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Letter

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Synthesis and Biological Evaluation of the First Example of NO-Donor Histone Deacetylase Inhibitor

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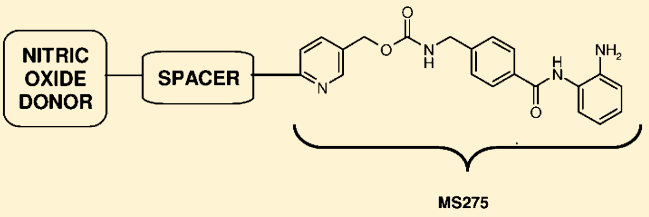
Supporting Information

ABSTRACT: The NO-donor histone deacetylase inhibitor **2**, formally obtained by joining Entinostat **1**, a moderately selective Class I histone deacetylases (HDACs) inhibitor, to a 4-(methylaminomethyl)furoxan-3-carbonitrile scaffold, is described and its preliminary biological profile discussed. This hybrid regulates Classes I and II HDACs. Nitric oxide (NO) released by the compound activates soluble guanylate cyclase (sGC), causing Class II nuclear shuttling and chromatin modifications, with consequences on gene expression. The hybrid affects a number of micro-RNAs not modulated by its individual components; it promotes myogenic differentiation, inducing the formation of larger myotubes with significantly more nuclei per fiber, in a more efficient manner than the 1:1 mixture of its two components. The hybrid is an example of a new class of NO-donor HDACs now being developed, which should be of interest for treating a number of diseases.



KEYWORDS: *HDAC, histone deacetylase inhibitors, NO-donor, multitarget drugs, epigenetics*

Histone deacetylases (HDACs) are a family of epigenetic enzymes that catalyze the hydrolysis of acetylated ε-amino groups of Lys in H3 and H4 histone tails.[1] Removal of the acetyl groups restores the positive charge of Lys residues, enabling them to interact with the negative phosphate groups of DNA, with consequent chromatin tightness and transcriptional repression.[2] The converse occurs under the action of another family of enzymes, the histone acetyltransferases (HATs), which catalyze the transfer of acetyl groups from acetyl-CoA to lysine residues in H3 and H4 histone tails inducing DNA unwinding and chromatin decondensation, with potential transcriptional activation.

Human HDACs are a family of 18 enzymes grouped into four classes (I, II, III, and IV).[3] Class III HDACs, also known as "sirtuins", are NAD-dependent deacetylase enzymes, whereas Classes I, II, and IV are zinc-dependent metalloproteins. Class I HDACs comprise HDAC1, 2, 3, and 8, which have conserved structure and similar functions, but rather different distributions: HDAC1 and 2 are mostly nuclear, while HDAC3 and HDAC8 are tissue-specific and present either in the nuclear or in the cytoplasmatic compartments.[1,3−5] Class II HDACs are more structurally complex, and their functions are not yet clear. Class II is further divided into subclasses IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10). The members of Class II are localized in the cytosol when they are phosphorylated, whereas their dephosphorylation enables them to translocate into the nucleus (nuclear shuttling) with consequent histone deacetylation and gene repression.[6] HDAC11, a poorly characterized enzyme, is the sole member of class IV and is primarily nuclear. Lastly, the members of Class III (the sirtuins) show no homology with the other classes and preferentially deacetylate nonhistone substrates.[7]

Nitric oxide (NO) is a well-known gaseous molecule that plays very important roles in mammalian physiology and pathophysiology. In the cardiovascular system, NO contributes to maintaining micro- and macrovascular homeostasis.[8] In the central nervous system, NO plays roles in learning and memory formation.[9] In the skeletal muscles, it controls the resting potential and modulates contraction−excitation coupling.[10] NO also plays very important roles in the biology of tumors and in inflammation.[11,12] It was early recognized that many of the effects triggered by NO are consequent on activation of the soluble guanylate cyclase (sGC)/cGMP pathway, following the nitrosylation of heme iron within sGC. Over the past decade, an increasing body of research has shown that many NO-based activities are due to modifications of proteins that regulate numerous physiological and physiopathological processes. These modifications include two chemical reactions: S-

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nitrosation, also known as S-nitrosylation, and tyrosine(Tyr)-nitration.[13] NO thus orchestrates different kinds of intracellular regulation, including functional regulation of epigenetic process enzymes[2] and microRNA expression.[14]

HDACs have been found to be both direct and indirect targets of NO. It has been shown that the Class I enzyme HDAC2 can be directly modified by NO, undergoing either Tyr-nitration or S-nitrosylation.[2] The Class II HDACs might also be indirectly activated by NO: NO induces Class IIa HDAC nuclear shuttling by activation of protein phosphatase 2A (PP2A) through the sGC/cGMP pathway, being known to contribute to endothelial cells functions[6] and mouse embryonic stem cell (mESc) differentiation along the mesoderm lineage. Moreover, we recently demonstrated that NO determines a functional crosstalk between Classes III and I histone deacetylases, enzymes that are crucial for appropriate skin-repair processes.[15] Abnormal HDAC activity has been found to be involved in the development of several kinds of tumors, and in recent years considerable attention has been paid to HDAC inhibitors (DIs) as anticancer agents.[16−19] Very recent results show the potential application of DIs in inflammatory, immunological, neurological, neuromuscular, and cardiovascular diseases.[5] Intriguingly, supplementation with appropriate amounts of exogenous NO might be useful in a number of disorders, in whose pathogenesis HDAC overexpression plays a significant role.

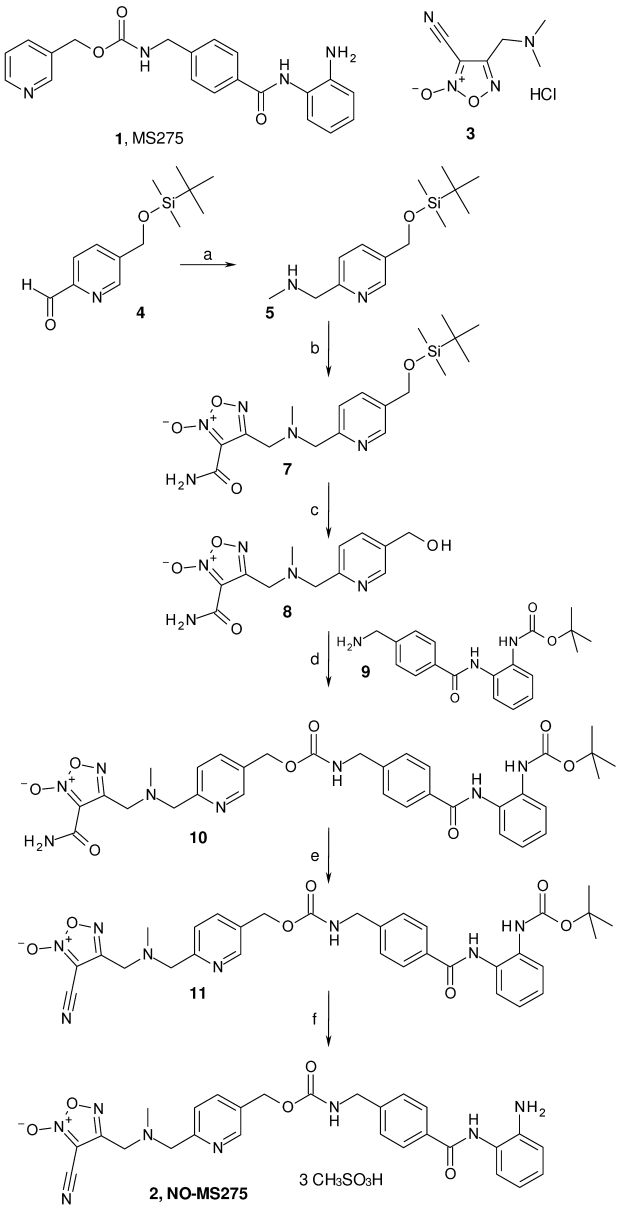
One example of how the use of DIs and NO-donors may be synergistic is in controlling cardiac hypertrophy.[20] It has recently been shown that, in this disease, Class I HDACs are pro-hypertrophic, in particular HDAC2, whereas Class II HDACs down-regulate hypertrophy.[20] Thus, a selective HDAC2 inhibitor capable of releasing NO and able to induce nuclear shuttling of Class IIa enzymes would be of interest.

Wound healing is another field in which NO and DIs synergism might be crucial to enhance the repair process. The importance of NO during wound healing is well-known,[21,22] and we recently demonstrated the role of Class I DIs during skin repair processes.[15] Local administration of DIs within the wound promoted the release of specific growth factors (GFs) including Fibroblast Growth Factor 10 (FGF10), Epidermal Growth Factor (EGF), and Insulin Growth Factor I (IGF-I) from activated keratinocyte, thus accelerating wound closure.[15]

In the light of these findings, we have developed a new class of hybrid drugs in which MS-275 (**1**, Entinostat; pyridin-3-ylmethyl 4-(2-aminophenylcarbamoyl)benzylcarbamate),[23] a moderately selective Class I DIs, was joined to different NO-donor substructures. This letter reports the synthesis, structural characterization and biochemical profile of (6-(((3-cyanofuroxan-4-yl)methyl)(methylamino)methyl)pyridin-3-yl)methyl(4-((2-aminophenylcarbamoyl)benzyl))carbamate **2** (NO-MS275), the first example of this class of products. It was obtained by joining the lead 1 with the NO-donor 3-CN-furoxan moiety,[24] through a methyl(aminomethyl)methyl bridge. Other kinds of hybrids in which DIs are linked to tyrosine kinase inhibitors, have recently been reported.[25]

The product was prepared via the synthetic pathway depicted in Scheme 1. The *tert*-butyldimethylsilyl protected 5-(hydroxymethyl)pyridine-2-carbaldehyde **4** was subjected to reductive amination by action of methylamine and sodium borohydride, to give the related 2-methylaminomethyl substituted pyridine **5**. This intermediate, treated with 4-bromomethylfuroxan-3-carboxyamide (**6**) in acetone solution in the presence of $NaHCO_3$, gave the furoxan intermediate 7,

Scheme 1[a]



[a](a) $MeNH_2$, MeOH; $NaBH_4$, MeOH; (b) 4-bromomethylfuroxancarboxyamide (**6**), $NaHCO_3$, acetone; (c) CH_3COOH, H_2O, THF 3/1/1; (d) CDI, THF; (e) $(CF_3CO)_2O$, Py, THF, 0 °C; (f) CH_3SO_3H, THF.

which was subsequently deprotected using acetic acid in THF, to afford the expected alcohol **8**. Activation of **8** with *N*,*N*′-carbonyldiimidazole (CDI), followed by coupling, in THF, with the *tert*-butoxycarbonyl (BOC) protected intermediate **9**, gave rise to the furoxancarboxamide derivative **10**. This latter product was dehydrated by action of trifluoroacetic anhydride to the related carbonitrile **11**, which was subsequently deprotected with methanesulfonic acid to give the target compound **2** as its methanesulfonate salt.

The hybrid **2** has been extensively characterized biochemically, and its ability to trigger skin repair processes has been explored.

To evaluate the biological proprieties of **2**, assays were performed using the transformed human keratinocytes

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(HaCaT). NO release was analyzed using a 4,5-diaminofluorescein diacetate (DAF-2D) probe (Alexis). In these experiments, HaCaT cells were treated with **2** (10 μM) for 1 or 5 h. The lead **1** (10 μM) and the NO donor **3** (10 μM) were used as controls, whereas DMSO was used as solvent and was present in equal amounts in all experiments. DAF-2D was added to complete medium for the entire duration of the experiments; cells were washed with PBS, trypsinized, centrifuged, and analyzed by FACS. Figure 1 shows the NO

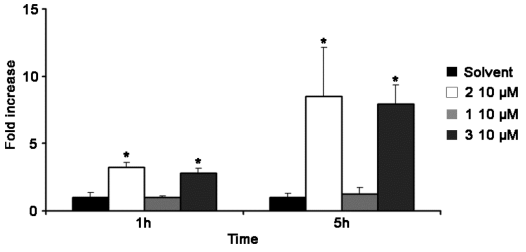

Figure 1. Evaluation of NO release by 4,5-diaminofluorescein diacetate (DAF-2D). Bars represent the increase in median fluorescence intensity. Data are presented as means ± SEM; * p < 0.05 versus the controls (t tests).

release observed after treatment with **2**: a significant increase occurred at 1 and 5 h compared to solvent alone. The lead **1** did not modulate NO production, whereas the NO donor **3** increased the number of NO positive cells to the same degree as did **2**, indicating that the hybrid is efficient at releasing NO.

When human HaCaT cells were incubated with SNP 1 mM, used as reference NO-donor compound, and with **2** at different concentrations, for 1 h, a highly significant dose-dependent production of cyclic GMP (cGMP) was observed, depending on the product's capacity to release NO (Figure 2).

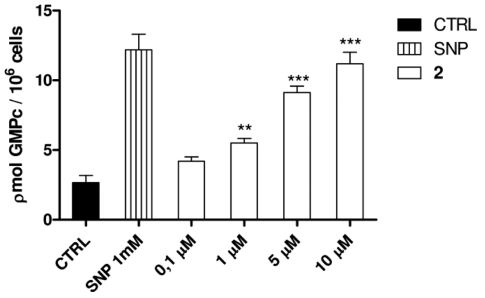

Figure 2. Production of cyclic GMP[3H] by **2**. Data are presented as means ± SEM; ** and *** indicate, respectively, p-values of <0.01 and <0.0001 versus controls (t tests).

The ability of **2** to inhibit HDAC activity was evaluated by a standard HDAC activity assay (BioVision). Fifty micrograms of HaCaT nuclear extracts were exposed to the hybrid **2** at three different concentrations (1, 10, and 50 μM) for 1 h. The lead **1**, the NO donor **3**, and trichostatin A (TSA) were used as controls, and DMSO as solvent. Figure 3 shows that **2** inhibits HDAC activity similarly to the same concentrations of **1**. Conversely, a larger amount of NO donor **3** is required to inhibit HDAC activity, indicating that HDAC inhibition of the hybrid **2** depends on the DI function. This shows that the hybrid **2** is simultaneously active as an NO donor and as a HDAC inhibitor.

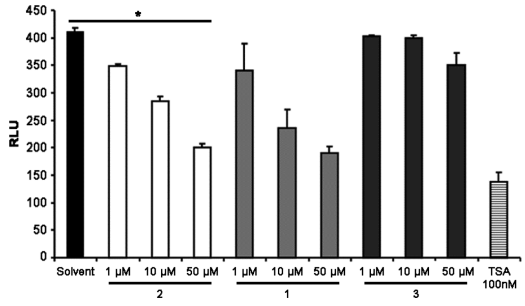

Figure 3. Evaluation of HDAC activity after treatment with **1**, **2**, **3**, and TSA. Data are presented as means ± SEM; * p < 0.05 versus the control group (t tests).

Studies have revealed NO-dependent regulation of the HDAC function.[2] NO affects Class I HDACs, especially HDAC2, by S-nitrosylation, which induces inhibition of the enzyme's activity.[26] Conversely, NO modulates Class II HDAC's nuclear shuttling, via an sGC/cGMP-dependent mechanism, which has been demonstrated in both endothelial cells (ECs)[6] and mouse embryonic stem cells (mES).[27] To better characterize the hybrid **2**, its effects on the regulation of different classes of HDAC were analyzed. To investigate HDAC2 S-nitrosylation, immunoprecipitation experiments were run with HaCaT cell extracts, obtained after treatment with **2** (10 μM) for 1 h. DMSO was used as solvent control and compound **3** (10 μM) as positive control for enzyme nitrosylation. Figure 4, panel A, shows that the NO released

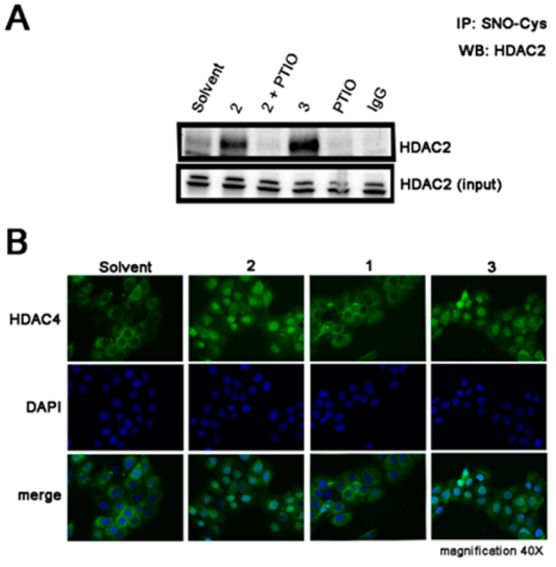

Figure 4. Different hybrid-dependent HDAC regulation. (A) Immunoprecipitation showing the effect of **2** on HDAC2 cysteine nitrosylation; concentration, **2** 10 μM, PTIO 100 μM, and **3** 10 μM. (B) Immunofluorescence panel showing the intracellular localization of HDAC4; green, HDAC4; blue, nuclei.

from **2** induces S-nitrosylation of HDAC2, thus demonstrating a significant effect on Class I HDACs. Notably, the effect of **2** on HDAC2 S-nitrosylation was abrogated by the use of the NO scavenger PTIO (100 μM) confirming the direct effect of NO on the HDAC target. In order to examine the effect of NO released by **2** on Class II HDACs, immunofluorescence analysis was employed, to establish the intracellular localization of HDAC4. HaCaT cells were treated independently with **2** (10

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μM), **1** (10 μM), or **3** (10 μM) for 1 h, and the localization of HDAC4 was determined at the end of treatment. DMSO was used as solvent control. Comparison of immunofluorescence images with those relating to solvent alone (Figure 4, panel B) showed that **2** promotes HDAC4 nuclear shuttling. Interestingly, compound **1** did not induce HDAC4 nuclear shuttling, whereas, as stated above, the NO donor **3** promoted Class IIa HDAC nuclear relocation. These results indicate that NO released from **2** is able to modulate Class IIa HDAC intracellular trafficking in HaCaT cells, similarly to what occurs in ECs and mES cells.[6,27]

Both NO and HDACs are able to modulate microRNA expression.[14,28] To investigate the additive contribution of the hybrid **2** compared to those made by its single components, we ran microRNA profiling, with TaqMan Human MicroRNA-B Arrays version 2.0 (Applied Biosystems, Foster City, CA). HaCaT cells were treated with **2** (10 μM) for 1 h. At the end of treatment, cells were collected and lysed with Trizol for total RNA extraction. The simple 4-(dimethylaminomethyl)furoxan-3-carbonitrile hydrochloride (**3**) (10 μM), taken as model of the furoxan component present in the hybrid, and **1** (10 μM) were also tested for comparison. DMSO was used as solvent control, and the solvent signal was subtracted from signals for each group. The Venn plot in Figure 5 shows microRNA

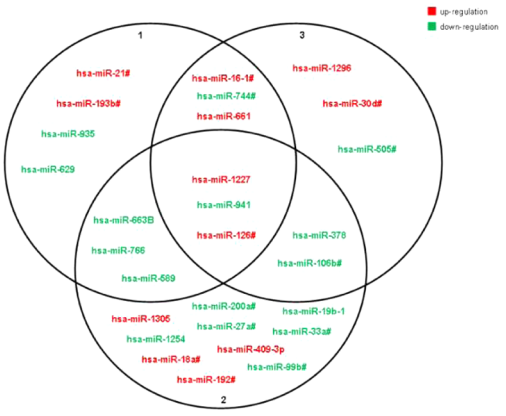

Figure 5. microRNAs profiling. The Venn plot represents microRNA modulation after 1 h of **1** (N = 4), **2** (N = 4), and **3** (N = 4) treatments.

modulation (red, up-regulation; green, down-regulation) produced by the distinct treatments. The intersection between the three groups indicates that **2** shares the ability to modulate miR1227, miR941, and miR126# with **1** and **3** but that it is unable to modulate certain other micro-RNAs, which conversely are modulated by these two latter products used separately. By contrast, **2** affects numerous micro-RNAs not modulated by its individual components. This picture suggests that the simultaneous regulation of NO- and HDAC-dependent pathways may induce additional specific effects.

In order to investigate whether the hybrid simultaneously displays both NO-dependent and HDAC-dependent activity in promoting skeletal muscle precursor differentiation, we investigated its in vitro capacity to modulate myotubes in mouse myoblast C2C12 cells. C2C12 cells were cultured in growth medium (20%) and treated for 24 h with compounds **1**, **2**, **3**, or **1** + **3** in combination. Differentiation was induced by reducing serum to 2% (differentiation medium). All compounds were used at 0.1 μM and renewed daily. Control cells were maintained in 20% serum (undifferentiated myoblast) or in the presence of solvent. During the first 72 h of differentiation, cell proliferation in response to treatments was analyzed. Figure 6 panel A shows that the hybrid **2**

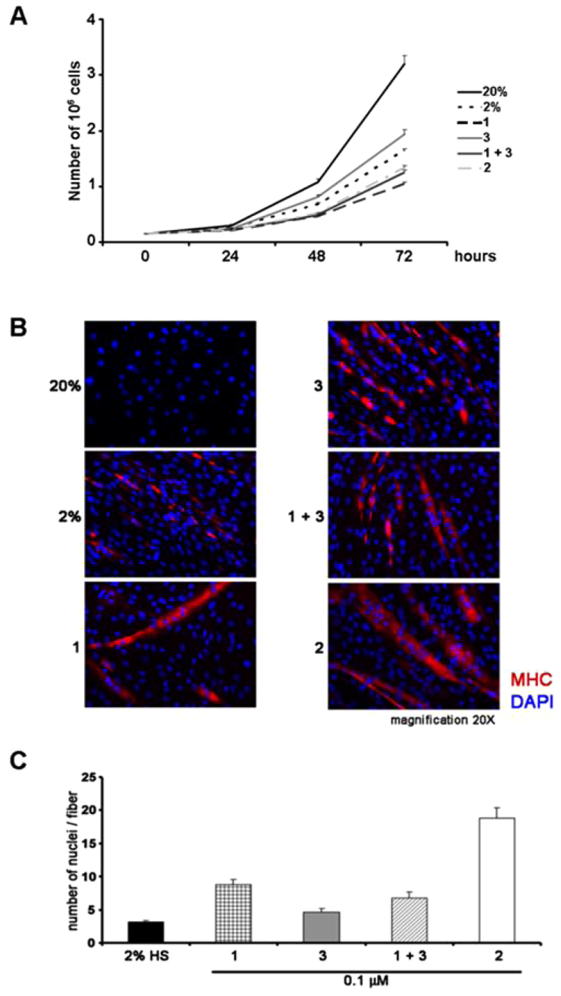

Figure 6. Evaluation of myogenic differentiation in C2C12 cells treated with **1**, **2**, **3**, or **1** + **3** in combination. (A) Growth curve of C2C12 in response to **1**, **2**, **3**, or **1** + **3** in combination (n = 4). (B) Immunofluorescence panels showing the rate of myogenic differentiation by MHC staining in response to **1**, **2**, **3**, or **1** + **3** in combination. Red, MHC; blue, nuclei; magnification 20×. (C) Number of nuclei per myotube in C2C12 cells after 7 days of differentiation, in the presence or absence of specific treatments. All compounds were used at 0.1 μM.

modulates cell proliferation similarly to **1** and to the combination of the two compounds, whereas the NO donor **3** enhances cell proliferation compared to solvent alone. To evaluate myogenic differentiation, at day 7, myosin heavy chain (MHC) positive fibers were determined by immunofluorescence, and the number of nuclei per myotube was counted manually. Figure 6 panel B shows that **2** promotes myogenic differentiation, inducing the formation of larger myotubes with a significantly larger number of nuclei per fibers (Figure 6, panel C) compared to the other conditions.

We developed the hybrid **2**, which is able to simultaneously release NO and regulate HDACs. The interaction with HDACs is the central core of this compound. It can differentially regulate two classes of HDACs: Class I and Class II. It strongly

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inhibits Class I HDACs either directly, as does the lead **1**, or indirectly, via S-nitrosylation, due to its ability to release NO. The NO released from the hybrid may activate Class II nuclear shuttling and chromatin modifications, with consequences on gene expression. The former process is regulated by the product's ability to activate the sGC/cGMP pathway. Finally, the microRNA profiling shows the ability of **2** to modulate micro-RNAs. Interestingly, it can affect numerous micro-RNAs that are not modulated by its individual components **1** and **3**. Further, the hybrid **2** showed a stronger differentiation potential compared to the mixture of the two compounds. Hybrid **2** is the first example of a new class of NO-donor HDACs under development in our laboratory. On the basis of the results described here, we conclude that this family of products is worthy of additional studies.

■ ASSOCIATED CONTENT

Supporting Information

Syntheses and characterization data for the compounds and procedures for biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

HDACs, hystone deacetylases; HATs, histone acetyltransferases; sGC, soluble guanylate cyclase; cGMP, cyclic guanylate cyclase; PP2A, protein phosphatase 2A; DIs, HDAC inhibitors; ECs, endothelial cells; mES, mouse embryonic stem cells

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