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Design, synthesis and preliminary biological evaluation of new hydroxamate histone deacetylase inhibitors as potential antileukemic agents

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ABSTRACT

This study concerns the synthesis of new histone deacetylase inhibitors (HDACi) characterized by a 1,4-benzodiazepine ring used as the cap, joined through an amide function or a triple bond as connection units, to a linear alkyl chain bearing the hydroxamate function as Zn^{2+}-chelating group. Biological tests performed in human acute promyelocytic leukemia NB4 cells showed that new hybrids can induce histone H3/H4 acetylation, growth arrest, and also apoptosis. Notably, chiral compounds exhibit stereoselective activity.

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Histone deacetylases (HDAC) and the other enzymes involved in chromatin remodeling are important targets for anticancer therapy, as inferred by the number of inhibitors (henceforth histone deacetylase inhibitors, HDACi) which have entered clinical trials as antineoplastic agents.[1,2] Several HDAC isoforms exist, classified into four distinct classes which differ for cellular localization, targeted acetylated proteins, and sensitivity to inhibitors.[3] The first HDACi to be studied was trichostatin-A (TSA, Fig. 1), a hydroxamic acid derivative: this functional group is important for activity since it coordinates the Zn^{2+} cation located at the bottom of the pocket for the *N*-acetylated residue of lysine.[4] Several other hydroxamic acid derivatives were independently developed, including SAHA (suberoyl anilide hydroxamic acid or Vorinostat, Fig. 1), recently approved by the FDA for the treatment of cutaneous T-cell lymphoma.[5] Other either natural or synthetic non-hydroxamate HDACi have also been developed and entered clinical trials.[6,7]

Classical HDACi share a common pharmacophore: a Zn^{2+}-chelating group joined, through a suitable linker, and connection unit, to a hydrophobic cap. The flexible part of the molecule enters the active site of the enzyme, while the cap should interact with the external domain of the enzyme and regulate the interaction between HDAC and the substrate, whether histone or non-histone protein. Much work has been done to optimize the chelating group, linker and connection unit, counting on (i) the availability of X-ray structures of the complexes between inhibitors and HDAC or analogous proteins, and also (ii) the high degree of homology, among the isoforms, of the catalytic domain, which has allowed the building of homology models.[8–11] In turn, there are more problems to optimize the cap through structure-based drug design: the modeling of the external domain of the proteins is hampered by the great variability in the surface regions, due to both the flexibility of the external loops and the differences in the sequences which allow specificity in the interaction with different substrate proteins.

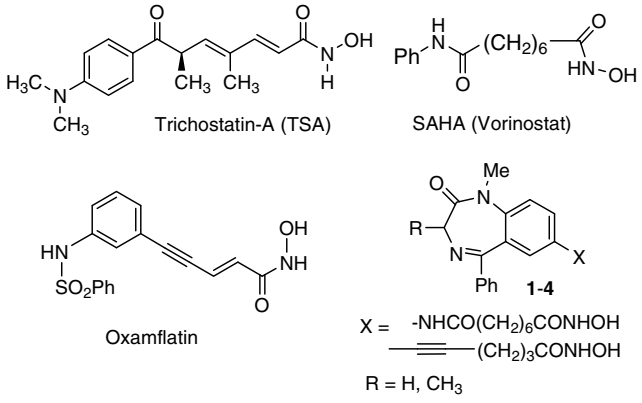

Figure 1. Structure of HDAC-inhibitors.

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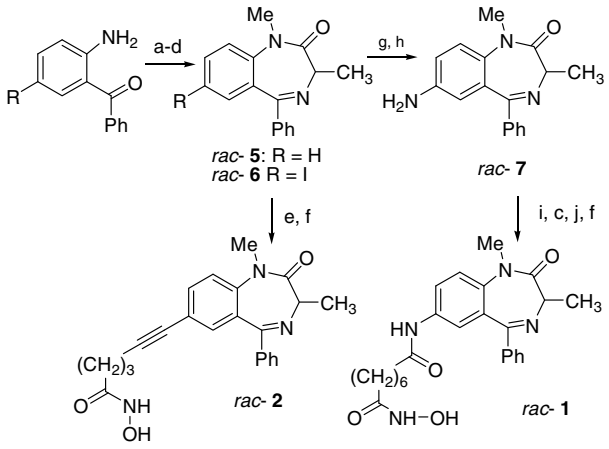
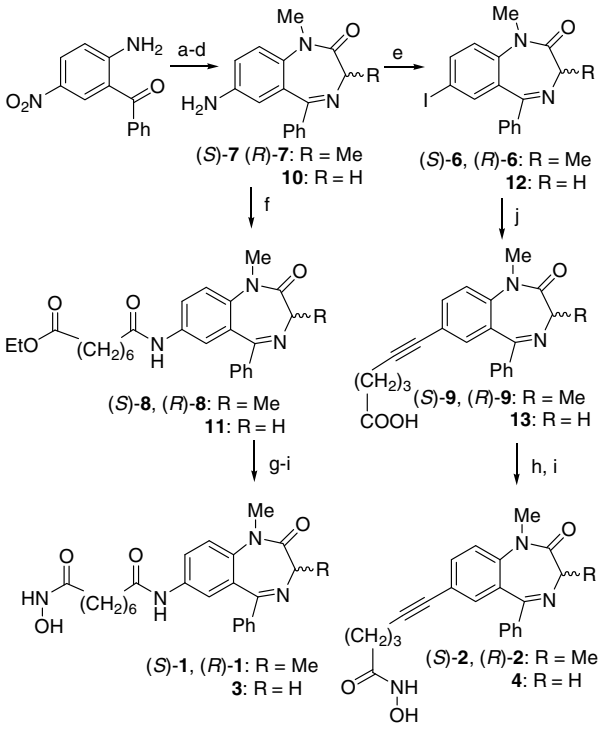
Previously, some of us showed that antagonists of platelet-activating factor receptor (PAF-R) such as WEB-2086 and WEB-2170 were capable of inducing erythroleukemia cell maturation, triggering apoptosis in human promyelocytic leukemia cells, and promoting differentiation in human breast cancer cells.[12–14] The chemical structure of these PAF-R antagonists is similar to that of anxiolytic benzodiazepines (BDZ), which were reported to exert cytostatic/differentiative effects in a variety of transformed cell types,[15,16] through mechanisms that, apparently, did not involve the peripheral BDZ receptor.[15,17]

Considering that the BDZ nucleus may be a versatile scaffold to design new anticancer agents (see for instance Refs. 18 and 19) and that HDACi were effective drugs to treat some forms of leukemia,[7,20,21] we thought it interesting to hybridize the structure of BDZ and SAHA to obtain HDACi with enhanced antineoplastic properties. Toward this aim, the 5-phenyl-1,3-dihydro-2-oxo-benzo[*e*][1,4]-diazepine ring was joined, using an amide function or a triple bond as connection units, to a linear alkyl chain bearing the hydroxamate function as chelating group, obtaining compounds **1**–**4** (Fig. 1). While the amide is a widely used connection unit,[6] the less frequent triple bond is found in compounds such as oxamflatin.[22] We initially chose to place the side chain in the 7-position of the BDZ ring, leaving the exploration of other positions to future work.[23] Finally, since the differentiation of Friend erythroleukemia cells induced by BDZ was reported to be stereoselective,[16] we introduced a chiral center in position 3 of the 1,4-diazepine ring (compounds **1** and **2**) and then compared their HDACi activities to those of the non-chiral analogues **3** and **4**.[24] During our work on this research, other HDACi, different from ours, and carrying a benzo[*e*][1,4]diazepin-2,5-dione nucleus as the cap, were reported.[25,26]

Racemics **1** and **2** were synthesized first (Scheme 1). Thus, *rac*-**5** and *rac*-**6** were prepared starting from, respectively, 2-aminobenzophenone or 4-iodo-2-aminobenzophenone.[27] Treatment of *rac*-**5** with potassium nitrate in sulfuric acid, followed by reduction with stannous chloride gave *rac*-**7** in 30% yield, which was treated with ethyl hydrogen suberate, followed by hydrolysis of the ester function and reaction with *N*-*tert*-butyldimethylsilylhydroxylamine. Chromatographic separation of the complex reaction mixture did not afford the protected hydroxamate but yielded a small amount of *rac*-**1** (10% overall yield starting from *rac*-**7**). For the synthesis of *rac*-**2**, the Sonogashira reaction between *rac*-**6** with *N*-(*tert*-butyldimethylsilyloxy)hex-5-ynamide, obtained by coupling commercially available 5-hexynoic acid with *N*-*tert*-butyldimethylsilylhydroxylamine, gave again a complex mixture, from which *rac*-**2** was obtained in low yield.



Scheme 1. Reagents: (a) N-BOC-alanine, WSC, HOBT; (b) HCl, MeOH; (c) NaOH; (d) NaOMe, Me_2SO_4; (e) *N*-(*tert*-butyldimethyl-silyloxy)hex-5-ynamide, CuI, $Pd(PPh_3)_4$, Et_3N; (f) chromatographic separation; (g) KNO_3, H_2SO_4; (h) $SnCl_2{\cdot}2H_2O$, HCl; (i) ethyl hydrogen suberate, WSC, HOBT; (j) $TBDMSONH_2$, BOP–Cl.



Scheme 2. Reagents: (a) Fmoc-alanine, or Fmoc-glycine, $SOCl_2$; (b) Et_3N, CH_2Cl_2; (c) Me_2SO_4, MeONa; (d) $SnCl_2{\cdot}2H_2O$, HCl; (e) $NaNO_2$, KI; (f) ethyl hydrogen suberate, WSC, HOBT; (g) NaOH; (h) $TBDMSONH_2$, BOP–Cl; (i) MeOH; (j) 5-hexynoic acid, CuI, $Pd(PPh_3)_4$, Et_3N. The synthesis of (*S*)-**1** and (*S*)-**2**, or (*R*)-**1** and (*R*)-**2**, started from enantiopure (*S*)-Fmoc-alanine of (*R*)-Fmoc-alanine, respectivley.

In order to improve yields, the synthesis of the enantiomers of **1** and **2** was performed in a different way (Scheme 2). Commercially available 2-amino-4-nitrobenzophenone was coupled with enantiopure Fmoc-alanine; deprotection, N-alkylation and reduction gave (*S*)-**7** and (*R*)-**7** in 50% yield. These compounds were converted into the ester (*S*)-**8** and (*R*)-**8** as described for the racemate; coupling with *N*-*tert*-butyldimethylsilylhydroxylamine, followed directly by deprotection gave (*S*)-**1** and (*R*)-**1** in 20% yield. Treatment of (*S*)-**7** and (*R*)-**7** with sodium nitrite and potassium iodide gave, respectively, (*S*)-**6** and (*R*)-**6**, which were reacted with 5-hexynoic acid to give (*S*)-**9** and (*R*)-**9**. Coupling with *tert*-butyldimethylsilylhydroxylamine, followed by deprotection gave (*S*)-**2** and (*R*)-**2** in 22% yield. The same pathway was applied to the synthesis of compounds **3** and **4**, starting from commercially available Fmoc-glycine (Scheme 2).

Biological activities of newly synthesized HDACi were assessed by using the human acute promyelocytic leukemia cell line NB4[28] as the experimental model. Procedures employed for cell culture, analysis of proteins and of apoptosis were as described previously.[13]

Initially, we tried to assess whether the new HDACi were capable of inducing acetylation of histone H3 and H4 isoforms. Results reported in Figure 2 concern samples of promyelocytic leukemia cells NB4 which were incubated in culture for 6 h with increasing drug concentrations to determine levels of acetylated histones by Western blot. All compounds tested were capable of promoting histone H3 acetylation in a dose-dependent manner and, on average, alkyne derivatives **2** and **4** were approximately two to three times more active than suberoyl derivatives **1** and **3**. This was valid also for drug-induced H4 acetylation by new HDACi, apart from compounds (*R*)-**2** and (*S*)-**1** which, under these experimental con-

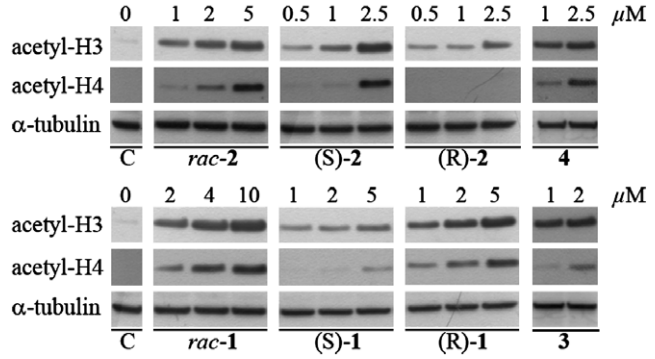


Figure 2. Dose-dependent acetylation of H3 and H4 histone isoforms. NB4 cells were incubated for 6 h with the drugs, then harvested and processed for immunodetection, by Western blot, of acetylated H3/H4, and of α-tubulin as the loading control (see Supporting information).

ditions, gave a null or a faint signal, respectively, to denote their low HDACi activity in contrast to the high activity of enantiomers (*S*)-**2** and (*R*)-**1**. Moreover, histone H3 appeared to be more sensitive than histone H4 to drug-induced acetylation. Apparently, the presence of the 3-methyl group in the BDZ cap did not interfere significantly with efficacy of new HDACi; on the contrary, the connection unit and length of the hydroxamic chain was relevant to HDACi activity, as the relatively short alkyne chain mimicking that of oxamflatin, was more effective than the longer six-methylene chain typical of SAHA (Fig. 1).

Subsequently, new hydroxamate-HDACi were assayed for their inhibitory effects on NB4 cell growth (Table 1). IC_{50} values indicated that (i) short-chain HDACi (**2** and **4**) were definitely more active than long-chain HDACi (**1** and **3**), and (ii) (*S*)-**2**, **4** and SAHA were the most effective compounds yielding overlapping values of approximately 0.6 μM.

Notably, compound **2** showed enantioselectivity as far as HDAC inhibition and antiproliferative effect were concerned. In fact, (*S*)-**2** was eight times more active than its *R*-enantiomer in decreasing NB4 cell growth (Table 1). Accordingly, Western blot analysis showed a clear difference in the amount of acetylated H3 and H4 histones induced by the two enantiomers (Fig. 2); (*S*)-**2** was more potent than (*R*)-**2** in inducing H3 acetylation, but only (*S*)-**2** was able to increase the amount of acetylated H4 histone, while the *R*-enantiomer seemed to be inactive. With regard to compound **1**, there was practically no enantioselectivity in terms of growth inhibition, although the *R*-enantiomer displayed higher potency than the *S*-one (Table 1); on the contrary, enantioselectivity was clearly evident looking at the different levels of acetylated H3 and H4 histones (Fig. 2), the eutomer being the (*R*)-enantiomer. These findings highlighted an inversion of the stereochemical preference between suberoyl and alkyne derivatives, thus suggesting a different selectivity for and/or a different way of binding to HDACs.

Enantioselectivity is not a rare event in the field of HDACi, although the majority of synthetic HDACi are non-chiral compounds.[3,6] Enantiopure compounds have been synthesized, mainly designed from cyclic peptides or derived from L-aminoacids.[29–32] In some instances both enantiomers have been synthesized and studied[33–37]: usually, the chiral center has been placed close to the rim of the HDAC active site channel. In our compounds, the chiral center placed on the cap was relatively far from the active site channel; while the addition of a small methyl group in position 3 on the BDZ nucleus was not so important for potency, since (*S*)-**2** and **4** showed similar IC_{50} values, its direction seemed to be crucial for activity as (*R*)-**2** was much less active than (*S*)-**2** or **4**. In addition, suberoyl and alkyne derivatives showed a different stereochemical preference. Apparently, the different connection unit (amide linkage or triple bond), possibly coupled with a different length of the methylene chain, allowed a different orientation of the cap on the surface of the enzyme; this enables compounds **1** and **2** to have access to spaces and/or to establish interactions, which have different stereochemical requirements.

To further characterize the compounds, *rac*-**2** and the two enantiomers (*S*)-**2** and (*R*)-**2** were examined by flow cytometry for their ability to decrease viability and to induce apoptosis in leukemic cells (Fig. 3). A 48-h treatment of NB4 with 5 μM *rac*-**2** or 2.5 μM (*S*)-**2** led to a massive apoptosis as indicated by the high percentages of cells in LR (lower right) and UR (upper right) quadrants and a few viable cells in LL (lower left) quadrants. Instead, in cultures either untreated (control) or treated with 2.5 μM (*R*)-**2**, viable cells in LL quadrants accounted for approximately 90% of the population with a negligible presence of apoptotic/necrotic bodies.

Table 1
Inhibition of NB4 cell growth by new HDACi

N	IC_{50} (μM)
rac-**1**	4.09 ± 0.37
(*S*)-**1**	5.83. ± 0.28
(*R*)-**1**	3.71 ± 0.15
rac-**2**	1.26 ± 0.11
(*S*)-**2**	0.61 ± 0.04
(*R*)-**2**	4.80 ± 0.31
3	2.50 ± 0.22
4	0.62 ± 0.03
SAHA	0.60 ± 0.04

Inhibitory effects of new HDACi on NB4 cell proliferation. Cells were incubated for 48 h with increasing amounts of listed compounds and then counted with the aid of a Bürker chamber. IC_{50} values—that is, the drug concentration (μM) necessary to reduce cell density by 50% relative to control—were the means ± SD of three separate experiments. Data relative to SAHA were reported for comparison.

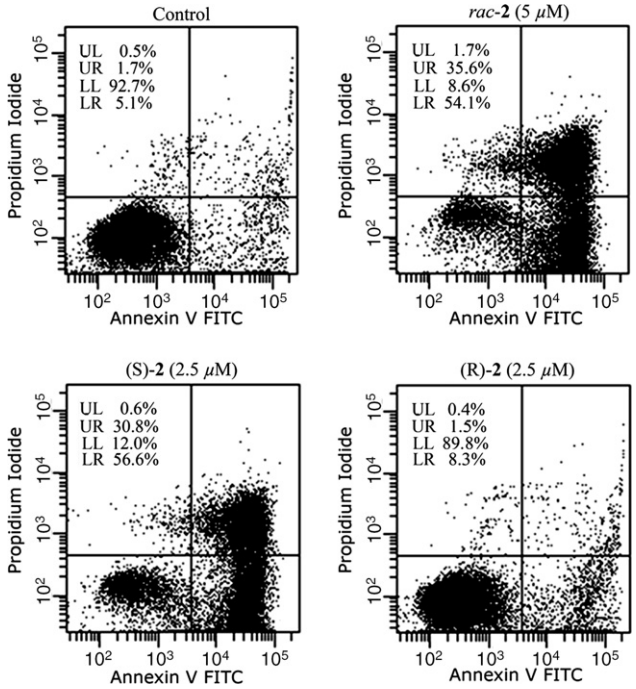


Figure 3. Cytofluorimetric analysis of apoptosis in HDACi-treated NB4 cells. Cells (0.5×10^6/ml) were incubated for 48 h either in the absence (control) or in the presence of *rac*-**2** (5 μM), (*S*)-**2** (2.5 μM) and (*R*)-**2** (2.5 μM). Cells were stained with Annexin-V-Fluos and PI using a binding buffer according to the manufacturer's instructions. *X*-axis, the fluorescent intensity of Annexin-V; *Y*-axis, the fluorescent intensity of PI. Values reported in each panel indicated the% of necrotic cells (upper left), late apoptotic cells (upper right), live cells (lower left), and early apoptotic cells (lower right). Results of a typical experiment out of three.

These data underlined once more the difference between active (*S*)-**2** and inactive (*R*)-**2** enantiomers.

In conclusion, newly synthesized hydroxamate-BDZ hybrids showed to be powerful HDACi regardless of the presence or absence of a chiral center, as activity of compounds **1** and **2** was comparable to that of their non-chiral analogues **3** and **4**. Even so, chiral compounds demonstrated to be stereoselective.

Compounds capable of promoting acetylation of histone H4, rather than histone H3, were also the most effective inhibitors of leukemia cell growth and the most active inducers of apoptosis. As regards growth inhibition activity (*S*)-**2**, **4** and SAHA yielded overlapping IC_{50} values. The length and structure of the hydroxamic chain can influence HDAC inhibitory properties: alkyne derivatives (short-chain) were approximately two to three times more active than suberoyl derivatives (long-chain). Work is underway to optimize the length of the polymethylene chain.

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Supplementary data

Experimental sections are available via the internet. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.07.119.

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