophic disease models and in humans.[15,17,18] Another physiopathological event associated with muscle dystrophies is the

reduced nitric oxide (NO) availability, due to the altered relocation of the neuronal and endothelial NO synthase to the cytoplasm,[19] leading to overproduction of oxygen and nitrogen reactive species. NO, an endogenous gaseous second messenger plays a pivotal role in a large number of physiopathological contexts. Its role in the vascular system is very well characterized: it contributes to maintain micro- and macro-vascular homeostasis inducing vasodilation, inhibition of platelet aggregation, modulation of leukocyte adherence to vessels and inhibition of smooth muscle cells proliferation.[20,21] NO is an important second messenger also in immunological and muscular systems. In the immune system, it works as one of the final effectors of the immune response triggering antimicrobial, antiparasitic and tumoricidal actions,[22,23] being fundamental during the struggle against tumorigenesis and inflammation.[24,25] The anti-cancer action of NO is still under investigation: low doses may facilitate whereas high dosage seems counteracting cancer progression.[20-23] Indeed, high levels of NO exert cytostatic and/or cytotoxic effects on cancer cells by activation of pro-apoptotic signalling[26,27] and suppress metastasis by inhibition of epithelial-mesenchymal transition and reduction of invasive properties.[28,29] At the moment, NO donors are the only pharmacologic tools to reach the dosage necessary to exert both the anti-metastatic effect and the sensitization of cancer cells to chemotherapy and immunotherapy.[30–33] The high dose of NO necessary to achieve an anticancer effect, however, sets the limits for the use of these drugs in consequence of possible adverse systemic effects associated to pronounced vasodilation and toxic metabolites accumulation.[34] It is in this frame that the design of NO-hybrids could help to overcome clinical problems enhancing the specificity of NO action and minimizing its side effects. In skeletal muscles, NO modulates the contractile force of fast twitching fibres[35,36] and regulates regenerative processes of damaged muscles directly activating satellite cells, usually responsible of muscular regeneration.[37–39] NO donors showed beneficial effects in dystrophic muscles, thanks to their ability to slow down the disease progression.[40–45] In this context, the beneficial effect of NO donors has been also attributed to the property of NO to directly inhibit class I HDAC2 by both cysteine (S)-nitrosylation and tyrosine (Tyr)-nitration.[3] Indeed, dystrophic mice treated either with HDACi or NO donors showed overlapping rescuing features prompting us to develop a new class of hybrid drugs in which MS-275 (Entinostat; pyridin-3-ylmethyl 4-(2-aminophenylcarbamoyl)benzylcarbamate **1**), a selective class I HDAC inhibitor,[46] was joined through an appropriate spacer to different NO-donor substructures, according to the scheme reported in Figure 1. In our previous work [47] we already characterized the first example of this kind of hybrid compounds, here indicated as compound **2** (Figure 1), which simultaneously released NO and regulated HDACs. In the present work we used as spacers either the methyl-aminomethyl- or the oxyethyloxy group, according to the synthetic pathway adopted. Either moieties bearing nitrooxy

(ONO₂) groups or appropriately substituted furoxan (1,2,5-oxadiazole 2-oxide) derivatives were chosen as NO-donor substructures. It is generally accepted, in fact, that nitrooxy derivatives require enzymatic bio-activation to release NO, whereas furoxans require only thiol cofactors to do that.[48,49] Specifically, the NO-donor moiety was joined to the α -position of the pyridine ring present on the surface recognition region of MS-275. In this paper we report the synthesis, the structural characterization and the biochemical profile as HDAC inhibitors and as NO-donors of this new class of hybrid drugs.

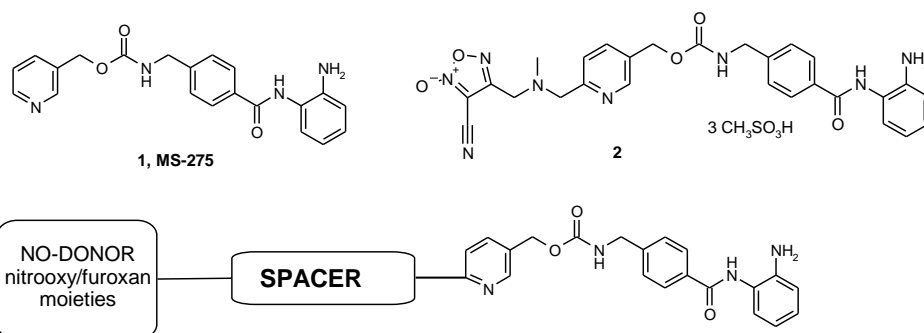


Figure 1. Structure of MS-275 (1) and block structure of NO-donor MS-275 analogues.

Material and methods

Compound synthesis. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance 300 at 300 and 75MHz, respectively, using SiMe₄ as the internal standard, and the following abbreviations were used to indicate the peak multiplicity: s = singlet, d = doublet, t = triplet, qt = quartet, qi = quintet, m = multiplet, br s = broad signal. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700 or with a Micromass Quattro microTM API (Waters Corporation, Milford, MA, USA). Melting points were determined with a capillary apparatus (Buchi 540). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM); PE stands for 40-60 petroleum ether. The progress of the reactions was followed by thin layer chromatography (TLC) on 5cm × 20cm plates with a layer thickness of 0.25mm. Anhydrous sodium sulfate was used as the drying agent for the organic phases. Organic solvents were removed under vacuum at 30°C. HPLC analyses were performed with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A), a diode-array detector (DAD) (model G1315B) integrated in the HP1100 system. Data analysis was done using a HP ChemStation system (Agilent Technologies). The analytical column was a Lichrospher C18 (4.6 × 250mm, 5 μ m) Merck. Preparative HPLC was performed on a Lichrospher C18 column (250mm × 25mm, 10 μ m) (Merck Darmstadt, Germany) with a Varian ProStar mod-210 with Varian UV detector mod-325. Highresolution mass spectra were recorded on a Bruker Bio Apex Fourier transform ion

cyclotron resonance (FT-ICR) mass spectrometer equipped with an Apollo I ESI source, a 4.7 T superconducting magnet, and a cylindrical infinity cell (Bruker Daltonics, Billerica, MA, USA).

Compounds **2**,^[47] **3**,^[50] **4**,^[51] **8**,^[52] **11**,^[53] **12**,^[53] **17**,^[47] **18**,^[54] **20**,^[55] 6-hydroxyhexane-1,2-diyl dinitrate ^[56] were synthesized according to literature.

6-[2-(*tert*-Butyldimethylsilanyloxy)ethoxy]nicotinic acid methyl ester (5**)**

To a stirred solution of PPh₃ (4.1 g, 15.6 mmol) in dry 1,4-dioxane (60 mL) under positive nitrogen pressure, DIAD (3.2 mL, 16.2 mmol) was added dropwise at 0°C. Stirring was continued for 15 minutes at room temperature (r.t.), then **3** (2.0 g, 13.1 mmol) was added. After 1h stirring at r.t., **4** (2.3 g, 13.1 mmol) was added and the mixture was stirred overnight. The reaction mixture was concentrated under reduced pressure. The resulting brown oil was purified by flash chromatography (eluent PE/EtOAc 99/1 then 90/10 v/v) to give the title compound as a white solid (30% yield). M.p. 48.5-49.5°C. ¹H-NMR (300 MHz, CDCl₃) δ = -0.08 (s, 6H, (CH₃)₂Si); 0.90 (s, 9H, (CH₃)₃CSi); 3.91 (s, 3H, COOCH₃); 3.97 (t, 2H, SiOCH₂CH₂O); 4.44 (t, 2H, SiOCH₂CH₂O); 6.78 (d, 1H, C₅H₃N); 8.14 (d, 1H, C₅H₃N); 8.80 (s, 1H, C₅H₃N). ¹³C-NMR (75 MHz, CDCl₃) δ = -5.2; 18.4; 25.9; 52.0; 61.7; 67.8; 110.9; 119.6; 139.5; 150.0; 165.9; 166.5. MS CI [M+H]⁺ *m/z* 312.

6-[2-(*tert*-Butyldimethylsilanyloxy)ethoxy]pyridin-3-ylmethanol (7**)**

To a suspension of LiAlH₄ (0.18 g, 4.80 mmol) in dry THF (30 mL), stirred under positive nitrogen pressure at -15°C, a solution of **5** (2.99 g, 9.60 mmol) in dry THF (20 mL) was added dropwise. After 2h, first H₂O (0.25 mL), second 15% NaOH (0.25 mL) and third H₂O (0.75 mL) were added. A rubbery white solid was filtered and washed with EtOAc (50 mL). The filtrate was washed with brine, dried, filtered and concentrated under reduced pressure. The yellow oil was purified by flash chromatography (eluent PE/EtOAc 80/20 v/v), to give the title compound as a pale yellow oil. (75% yield). ¹H-NMR (300 MHz, CDCl₃) δ = 0.08 (s, 6H, (CH₃)₂Si); 0.90 (s, 9H, (CH₃)₃CSi); 2.18 (br s, 1H, OH); 3.96 (t, 2H, SiOCH₂CH₂O); 4.36 (t, 2H, SiOCH₂CH₂O); 4.60 (s, 2H, CH₂OH); 6.75 (d, 1H, C₅H₃N); 7.59-7.63 (m, 1H, C₅H₃N); 8.06 (d, 1H, C₅H₃N). ¹³C-NMR (75 MHz, CDCl₃) δ = -5.2; 18.4; 25.9; 61.9; 62.5; 67.3; 111.2; 129.0; 138.5; 145.6; 163.5. MS CI [M+H]⁺ *m/z* 284.

General procedure for the preparation of Boc protected derivatives **9, **27-29**.**

To a suspension of CDI (0.20 g, 1.1 eq) in dry THF (20 mL) under positive nitrogen pressure, the appropriate alcohol (1.10 mmol) was added dropwise. The mixture was stirred at r.t. for 3h, then **8** (0.38 g, 1.10 mmol) was added. The mixture was stirred at r.t. overnight then was concentrated under reduced pressure and the residue was dissolved in CH₂Cl₂ (50 mL). The organic phase was washed twice with H₂O (30 mL) and brine (30 mL), dried, filtered and concentrated under reduced pressure.

The crude products so obtained were purified by flash chromatography to give compounds **9**, **27-29** as white foams. Chromatographic eluents and yields of the products were as follow.

{2-[4-((6-[2-(*tert*-Butyldimethylsilanyloxy)ethoxy]pyridin-3-ylmethoxycarbonylamino)methyl)benzoylamino]phenyl}carbamic acid *tert*-butyl ester (9**)**

Eluent: CH₂Cl₂/MeOH 95/5 v/v; 70% yield.

¹H-NMR (300 MHz, CDCl₃) δ = 0.08 (s, 6H, (CH₃)₂Si); 0.90 (s, 9H, (CH₃)₃CSi); 1.50 (s, 9H, (CH₃)₃CO); 3.96 (t, 2H, SiOCH₂CH₂O); 4.36-4.43 (m, 4H, SiOCH₂CH₂O + C₆H₄CH₂); 5.06 (s, 2H, C₅H₃NCH₂); 5.27 (br s, 1H, NH); 6.75 (d, 1H, C₅H₃N); 6.93 (s, 1H, C₆H₄); 7.12-7.35 (m, 5H, C₆H₄ + NH); 7.61 (d, 1H, C₆H₄); 7.76 (d, 1H, C₅H₃N); 7.90 (d, 2H, C₆H₄); 8.14 (s, 1H, C₅H₃N); 9.22 (br s, 1H, NH). ¹³C-NMR (75 MHz, CDCl₃) δ = -5.2; 18.4; 25.9; 28.3; 44.7; 61.8; 64.3; 67.3; 81.4; 111.2; 124.5; 124.8; 125.8; 126.0; 127.5; 127.8; 130.0; 130.8; 133.4; 139.4; 142.5; 147.1; 154.7; 156.4; 163.9; 165.3. MS CI [M+H]⁺ *m/z* 651.

(2-{4-[(6-[[Methyl-(3-nitrooxypropyl)amino]methyl]pyridin-3-ylmethoxycarbonylamino)methyl]benzoylamino}phenyl)carbamic acid *tert*-butyl ester (27**)**

Eluent: CH₂Cl₂/MeOH 98/2 v/v; 80% yield.

¹H-NMR (300 MHz, DMSO-d₆) δ = 1.45 (s, 9H, (CH₃)₃CO); 1.86 (qi, 2H, CH₂CH₂CH₂); 2.17 (s, 3H, CH₃); 2.45 (t, 2H, NCH₂); 3.60 (s, 2H, NCH₂Py); 4.30 (d, 2H, NHCH₂); 4.57 (t, 2H, CH₂ONO₂); 5.08 (s, 2H, CH₂O); 7.13-7.23 (m, 2H), 7.40-7.44 (m, 3H), 7.53-7.57 (m, 2H), 7.76 (d, 1H), 7.90-7.98 (m, 3H), 8.50 (s, 1H) (11 aromatic protons + 1H NH); 8.68 (s, 1H), 9.81 (s, 1H) (2H NH). ¹³C-NMR (75 MHz, DMSO-d₆): δ = 23.9; 27.9; 41.8; 43.5; 52.7; 62.7; 63.1; 72.1; 79.6; 122.2; 123.8; 124.0; 125.5; 125.9; 126.9; 127.6; 129.7; 130.8; 131.6; 132.8; 136.1; 143.6; 148.2; 153.4; 156.2; 158.7; 165.0. MS ESI [M+H]⁺ *m/z* 623.

(2-{4-[(6-[[5,6-Bisnitrooxyhexyl)methylamino]methyl]pyridin-3-ylmethoxycarbonylamino)methyl]benzoylamino}phenyl)carbamic acid *tert*-butyl ester (28**)**

Eluent: CH₂Cl₂/MeOH 98/2 v/v; 40% yield.

¹H-NMR (300 MHz, DMSO-d₆) δ = 1.39-1.49 (m, 13H, (CH₃)₃CO + 2 CH₂); 1.67-1.72 (m, 2H, CH₂) (NCH₂CH₂CH₂CH₂); 2.15 (s, 3H, CH₃); 2.37 (t, 2H, NCH₂); 3.59 (s, 2H, NCH₂Py); 4.29 (m, 2H, NHCH₂); 4.66-4.72 (m, 1H, CH_aH_bONO₂); 4.90-4.95 (m, 1H, CH_aH_bONO₂), 5.08 (s, 2H, CH₂O); 5.38-5.44 (m, 1H, CHONO₂); 7.15-7.20 (m, 2H), 7.39-7.45 (m, 3H), 7.52-7.56 (m, 2H), 7.74-7.77 (m, 1H), 7.89-7.95 (m, 3H), 8.49 (s, 1H) (11 aromatic protons + 1H NH); 8.67 (s, 1H, NH), 9.81 (s, 1H, NH). ¹³C-NMR (75 MHz, CDCl₃): δ = 22.6; 26.6; 28.3; 29.3; 42.5; 44.6; 56.9; 63.4; 64.2; 71.3; 79.3; 81.1; 123.0; 124.5; 124.8; 125.5; 125.7; 126.0; 127.3; 127.8; 130.5; 133.2; 136.5; 137.1; 142.7; 148.7; 156.5; 158.8; 159.0; 165.7. MS ESI [M+H]⁺ *m/z* 726.

(2-{4-[(6-[(3-Carbamoylfuroxan-4-ylmethyl)methylamino]methyl}pyridin-3-ylmethoxycarbonylamino)methyl]benzoylamino}phenyl)carbamic acid *tert*-butyl ester (29)

Eluent: CH₂Cl₂/MeOH 95/5 v/v; 70% yield.

¹H-NMR (300 MHz, DMSO-d₆) δ = 1.45 (s, 9H, (CH₃)₃CO); 2.24 (s, 3H, CH₃); 3.79 (s, 2H, NHCH₂Ph); 3.96 (s, 2H); 4.30 (m, 2H) (CH₂CH₃NCH₂); 5.10 (s, 2H, CH₂O); 7.13-7.23 (m, 2H, aromatic protons); 7.40-7.55 (m, 5H, aromatic protons); 7.77-7.98 (m, 4H, aromatic protons); 8.33 (br s, 1H, aromatic protons); 8.53 (br s, 2H, aromatic protons); 8.82 (br s, 1H, CONH₂); 9.82 (bs, 1H, CONH₂). ¹³C-NMR (75 MHz, DMSO-d₆) δ = 27.9; 41.2; 43.5; 51.7; 61.8; 63.0; 66.7; 79.6; 110.7; 122.6; 123.8; 124.0; 125.5; 125.9; 126.9; 127.6; 129.7; 131.0; 132.8; 136.3; 143.6; 148.5; 153.4; 155.2; 155.7; 156.2; 157.5; 165.0. MS ESI [M+H]⁺ m/z 661.

[2-(4-[(6-(2-Hydroxyethoxy)pyridin-3-ylmethoxycarbonylamino)methyl]benzoylamino)phenyl]carbamic acid *tert*-butyl ester (10)

To a solution of **9** (0.35 g, 0.50 mmol) in distilled THF (10 mL), 1M tetrabutylammonium fluoride in THF (0.78 mL, 0.70 mmol) was added. The solution was kept under stirring at r.t. for 3h, then it was poured in H₂O and extracted with EtOAc (2 × 30 mL). The organic phases were washed with brine, dried, filtered and concentrated under reduced pressure. The yellow oil was purified by flash chromatography (eluent CH₂Cl₂/iPrOH 99/1 v/v), to give the title compound as a white foam (65% yield). ¹H-NMR (300 MHz, CDCl₃) δ = 1.49 (s, 9H, (CH₃)₃CO); 3.91 (m, 2H, HOCH₂CH₂O); 4.38-4.43 (m, 4H, HOCH₂CH₂O + C₆H₄CH₂); 5.05 (s, 2H, C₅H₃NCH₂); 5.50 (br s, 1H, NH); 6.77 (d, 1H, C₅H₃N); 7.08-7.30 (m, 5H, C₆H₄ + NH); 7.62 (d, 1H, C₆H₄); 7.70 (d, 1H, C₅H₃N); 7.87 (d, 2H, C₆H₄); 8.09 (s, 1H, C₅H₃N); 9.28 (br s, 1H, NH). ¹³C-NMR (75 MHz, CDCl₃) δ = 28.3; 44.6; 62.3; 64.1; 68.9; 81.2; 111.3; 124.5; 125.4; 125.7; 126.0; 127.3; 127.8; 130.3; 130.6; 133.3; 139.9; 142.5; 146.6; 154.6; 156.4; 163.8; 165.5. MS CI [M+H]⁺ m/z 537.

General procedure for the preparation of BOC protected derivatives 13, 14.

To a solution of **10** (0.40 g, 0.70 mmol) in CH₂Cl₂ or CH₃CN (5 mL), the appropriate furoxan derivative (0.70 mmol) and DBU (0.22 mL, 1.4 mmol) were added. The mixture was kept under stirring at r.t. for 18h, then was poured in H₂O and extracted with CH₂Cl₂ (30 mL). The organic phase was washed with H₂O (2 × 20 mL) and brine, dried, filtered and concentrated under reduced pressure. The crude products so obtained were purified by flash chromatography to give compounds **13**, **14** as white foams. Chromatographic eluents and yields of the products were as follow.

{2-[4-[(6-[2-(3-Phenylfuroxan-4-yloxy)ethoxy]pyridin-3-ylmethoxycarbonylamino)methyl]benzoylamino]phenyl}carbamic acid *tert*-butyl ester (13)

Eluent: CH₂Cl₂/ MeOH 99/1 v/v; yield: 74% yield.

¹H-NMR (300 MHz, CDCl₃) δ = 1.51 (s, 9H, (CH₃)₃CO); 4.42-4.44 (m, 2H, FxOCH₂CH₂O); 4.83 (s, 4H, FxOCH₂CH₂O + C₆H₄CH₂); 5.08 (s, 2H, C₅H₃NCH₂); 5.17 (t, 1H, NH); 6.78-6.81 (m, 2H, C₅H₃N + aromatic protons); 7.14-7.23 (m, 4H, aromatic protons + NH); 7.36 (d, 2H, aromatic protons); 7.41-7.50 (m, 2H, aromatic protons); 7.64 (d, 1H, C₅H₃N); 7.80 (1d, H, aromatic protons); 7.92 (d, 2H, aromatic protons); 8.09-8.16 (m, 3H, aromatic protons + C₅H₃N); 9.18 (br s, 1H, NH). ¹³C-NMR (75 MHz, CDCl₃): δ = 28.3; 44.7; 63.1; 64.2; 69.1; 81.4; 107.7; 111.1; 122.3; 124.5; 124.8; 125.6; 125.7; 126.0; 126.2; 127.1; 127.4; 127.8; 128.8; 129.9; 130.4; 130.8; 133.4; 139.7; 142.4; 147.0; 147.2; 154.6; 156.3, 162.2; 163.1; 165.2. MS ESI [M+H]⁺ *m/z* 697.

{2-[4-({6-[2-(3-Phenylsulfonylfuroxan-4-yloxy)ethoxy]pyridin-3-ylmethoxycarbonylamino)methyl}benzoylamino)phenyl]carbamic acid *tert*-butyl ester (14)

Eluent: CH₂Cl₂/ MeOH 99/1 v/v; yield: 70% yield.

¹H-NMR (300 MHz, CDCl₃) δ = 1.49 (s, 9H, (CH₃)₃CO); 4.40 (m, 2H, FxOCH₂CH₂O); 4.74 (br s, 4H, FxOCH₂CH₂O + C₆H₄CH₂); 5.08 (s, 2H, C₅H₃NCH₂); 5.49 (t, 1H, NH); 6.76 (d, 1H, C₅H₃N); 7.10-7.19 (m, 3H, aromatic protons); 7.27-7.32 (m, 3H, aromatic protons); 7.54 (t, 2H, aromatic protons); 7.64-7.73 (m, 3H, aromatic protons); 7.88 (d, 2H, aromatic protons); 8.01 (d, 2H, aromatic protons); 8.15 (br s, 1H, C₅H₃N); 9.35 (bs, 1H, NH). ¹³C-NMR (75 MHz, CDCl₃): δ = 28.3; 44.7; 62.9; 64.4; 69.6; 81.3; 110.4; 111.0; 124.5; 125.7; 126.0; 127.4; 127.8; 128.5; 129.6; 130.2; 130.7; 131.7, 133.3, 135.6, 138.0; 139.7; 142.5; 147.0; 150.6; 154.6; 156.4; 158.8; 163.0; 165.4. MS ESI [M+H]⁺ *m/z* 761.

5,6-Bisnitrooxy-1-hexanmethansulfonate (19)

To a solution of 5,6-bisnitrooxy-1-hexanol (0.50 g, 2.20 mmol) in CH₂Cl₂ (20 mL), pyridine (0.36 mL, 4.40 mmol) was added. The mixture was stirred at 0°C and methansulfonylchloride (0.51 mL; 6.60 mmol) was added dropwise. The mixture was stirred at r.t. for 6h, then it was poured in H₂O and extracted with CH₂Cl₂ (3 × 20 mL). The organic phases were washed with 1M HCl (30 mL) and brine, dried, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (eluent PE/EtOAc 80/20 then 60/40 v/v), to give the title compound as a yellow oil (80% yield).

¹H-NMR (300 MHz, CDCl₃) δ = 1.54-1.71 (m, 4H), 1.78-1.88 (m, 4H) (OCH₂CH₂CH₂CH₂); 3.03 (s, 3H, CH₃); 4.26 (t, 2H, OCH₂CH₂); 4.47-4.53 (m, 1H, CH_aH_bONO₂), 4.75-4.80 (m, 1H, CH_aH_bONO₂); 5.27-5.34 (m, 1H, CHONO₂). ¹³C-NMR (75 MHz, CDCl₃) δ = 21.1; 28.7; 37.4; 69.0; 71.1; 78.9. MS CI [M+H]⁺ *m/z* 303.

General procedure for the preparation of alcohols 24-26.

To a solution of **17** (0.88 g, 3.30 mmol) in acetone (10 mL), the appropriate NO-donor moiety (3.30 mmol) and NaHCO₃ (0.28 g, 3.30 mmol) were added. The mixture was stirred at the appropriate temperature until the completion of the reaction (6-24h). The solvent was then evaporated under reduced pressure and the residue was dissolved in CH₂Cl₂ (50 mL). The organic phase was washed with water (2 × 30 mL) and brine, dried, filtered and concentrated under reduced pressure. Crude products (**21-23**) so obtained were partially purified by flash chromatography and use in the next synthetic step.

Appropriate *tert*-butyldimethylsilyl protected compound (1.1 mmol) was dissolved in CH₃COOH/H₂O/THF 3/1/1 v/v/v (15 mL). The mixture was kept under stirring at r.t. for 4 days. Then 2M NaOH was added to reach pH 8. The mixture was extracted with CH₂Cl₂ (3 × 30 mL). The organic phases were washed with H₂O (2 × 30 mL), brine, dried and concentrated under reduced pressure. Crude products so obtained were purified by flash chromatography to give compounds **24-26** as oils. Chromatographic eluents and yields of the products were as follow.

(6-[[Methyl-(3-nitrooxy-propyl)amino]methyl]pyridin-3-yl)methanol (24)

First step. 24h at r.t.; eluent: CH₂Cl₂/MeOH 99/1 v/v then 95/5 v/v; yield: 45%; pale yellow oil.

Second step. Eluent CH₂Cl₂/MeOH 95/5 v/v; yield 80%; yellow oil.

¹H-NMR (300 MHz, CDCl₃) δ = 1.90 (qi, 2H, CH₂CH₂CH₂); 2.23 (s, 3H, CH₃); 2.50 (t, 2H, NCH₂); 3.61 (s, 2H, NCH₂Py); 4.51 (t, 2H, CH₂ONO₂); 4.67 (s, 3H, CH₂O + OH); 7.38 (d, 1H, C₅H₃N); 7.68-7.72 (m, 1H, C₅H₃N); 8.40 (m, 1H, C₅H₃N). ¹³C-NMR (75 MHz, CDCl₃): δ = 24.6; 42.3; 53.1; 61.9; 63.3; 71.5; 123.0; 135.5; 135.7; 147.5; 157.8. MS CI [M+H]⁺ *m/z* 256.

(6-[[5,6-Bisnitrooxyhexyl)methylamino]methyl]pyridin-3-yl)methanol (25)

First step. 48h at 50°C; eluent: CH₂Cl₂ then CH₂Cl₂/MeOH 95/5 v/v; yield: 50%; yellow oil.

Second step. Eluent CH₂Cl₂/MeOH 95/5 v/v; yield: 65%; yellow oil.

¹H-NMR (300 MHz, CDCl₃) δ = 1.41-1.62 (m, 4H), 1.68-1.79 (m, 2H) (NCH₂CH₂CH₂CH₂); 2.24 (s, 3H, CH₃); 2.44 (t, 2H, NCH₂); 3.63 (s, 2H, NCH₂Py); 4.36 (br s, 1H, OH); 4.43-4.50 (m, 1H, CH_aH_bONO₂); 4.70 (s, 2H, CH₂O); 4.72-4.77 (m, 1H, CH_aH_bONO₂), 5.24-5.32 (m, 1H, CHONO₂); 7.38 (d, 1H, C₅H₃N); 7.67-7.71 (m, 1H, C₅H₃N); 8.41 (m, 1H, C₅H₃N). ¹³C-NMR (75 MHz, CDCl₃) δ = 22.6; 26.5; 29.0; 42.3; 56.8; 61.9; 63.2; 71.3; 79.3; 123.3; 135.5; 135.7; 147.6; 157.5. MS CI [M+H]⁺ *m/z* 359.

4-[(5-Hydroxymethylpyridin-2-yl)methyl)methylamino]methyl-3-furoxancarboxamide (26)

First step. 6h at r.t.; eluent: CH₂Cl₂/MeOH 99/1 v/v then 95/5 v/v; yield: 70%; pale yellow oil.

Second step. Eluent CH₂Cl₂/MeOH 99/1 v/v then 95/5 v/v; yield 80%; yellow oil.

¹H-NMR (300 MHz, CDCl₃) δ = 2.27 (s, 3H, CH₃); 3.81 (s, 2H, NCH₂Py); 3.98 (s, 2H, FxCH₂N); 4.56 (s, 2H, CH₂O); 5.35 (br s, 1H, OH); 7.41 (d, 1H, C₅H₃N); 7.74 (d, 1H, C₅H₃N); 8.36 (br s, 1H,

CONH₂); 8.49 (s, 1H, C₅H₃N), 8.63 (br s, 1H, CONH₂). ¹³C-NMR (75 MHz, CDCl₃): δ = 41.3, 51.8, 60.5, 62.0, 110.8, 122.6, 135.0, 136.1, 147.4, 155.3, 155.8, 156.2, . MS CI [M+H]⁺ *m/z* 294.

General procedure for the preparation of chloride salts 15, 16, 30-32.

To a solution of the appropriate Boc-protected derivatives (0.47 mmol) in dry 1,4-dioxane (4 mL), 5.30M HCl solution in dry 1,4-dioxane (0.50 mL) was added. The mixture was kept under stirring at r.t. until the completion of the reaction (6-24h). The solvent was removed and the obtained solid was triturated with dry CH₂Cl₂ until the formation of a white solid, which was filtered and dried to give compounds **15, 16, 30-32**. Crude products so obtained were purified, if necessary, by preparative HPLC. Chromatographic eluents and yields of the products were as follow.

[4-(2-Aminophenylcarbamoyl)benzyl]carbamic acid 6-[2-(3-phenylfuroxan-4-yloxy)ethoxy]pyridin-3-ylmethyl ester dihydrochloride (15)

Crude product was purified by preparative HPLC (eluent CH₃CN/H₂O/HCl 60/40/0.05 v/v/v, flow = 20 mL/min, λ = 226 nm, injected 100 mg in 2 mL of CH₃CN/H₂O/DMSO 50/50/0.01). M.p. 113°C (with decomposition). Yield: 68%.

¹H-NMR (300 MHz, DMSO-d₆) δ = 4.29 (d, 2H, FxOCH₂CH₂O); 4.79 (m, 4H, FxOCH₂CH₂O + C₆H₄CH₂); 5.02 (s, 2H, C₅H₃NCH₂); 6.91 (d, 1H, C₅H₃N); 7.34-7.65 (m, 10H, aromatic protons + NH₂); 7.77 (d, 1H, C₅H₃N); 7.94-7.99 (m, 3H, aromatic protons); 8.11 (d, 2H, aromatic protons); 8.21 (s, 1H, C₅H₃N); 10.61 (s, 1H, NH). ¹³C-NMR (75 MHz, DMSO-d₆) δ = 43.6; 62.9; 63.2; 69.5; 107.6; 110.7; 121.9; 124.4; 126.0; 126.1; 126.3; 126.5, 126.8; 127.3; 128.2; 128.5; 129.0; 130.7; 132.0; 132.1; 140.2; 144.0; 146.7; 156.4; 162.2; 162.6; 165.5. MS ESI [M+H]⁺ *m/z* 597.3. **ESI-HRMS [M+H]⁺: 597.2096, C₃₁H₂₉N₆O₇ requires 597.2092; HPLC purity > 95%.**

[4-(2-Aminophenylcarbamoyl)benzyl]carbamic acid 6-[2-(3-phenylsulfonylfuroxan-4-yloxy)ethoxy]pyridin-3-ylmethyl ester dihydrochloride (16)

Crude product was purified by preparative HPLC (eluent CH₃CN/H₂O/HCl 30/70/0.05 v/v/v, flow = 20 mL/min, λ = 226 nm, injected 100 mg in 2 mL of CH₃CN/H₂O/DMSO 50/50/0.01). M.p. 130°C (with decomposition). Yield: 30%.

¹H-NMR (300 MHz, DMSO-d₆) δ = 4.27 (m, 2H, FxOCH₂CH₂O); 4.64 – 5.04 (m, 8H, 3CH₂ + NH₂); 5.03 (s, 2H, C₅H₃NCH₂); 5.77 (s, 1H, NH); 6.60 (m, 1H, aromatic proton); 6.77-6.97 (m, 3H, aromatic protons); 7.16 (d, 1H, aromatic proton); 7.37 (d, 2H, aromatic protons); 7.66-7.95 (m, 9H, aromatic protons); 8.21 (s, 1H, NH); 9.63 (s, 1H, NH). ¹³C-NMR (75 MHz, DMSO-d₆) δ = 43.6; 62.9; 63.1; 69.8; 110.5; 110.6; 116.1; 116.2; 123.3; 126.2; 126.7; 126.8; 127.8, 128.2; 130.0; 133.2; 136.1; 137.2; 140.1; 143.1; 146.8; 156.4; 158.8; 162.5; 165.1. MS ESI [M+H]⁺ *m/z* 661.2. **ESI-HRMS [M+H]⁺: 661.1714, C₃₁H₂₉N₆O₉S requires 661.1711.2092; HPLC purity > 95%.**

[4-(2-Aminophenylcarbamoyl)benzyl]carbamic acid 6-[[methyl-(3-nitrooxypropyl)amino]methyl]pyridin-3-ylmethyl ester trihydrochloride (30)

Pale yellow foam; yield: 80%.

$^1\text{H-NMR}$ (300 MHz, DMSO- d_6) δ = 2.23 (qi, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$); 2.76 (s, 3H, CH_3); 3.22 (t, 2H, NCH_2CH_2); 4.30 (d, 2H, NHCH_2); 4.50 (s, 2H, NCH_2Py); 4.61 (t, 2H, CH_2ONO_2); 5.16 (s, 2H, CH_2O); 7.32-7.48 (m, 4H), 7.62-7.80 (m, 3H), 7.97 (d, 1H), 8.07-8.16 (m, 3H) (aromatic protons); 8.69 (s, 1H, NH), 10.63 (s, 1H, NH). $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) δ = 21.3; 40.3; 43.6; 51.9; 58.1; 62.8; 70.8; 124.4; 125.5; 125.8; 126.3; 126.8; 127.3; 128.3; 128.4; 132.1; 132.2; 133.5; 137.4; 143.8; 148.4; 149.6; 156.2; 165.4. MS ESI $[\text{M}+\text{H}]^+$ m/z 523.3. ESI-HRMS $[\text{M}+\text{H}]^+$: 523.2304, $\text{C}_{26}\text{H}_{31}\text{N}_6\text{O}_6$ requires 523.2300; HPLC purity > 95%.

[4-(2-Aminophenylcarbamoyl)benzyl]carbamic acid 6-[[5,6-bisnitrooxyhexyl)methylamino]methyl]pyridin-3-ylmethyl ester trihydrochloride (31)

Pale yellow foam; yield: 68%.

$^1\text{H-NMR}$ (300 MHz, DMSO- d_6) δ = 1.40 (qi, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$); 1.74 (m, 4H, $\text{CH}_2\text{CH}_2\text{CH}_2$); 2.74 (s, 3H, CH_3); 3.10 (m, 2H, CH_2); 4.31 (d, 2H, NHCH_2); 4.46 (s, 2H, NCH_2Py); 4.68-4.74 (m, 1H, $\text{CH}_a\text{H}_b\text{ONO}_2$), 4.92-4.97 (m, 1H, $\text{CH}_a\text{H}_b\text{ONO}_2$); 5.16 (s, 2H, CH_2O); 5.37-5.46 (m, 1H, CHONO_2); 7.32-7.54 (m, 5H), 7.53-7.70 (m, 2H), 7.91-7.95 (m, 1H), 8.04-8.11 (m, 3H); 8.68 (s, 1H, NH); 10.57 (s, 1H, NH); 10.92 (bs, 1H, exchangeable proton). $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) δ = 21.4; 22.8; 27.7; 39.7; 43.6; 54.7; 58.0; 62.8; 71.8; 80.0; 124.3; 125.5; 125.8; 126.2; 126.7; 127.2; 128.2; 128.3; 132.0; 132.1; 133.4; 137.3; 143.8; 148.3; 149.7; 156.1; 165.3. MS ESI $[\text{M}+\text{H}]^+$ m/z 626. ESI-HRMS $[\text{M}+\text{H}]^+$: 626.2563, $\text{C}_{29}\text{H}_{36}\text{N}_7\text{O}_9$ requires 626.2569; HPLC purity > 95%.

[4-(2-Aminophenylcarbamoyl)benzyl]carbamic acid 6-[[3-carbamoylfuroxan-4-ylmethyl)methylamino]methyl]pyridin-3-ylmethyl ester trihydrochloride (32)

Crude product was purified by preparative HPLC (eluent $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{HCl}$ 60/40/0.05 v/v/v, flow = 20 mL/min, λ = 226 nm, injected 100 mg in 2 mL of $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{DMSO}$ 50/50/0.01). M.p. 102-106°C (with decomposition). Yield: 52%.

$^1\text{H-NMR}$ (300 MHz, DMSO- d_6) δ = 2.87 (s, 3H, CH_3); 4.30 (d, 2H, NHCH_2Ph); 4.65 (s, 2H), 4.78 (s, 2H) ($\text{CH}_2\text{CH}_3\text{NCH}_2$); 5.17 (s, 2H, CH_2O); 7.32-7.48 (m, 4H), 7.58-7.74 (m, 3H), 7.95-8.14 (m, 5H) (11 aromatic protons, 1H CONH); 8.79 (bs, 2H, 2 CONH); 10.63 (s, 1H, CONH). $^{13}\text{C-NMR}$ (75 MHz, DMSO- d_6) δ = 41.0; 43.6; 49.6; 58.8; 62.7; 111.1; 124.4; 125.2; 125.8; 126.3; 126.8; 127.2; 128.3; 128.4; 132.2; 133.7; 138.0; 143.9; 147.8; 149.9; 151.3; 156.2; 156.3; 165.4. MS ESI $[\text{M}+\text{H}]^+$ m/z 561.3. ESI-HRMS $[\text{M}+\text{H}]^+$: 561.2208, $\text{C}_{27}\text{H}_{29}\text{N}_8\text{O}_6$ requires 561.2205; HPLC purity > 95%.

5,6-Bisnitrooxyhexyl-N,N-dmimethylamine oxalate (33)

To a solution of **19** (0.60 g, 2.00 mmol) in EtOH (15 mL) a solution 5.6M of N,N-dimethylamine in MeOH (0.71 mL, 4.00 mmol) was added and the reaction mixture was heated at 50°C for 24h. The mixture was poured in water and 1M NaOH was added (20 mL), then extracted with CH₂Cl₂ (2×15 mL), the organic phases were washed with brine, dried, filtered and concentrated under reduced pressure. The crude product was purified by flash chromatography (eluent CH₂Cl₂/MeOH 95/5 v/v). The resulting amine was dissolved in EtOAc (15 mL), and a solution of oxalic acid (0.09 mg, 1.00 mmol) in EtOAc (5 mL) was added. The precipitate was filtered and washed with dry Et₂O to give the title compound as white solid. **M.p. 95-96°C**. Yield: 49%.

¹H-NMR (300 MHz, DMSO-d₆) δ = 1.49 (m, 2H, OCH₂CH₂CH₂CH₂CH₂N); 1.61-1.78 (m, 4H, OCH₂CH₂CH₂CH₂CH₂N); 2.70 (s, 6H, 2CH₃); 2.98 (t, 2H, OCH₂CH₂CH₂CH₂CH₂N); 4.71 (dd, 1H, CH_aH_bONO₂); 4.93 (dd, 1H, CH_aH_bONO₂); 5.41 (m, 1H, CHONO₂). ¹³C-NMR (75 MHz, DMSO-d₆) δ = 21.4; 23.3; 27.8; 42.1; 56.1; 71.9; 80.1; 164.7. MS ESI [M+H]⁺ *m/z* 252. **ESI-HRMS [M+H]⁺: 252.1189, C₈H₁₈N₃O₆ requires 252.1190**; HPLC purity > 95%.

3-Phenylsulfonyl-4-(2-methoxyethoxy)furoxan (34)

To a solution of 3,4-bisphenylsulfonylfuroxan (0.73 g, 2.00 mmol) in CH₂Cl₂ (30 mL) 2-methoxyethanol (0.24 mL, 3.00 mmol) and DBU (0.90 mL, 4.00 mmol) were added and the solution stirred at r.t. for 2h. Then the reaction mixture was washed with H₂O (20 mL) and 1M HCl (20 mL), dried, filtered and concentrated under reduced pressure. The crude product was purified by crystallization from *i*Pr₂O to give the title compound as white solid. **M.p. 111°C**. Yield: 52%

¹H-NMR (300 MHz, DMSO-d₆) δ = 3.46 (s, 3H, OCH₃); 3.81 (t, 2H, CH₂OCH₃); 4.57 (t, 2H, FxOCH₂CH₂); 7.62 (m, 2H, C₆H₅); 7.76 (m, 1H, C₆H₅); 8.08 (m, 2H, C₆H₅). ¹³C-NMR (75 MHz, DMSO-d₆) δ = 59.3; 69.7; 70.5; 110.0; 128.6; 139.6; 135.6; 138.1; 159.0. MS ESI [M+Na]⁺ *m/z* 323. **ESI-HRMS [M+Na]⁺: 323.0310, C₁₁H₁₂N₂NaO₆S requires 323.0308**; HPLC purity > 95%.

Vasodilating properties. Vasodilator activity was studied as previously described.[57] Briefly, thoracic aortas strips deprived of endothelium were isolated from male Wistar rats weighing 180-200 g. The tissues were mounted under 1.0g tension in organ baths containing 30 mL of Krebs-bicarbonate buffer, maintained at 37°C and continuously gassed with 95% O₂-5% CO₂ (pH = 7.4). After an equilibration period the aortic strips were contracted with L-phenylephrine (1 μM). When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. Effect of ODQ (1 μM) was evaluated in a separate series of experiments in which it was added to the organ bath 5min before the contraction. Results were expressed as EC₅₀ values; data are the mean ± SEM of at least three experiments. Responses were recorded by an isometric transducer

connected to the MacLab System PowerLab. Addition of drug vehicle (1% DMSO) had not appreciable effect on contraction.

Cell culture. Transformed human keratinocytes (HaCaT) were grown at 37°C in a 5% CO₂ atmosphere in complete Dulbecco's Modified Eagle Medium (DMEM, LONZA) supplemented with 5% (v/v) L-Glutamine (SIGMA), 5% (v/v) Penicillin-Streptomycin (SIGMA), 10% (v/v) Fetal Calf Serum (FCS, GIBCO). Cells were treated with the 2 hybrids at different concentrations (1, 10, 25, and 50 µM) for 1, 3 and 5h to select the best concentration for biological evaluations. In all experiments, **1** and **33** or **34** were used as reference compounds and DMSO was used as solvent control.

Myoblast differentiation experiments. A cell line of murine myoblast (C2C12) was obtained by Cell Lines Service GmbH (CLS) and was cultured at 37°C in a 5% CO₂ atmosphere in complete DMEM (SIGMA) containing 5% (v/v) L-Glutamine (LONZA), 5% (v/v) Penicillin-Streptomycin (SIGMA), 20% (v/v) Fetal Bovine Serum (FBS, Millipore). For differentiation experiments cells were kept in DMEM (SIGMA) with 2% Horse Serum (HS, SIGMA) for 5 days in presence or in absence of compounds **1**, **2**, **16**, **31**, **33**, **34** or **1 + 33**, **1+ 34** in combination. All compounds were used at 0.5µM and renewed daily. Control cells were maintained in 20% serum (undifferentiated myoblast) or in the presence of vehicle (DMSO).

Nitric oxide production. Nitric oxide production was evaluated by adding 4,5-diaminofluorescein diacetate (DAF-2D) (Alexis), a sensitive fluorescent dye for detection of intracellular NO, to the complete medium 5 µM. At the end of treatment, cells were collected and analysed by fluorescence-activated cell sorting (FACS) to detect intracellular NO production. Upon entry into the cell, DAF-2D is transformed into the less cell-permeable DAF-2 by cellular esterases, thus preventing loss of signal due to diffusion of the molecule from the cell. In the presence of oxygen, DAF-2 reacts with NO to yield the highly fluorescent triazolofluorescein (DAF-2T).

HDAC assays. HDAC assays were performed with the HDAC colorimetric activity assay Kit (BioVision) according to manufacturer instructions. Briefly, HaCaT total cell lysates were obtained by mechanical lysis, using lysis buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% Triton x-100, 10% glycerol supplemented with 1mM PMSF and protease inhibitor mix); 50µg total extract were used in each experiment. Total extract was spotted directly onto a 96 multiwell plate provided in the kit, in the presence of HDAC assay buffer and HDAC

substrate (acetylated lysine side chain). Control wells contained the same mixture plus TSA. Samples were assayed in duplicate. The mixture was incubated for 1h at 37°C; a lysine developer solution was then added and the plate was incubated for 30min at room temperature (r.t.). The plate was read by colorimetric detection at wavelength 405 nm, using an ELISA reader (EnSpire Perkinelmer). Percent HDAC activity is shown with respect to the activity of HDAC alone (100%). The kit contains positive controls that consist of nuclear extract of HeLa. TrichostatinA (TSA) on HaCaT total cell lysate was used as a negative control.

Immunofluorescence analysis. Treated HaCaT cells were fixed in 4% paraformaldehyde solution and blocked for 1h in PBS containing 10% bovine serum albumin (BSA). Cell fields were incubated with primary and secondary antibody in PBS containing 1% BSA . Nuclei were detected with Hoechst. Staining was visualized via an Apotome microscope; pictures were obtained using an Axiocam and analysed with KS 300 3.0 acquisition software (Zeiss). Myosin Heavy Chain staining: After 5 days of treatment, C2C12 cells were fixed and permeabilized in 4% paraformaldehyde. Fixed cells were blocked with 10% BSA/PBS, and incubated with MHC (anti-Myosin Heavy Chain (Millipore); diluted 1:50 in 1% BSA/PBS) and anti-mouse IgG-AlexaFluor-594 conjugated (Thermo Fisher Scientific). Cells were stained with DAPI to visualize cell nuclei. Images were captured on Axio Observer Z1 microscope (20X magnification) and analysed using digital image analysis software (AxioVision Rel. 4.8, Carl Zeiss).

Immunoprecipitation and Western blotting. HDAC2 S-nitrosylation was determined by immunoprecipitation using anti-S-nitrosylation (SNOCys) (4 µg for 500 µg total proteins, polyclonal; Alpha Diagnostic) analysed by Western blotting with HDAC2 antibody (1:500, Santa Cruz). Immunoprecipitation experiments were performed using 500µg total cell extract obtained by lysis in RIPA buffer (50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% triton X100, 2 mM MgCl₂, and 1% sodium deoxycholate supplemented with 1 mM PMSF and protease/ phosphatase inhibitor mix). Paramagnetic beads (Ademtech's Bioadembeads) were used for specific protein separation. Negative controls were run using the same amount of protein extract from WT samples immunoprecipitated with the corresponding purified IgG antisera (Santa Cruz) in the absence of primary antibody.

RNA isolation and qRT-PCR. The analysis was performed on four independent experiments. Total RNA was extracted from C2C12 cells using Tri-Reagent (SIGMA) following supplier's instructions. The concentration and purity of the RNA samples were determined by Nanodrop1000. cDNA synthesis for quantitative real-time PCR (qRT-PCR) was carried out with SuperScript III First-Strand

Synthesis Super Mix for qRT-PCR (Invitrogen) according to the manufacturer's protocol. All reactions were performed in 96-well format in the StepOne Plus Real-Time PCR System (Applied Biosystems) using PerfeCTa® SYBRGreen® FastMix®, ROX™ (Quanta BIOSCIENCES™). For each gene of interest, qRT-PCR was performed as follows: each RNA sample was tested in duplicate and P0 was used to normalize transcript abundance. mRNA expression levels were calculated by Comparative Ct Method by using the Applied Biosystem software (Applied Biosystem) and were presented as fold induction of transcripts for target genes. Fold change above 1 denotes upregulated expression, and fold change below 1 denotes downregulated expression versus the solvent.

List of forward and reverse primers:

Gene	Species	Forward (5'-3')	Reverse (5'-3')
<i>Myomaker</i>	<i>Mus Musculus</i>	ATCGCTACCAAGAGGCGTT	CACAGCACAGACAAACCAGG
<i>Myogenin</i>	<i>Mus Musculus</i>	GGGCAATGCACTGGAGTT	GACATATCCTCCACCGTGATG
<i>MRF4</i>	<i>Mus Musculus</i>	CAGCAAGAGAAGATGCAGGAG	CCTGGAATGATCCGAAACAC
<i>P0</i>	<i>Mus Musculus</i>	GCGTCCTGGCATTGTCTGT	GAAGGCCTTGACCTTTTCAGTAAG

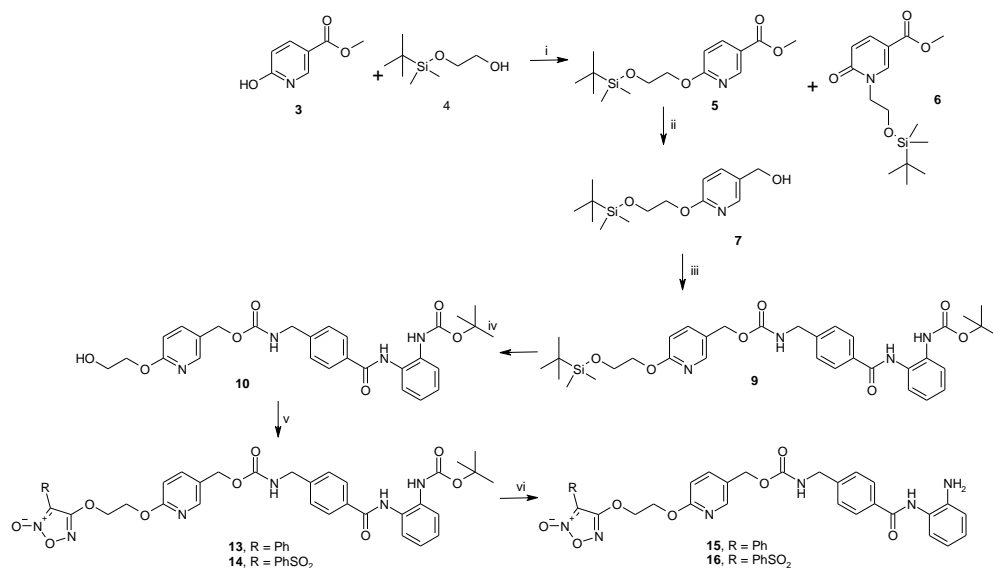
Statistical analyses. Student's T test was used to assess statistically significant differences among different groups. A value of at least $P < 0.05$ was considered statistically significant.

Results and Discussion

1. Chemistry

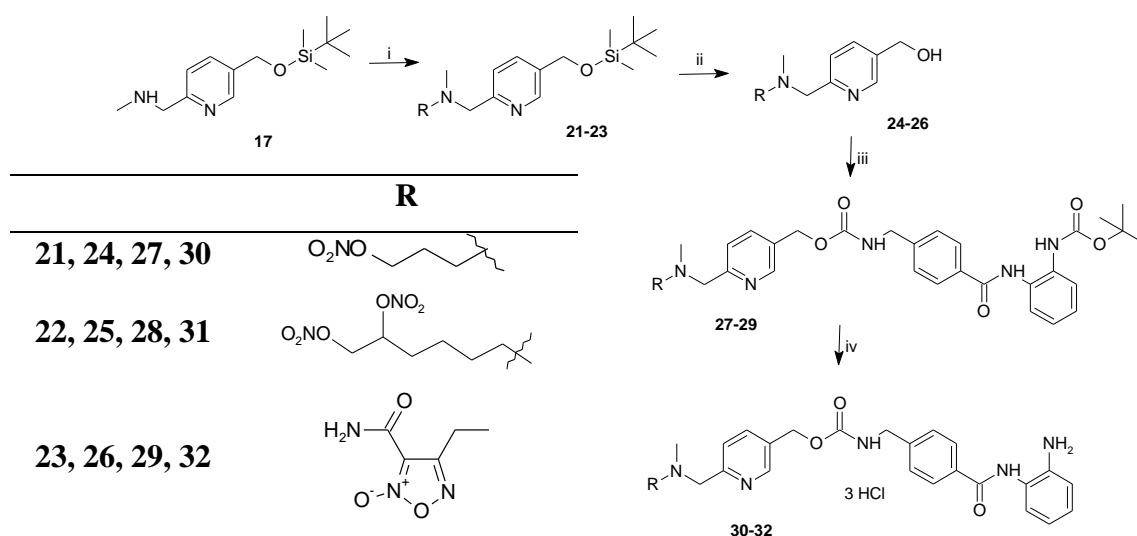
Synthesis. The synthetic strategy to obtain the final products **15**, **16** in which the oxyethyloxy group is present as linker is reported in Scheme 1. The 6-hydroxynicotinic acid methyl ester **3** was coupled in 1,4-dioxane solution with *tert*-butyldimethylsilyl-protected ethyleneglycol **4** in the presence of the adduct between Ph_3P and diisopropylazodicarboxylate (DIAD) (Mitsunobu conditions), to afford a mixture of compounds in which the O-alkylated **5** and the N-alkylated **6** products were present. After chromatographic separation, **5** was reduced with LiAlH_4 to the related alcohol **7**. Activation of **7** with *N,N'*-carbonyldiimidazole (CDI), followed by coupling in THF with [2-(4-aminomethylbenzoylamino)phenyl]carbamic acid *tert*-butyl ester (**8**) gave rise to the intermediate **9**. *tert*-Butyldimethylsilyl-protection was removed using tetrabutylammonium fluoride in THF to give the alcohol **10**. Nucleophilic displacement of the phenylsulfonyl-group in the furoxan derivatives **11** (3-phenyl-4-phenylsulfonylfuroxan) or **12** (3,4-bisphenylsulfonylfuroxan) gave rise to the *Boc*-

protected final products **13**, **14** which, treated with 5.30M HCl in 1,4-dioxane, produced the expected target compounds **15**, **16**.



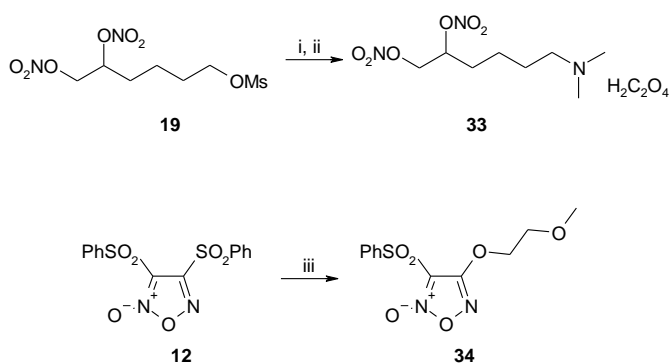
Scheme 1. Synthesis of compounds 15-16. Regents and conditions. i) Ph_3P , DIAD, 1,4-dioxane; ii) LiAlH_4 , dry THF; iii) CDI, **8** ([2-(4-aminomethylbenzoylamino)phenyl]carbamic acid *tert*-butyl ester), dry THF; iv) TBAF, THF; v) **11** (3-phenyl-4-phenylsulfonylfuroxan), DBU, CH_3CN or **12** (3,4-bisphenylsulfonylfuroxan), DBU, CH_2Cl_2 ; vi) 5.30M HCl in 1,4-dioxane, THF.

The synthetic pathway to obtain the final products **30-32** in which the group methyl-aminomethyl- is present as linker is reported in Scheme 2. The *tert*-butyl(dimethyl)silyl-protected 5-(methylaminomethyl)pyridine-2-carbaldehyde **17** was prepared according to literature procedure.[49] Different NO-donor moieties were inserted on this scaffold using opportune alkylating agents: 3-bromopropylnitrate (**18**), 5,6-bisnitrooxyhexylmethanesulfonate (**19**), 4-bromomethylfuroxan-3-carboxamide (**20**) for the preparation of **21-23**, respectively. These products were deprotected with a mixture of $\text{CH}_3\text{COOH}/\text{THF}/\text{H}_2\text{O}$ (3/1/1 v/v/v) to afford the free alcohols **24-26**. Reaction of these intermediates with the *Boc*-protected amine **8** utilizing CDI in THF as activating reagent, gave rise to the *Boc*-protected products **27-29**, which, treated with 1,4-dioxane saturated with HCl, produced the expected target compounds **30-32**.



Scheme 2. Synthesis of compounds 30-32. Regents and conditions. i) NaHCO_3 , acetone, **18** (3-bromopropyl nitrate) or **19** (5,6-bisnitrooxyhexylmethanesulfonate) or **20** (4-bromomethylfuroxan-3-carboxamide); ii) $\text{CH}_3\text{COOH}/\text{THF}/\text{H}_2\text{O}$ (3/1/1 v/v/v), 4 days, r.t.; iii) CDI, **8**, dry THF; iv) 5.30M HCl in 1,4-dioxane, THF.

Compounds **33** and **34** were synthesized as reference compounds able to release NO only, as depicted in the scheme 3. The dinitrooxy derivative **33** was obtained by reaction of **19** with N,N-dimethylamine in EtOH, and was isolated as oxalate. Furoxan derivative **34** was obtained from the reaction of 3,4-bisphenylsulfonylfuroxan **12** and 2-methoxyethanol in CH_2Cl_2 using DBU as base.



Scheme 3. Synthesis of compounds 33, 34. Regents and conditions. i) 5,6M N,N-dimethylamine in EtOH, 50°C; ii) $\text{H}_2\text{C}_2\text{O}_4$, EtOAc; iii) 2-methoxyethanol, DBU, CH_2Cl_2 .

2. Biology

Single moiety activity preservation

Hybrid molecules were designed to avoid alteration of both the affinity for the active site of HDAC enzymes and the NO release ability. HDAC inhibition activity was preserved putting the linker on the most extremely variable part of the MS-275 pharmacophore (*Figure 1*) and tested by HDAC activity assay. The assay was performed in HaCaT cell lysates after 1 hour of treatment with 10 μM

of MS-275 alone or with hybrids **15**, **16**, **32**, **30** and **31**. 100nM of TSA, the most known potent HDACi,⁷ and the previously studied hybrid **2** [47] were used as control reference.

In vitro all hybrids significantly inhibited total HDAC activity determining a decrease from 20% to 50% compared to controls (*Figure 2*). These results confirm previous prediction indicating that the conjugation of NO donor moieties does not significantly alter the affinity of the hybrid molecules to HDAC enzymes.

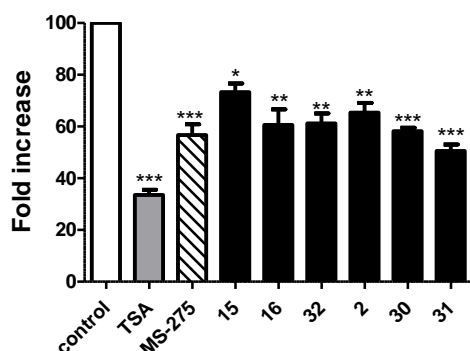


Figure 2. Hybrids inhibition of total HDAC activity. Data are represented as means \pm SEM; * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$; versus the control group (*t test*).

The ability to release NO was evaluated in HaCaT cells by of 4,5-diaminofluorescein (DAF-2 DA) fluorescence and FACS analysis after 1h (*Figure 3A*) to 5h (*Figure 3B*) of treatment with the synthesized hybrid molecules. As the balance is crucial in hybrid molecules, NO release was tested at the same concentration at which hybrid molecules displayed HDAC inhibition activity (10 μ M). For this experiment, diethylenetriamine NONOate (DETA/NO), a spontaneous NO donor, and the previously characterized hybrid **2** [47] were used as positive controls. Results were consistent with the hybrid nature of the chemical structures (*Figure 3*). Among nitrates, mononitrooxy compound **30** displayed hardly any ability to release NO at 10 μ M, whereas, as expected, dinitrooxy compound **31**, at the same concentration, released more NO (*Figure 3*). Among furoxans, compound **15**, bearing a phenylfuroxan moiety, displayed a weak NO donor ability, whereas compounds **16**, a phenylsulfonylfuroxan moiety, showed the highest NO release rate among furoxans and all the synthesized hybrids with a progressive time dependent increase (*Figure 3*). Furoxancarboxamide **32** did not release NO neither at 10 μ M (*Figure 3*) nor at 50 μ M or 100 μ M (data not shown). This behaviour was unexpected because furoxancarboxamide moiety has been previously reported as a good NO donor- better than phenylfuroxan moiety- displaying vasodilating properties in rat aorta strips pre-contracted with phenylephrine.[58] At present, we cannot provide an explanation for this anomalous behaviour; further evaluations are required but they are not object of the present manuscript.

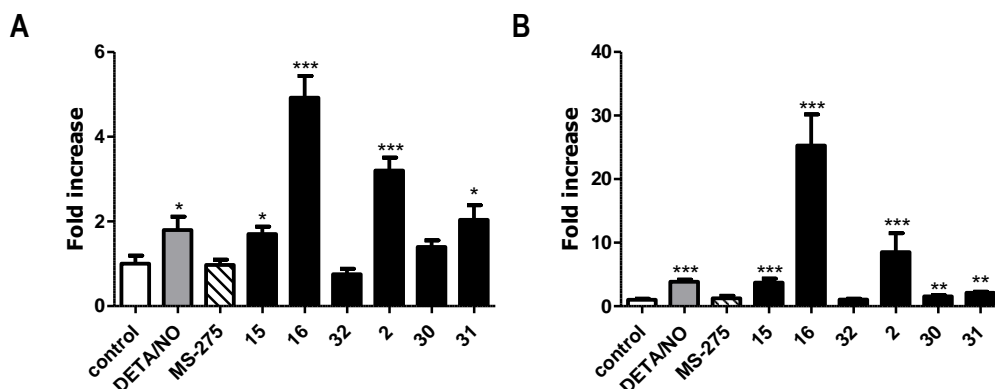


Figure 3. Intracellular nitric oxide release of the designed hybrids. (A) Nitric oxide release evaluation after 1h of treatment. **(B)** Nitric oxide release evaluation after 5h of treatment. Data are represented as means \pm SEM; * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ versus control group (*t test*).

Taken together these results obtained from HDAC activity assay and DAF analysis showed pharmacologic potential for the synthesized molecules **16** and **31**. To further investigate the NO release ability, we performed some biochemical analysis to show their effect on both class I and class II HDACs. Indeed, NO directly induced class I HDAC2 S-nitrosylation [59,60] and class II HDAC4/5 shuttling into the nucleus with consequent histone deacetylation and gene repression.[10,61] S-nitrosylation of HDAC2, able to influence both the catalytic activity and the enzyme association to chromatin, has been tested in immunoprecipitation experiments after 1h of HaCaT cells treatment with compounds **16** and **31**, the best NO donors according to FACS analysis (*Figure 4*). In this experiment hybrid **2** [47] was used as positive control. All compounds were able to induce HDAC2 S-nitrosylation, suggesting that NO released from these hybrid molecules was able to chemically modify HDAC2 (*Figure 4*).

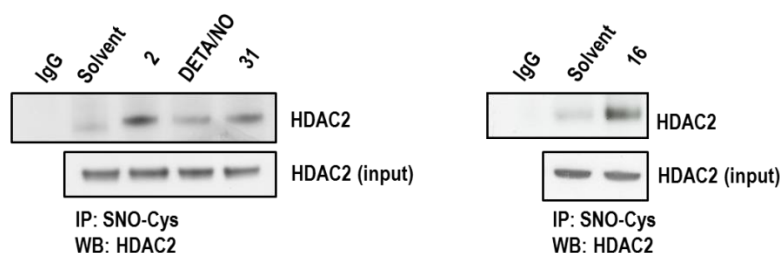


Figure 4. Hybrid induction of HDAC2 S-nitrosylation. Representative immunoprecipitation for S-nitrosocysteine revealed for HDAC2.

The NO-dependent translocation of class II HDACs was analysed by immunofluorescence on HaCaT cells treated for 1h with 10 μ M of compounds **16** and **31** (*Figure 5*). As shown in *Figure 5*, class II HDAC (HDAC4 and HDAC5) nuclear translocation was significantly promoted by treatments compared to control condition. This result suggests that the hybrids are still able to activate the nuclear shuttling mechanism of class II HDACs.

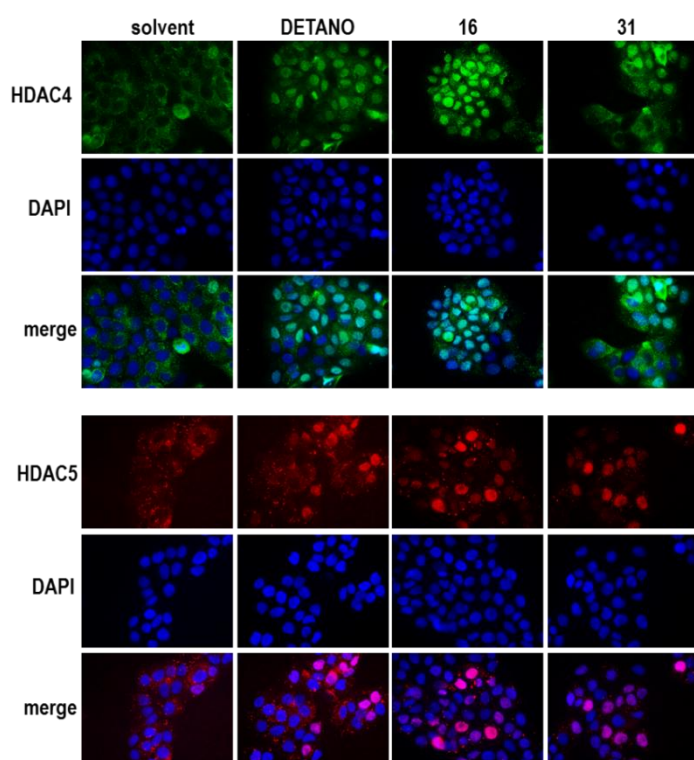


Figure 5. Hybrid induction of class II HDAC shuttling from cell cytoplasm to the nucleus. Representative immunofluorescence panels revealed for HDAC2 (green) and HDAC5 (red). Nuclei counterstained with DAPI (blue). Magnification 40x.

Vasodilating properties

The capacity of the hybrid NO-donor MS-275 analogues and of the reference compounds **33** and **34** to activate the soluble guanylyl cyclase (sGC) was evaluated through the ability of the products to induce vasodilation of rat aorta strips precontracted with phenylephrine, in the absence and in the presence of 1 μ M ODQ (1*H*-1,2,4-oxadiazolo-4,3-*a*-quinoxalin-1-one), a well-known inhibitor of this enzyme. All the products were able to dilate the precontracted tissue in a concentration-dependent manner. The vasodilator potencies, expressed as EC₅₀ values, are collected in Table 1. The most potent vasodilators were the phenylsulfonyl and the cyano substituted furoxan derivatives **16**, **34** and **2**, whose EC₅₀ values fall in the sub-micromolar range. These products were about tenfold and one hundred fold more potent than the dinitrooxy substituted analogue **31** and **33** and the remaining compounds **15**, **30**, **32**, respectively. When the experiments were repeated in the presence of ODQ a marked decrease in the potencies was observed, so indicating the involvement of the sGC activation in the vasodilator action.

Compound	Vasodilator activity	
	EC ₅₀ (μM) ± SE	EC ₅₀ (μM) ± SE + ODQ 1μM
15	3.4 ± 0.5	> 100
16	0.031 ± 0.004	0.35 ± 0.08
30	2.6 ± 0.8	44 ± 1
31	0.29 ± 0.07	11 ± 3
32	3.4 ± 0.6	63 ± 12
33	0.80 ± 0.20	> 100
34	0.0045 ± 0.0001	0.43 ± 0.02
2	0.024 ± 0.005	0.96 ± 0.20

Table 1. Vasodilator activity of the designed hybrids.

Functional analysis

Recently, we reported the ability of hybrid **2** to promote skeletal muscle differentiation promoting formation of large fused myotubes with a high nuclei/fibre ratio.[47] This effect was significantly higher than that obtained by the single hybrid components possibly in consequence of the combined effect of NO on class II HDACs, the nitrosylation of HDAC2 and the overall inhibition of class I HDAC activity.[47] According to the experimental evidences, the newly synthesized hybrid molecules **16** and **31** were the most promising compounds for skeletal muscle differentiation. C2C12 cells, a mouse myoblast cell line, were cultured in 20% serum (growth medium; GM) until differentiation induction by serum lowering to 2% (differentiation medium; DM). Differentiation was stimulated in the presence of 0.5μM hybrid compounds **16** or **31**. Control cells were differentiated in the presence of separated or combined pharmacophores (phenylsulfonylfuroxan - **34** - and MS275 for compound **16** and dinitrooxy - **33** - and MS275 for compound **31**) (*Figure 6*). After 5 days, differentiation response was evaluated by myosin heavy chain (MHC) expression (*Figure 6A*), by evaluation of nuclei/fiber ratio (*Figure 6B*) and by a panel of muscle differentiation markers, such as myomaker (MyoM), myogenin (MyoG) and myogenic regulatory factor 4 (MRF4) (*Figure 6C*). *Figure 6* panel A shows that **31** promotes formation of larger myotubes in comparison to **16**. The evaluation of the number of nuclei per fiber points out that compound **31** induces more multinucleated fibers than the combined pharmacophores (*Figure 6B*). The ability of **31** to stimulate fusion among myoblasts is further confirmed by the high expression of MyoM, the membrane activator of myoblast fusion, [62] depicted in *figure 6*, panel C.

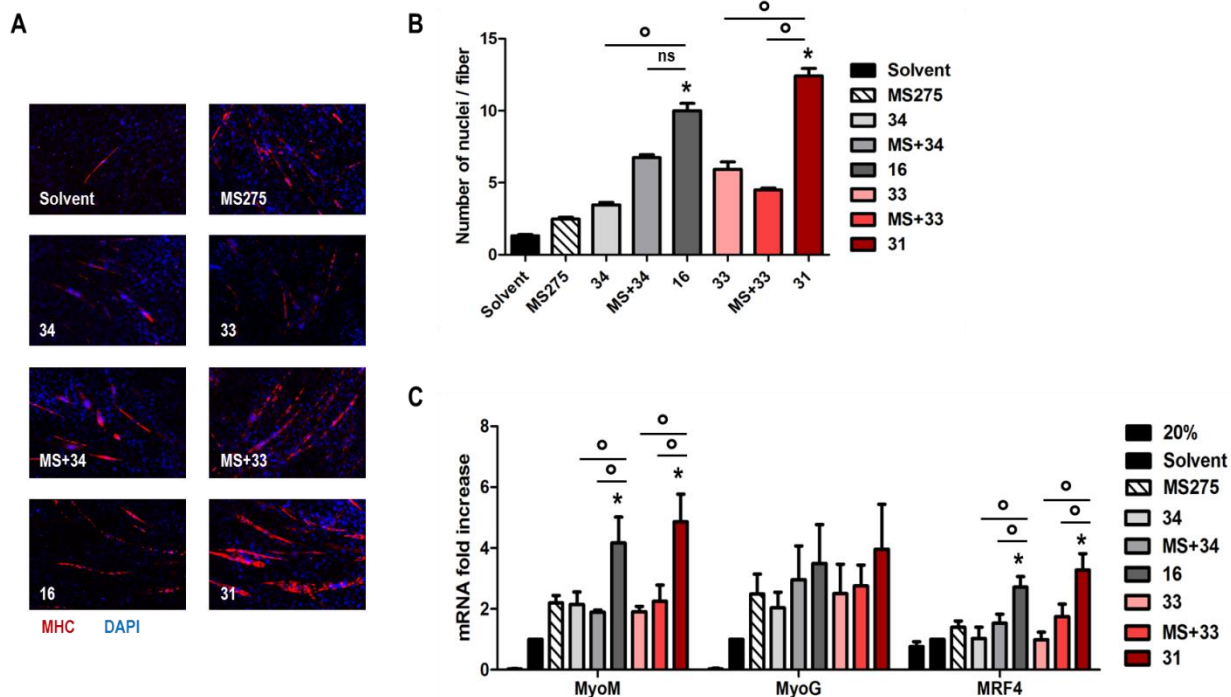


Figure 6. Hybrid induction of skeletal muscle differentiation. (A) Representative immunofluorescence panels revealed for MHC (red). Nuclei counterstained with DAPI (blue). Magnification 20x. (B) Number of nuclei per myofiber quantification in C2C12 after 5 days of differentiation in the presence of 0.5 μ M hybrid. Data are represented as means \pm SEM; * $p < 0.05$ versus MS275 group (*t test*); $^{\circ}$ $p < 0.05$ (*t test*). (C) Myomaker (MyoM), myogenin (MyoG) and myogenic regulatory factor 4 (MRF4) mRNA expression in C2C12 after 5 days of differentiation in the presence of 0.5 μ M hybrid. Data are represented as fold increase in comparison to solvent condition group. Data are represented as means \pm SEM; * $p < 0.05$ versus MS275 group (*t test*); $^{\circ}$ $p < 0.05$ (*t test*).

Conclusions

These data describe a group of novel hybrid compounds where various NO-donor groups (phenylsulfonylfuroxan, dinitrooxy) are coupled with the class I HDAC inhibitor MS-275. Interestingly, these hybrids distinctly affect HDAC activity inhibiting class I by MS275- and NO-dependent (S-nitrosylation) effect and activating class II via NO-dependent cytosol-nuclear shuttling. Functionally, this family of compounds exhibit specific and unique biological features to control muscle differentiation and *ex vivo* aortic vasodilatation. Overall we identified novel promising compounds that may be apply in different pathological context such as cardiovascular, neuromuscular and inflammatory disorders.

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Author contribution

SA carried out experiments and data analysis; KC carried out the synthesis; CC carried out experiments and data analysis and wrote the manuscript; SG carried out the synthesis, EM carried out experiments and data analysis, CG revised data analysis and the manuscript; RF revised the manuscript; FS conceived and carried out the experiments and wrote the manuscript; LL conceived and carried out the synthesis and wrote the manuscript. All the authors contributed critical discussion and approved the final version of the manuscript.

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