DOI: 10.1002/cmdc.200900082

Pyrrole-Based Hydroxamates and 2-Aminoanilides: Histone Deacetylase Inhibition and Cellular Activities

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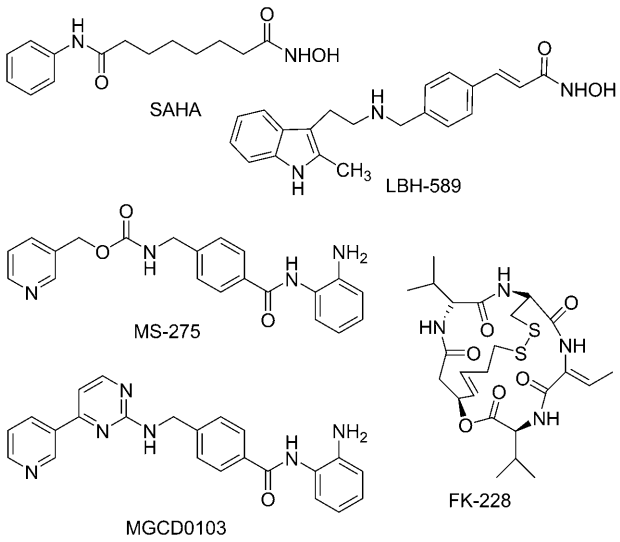
Histone acetylation/deacetylation is essential for chromatin remodeling and epigenetic regulation of gene transcription in eukaryotic cells. The acetylation level of the histone lysine residues is controlled by two enzyme superfamilies with opposite actions, histone acetyltransferases (HATs) and histone deacetylases (HDACs), and a shift in the balance of chromatin acetylation may result in changes in the regulation of patterns of gene expression.[1] HDACs have been implicated in several processes such as neoplasias, cardiac hypertrophy, T-cell differentiation and neuronal survival.[2,3] In cancer cells, HDAC inhibition causes histone hyperacetylation and leads to transcriptional activation of genes associated with growth arrest, terminal differentiation and/or apoptosis, both in vitro and in vivo.[2,4–6]

At the present there are 18 known human HDACs grouped into four classes based on the structure of their accessory domains. Classes I (HDAC1–3, 8), II (HDAC4–7, 9, and 10, of which HDAC4, 5 , 7, and 9 form the class IIa, and HDAC6 and 10 are considered as belonging to class IIb), and IV (HDAC11) enzymes are zinc(II)-dependent enzymes, while class III enzymes (also known as sirtuins) are defined by their dependency on NAD^+ for catalytic action. In addition to histones, HDACs have been shown to deacetylate a growing number of nonhistone proteins such as transcriptional activators, basal transcription factors (i.e., p53), and structural proteins (i.e., α-tubulin). While class I HDACs are ubiquitously expressed in tissues, located mainly in the nucleus of the cells, and often act as transcriptional corepressors, class II HDACs are present both in nucleus and cytoplasm with or without the shuttling system, have a tissue-specific expression, and appear to target selected cell physiological programs.

Generally, elevated class I (HDAC1–3) and more specifically HDAC1 expression is associated with poor prognosis and enhanced proliferation of gastric,[7,8] pancreatic,[9,10] colorectal,[11,12] prostate,[13] hepatocellular,[14] lung[15] and breast[16,17] cancer, and chemotherapy-resistant neuroblastoma in vitro.[18] The role of class II HDACs in cancer is less clear. Indeed, class II HDACs tended to be downregulated in human tumors, and high expression in some tumors has been linked to improved prognosis.[19,20] On the other hand, HDAC4 expression has been shown to be upregulated in breast cancer compared with renal, bladder, and colorectal cancer,[11] and in acute promyelocytic leukemia (APL) cells HDAC4 interacts with the PLZF-RARα fusion protein to repress differentiation.[21] HDAC5 and HDAC7 were highly expressed in colorectal cancer in contrast to bladder, renal, and breast cancer.[11] HDAC6 was upregulated in oral squamous cell carcinoma,[22] and its inhibition depleted the Bcr-Abl oncoprotein in K562 leukemia cells through acetylation of heat shock protein 90 (Hsp90) and subsequent disruption of its chaperone function.[23] HDAC6 inhibition also caused antiangiogenic effects through the regulation (with HDAC4) of HIF-1α transcriptional activity,[24] and Hsp90-mediated proteasomal degradation of vascular endothelial growth factor receptors.[25] HDAC6 inhibition by the selective inhibitor tubacin[26,27] causes growth inhibition of multiple myeloma cells,[28] activation of the caspases and apoptosis. Moreover, α-tubulin hyperacetylation has been reported to increase vesicular transport of brain-derived neurotrophic factor (BDNF).[29]

A number of small-molecule HDAC inhibitors (HDACi), including the hydroxamates suberoylanilide hydroxamic acid (SAHA, vorinostat),[30] recently approved by the FDA for the treatment of advanced cutaneous T-cell lymphoma (CTCL),[31] and LBH-589 (panobinostat),[32] the benzamides MS-275[33] and MGCD0103,[34] and the depsipeptide FK-228 (romidepsin),[35]



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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200900082.

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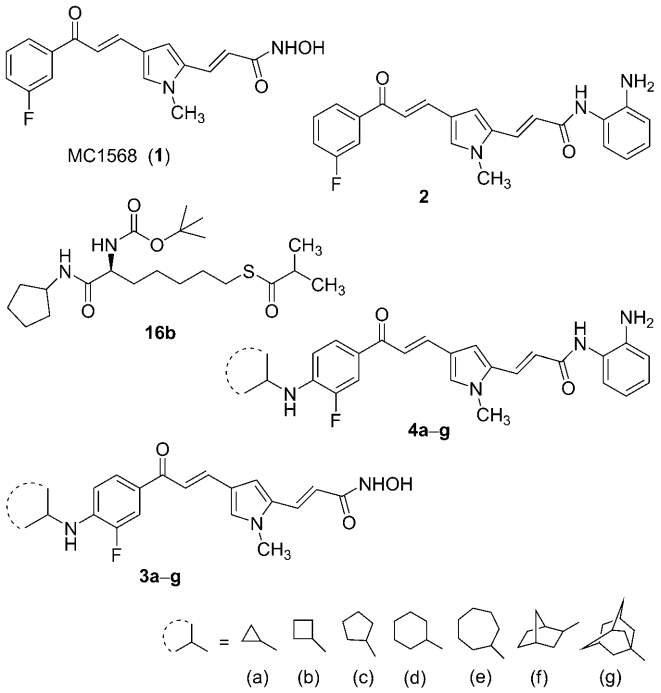
displayed in vivo antitumor efficacy and are in various phases of clinical trials for anticancer therapy.

In 2005, we identified MC1568 (**1**) as a class IIa-selective HDACi.[36,37] In immunoprecipitation (IP) assays, MC1568 (5 μM) displayed 54.9% inhibitory activity against HDAC4 (ZR-75.1 cells) and 0% against HDAC1 (U937 cells). It was subsequently shown to increase the level of α-tubulin acetylation (functional test for HDAC6 inhibition) in Jurkat, K562, chronic lymphocytic leukemia (CLL), and MCF-7 cells.[38,39] Therefore, MC1568 can be considered a pan-class II-selective HDACi. In 2007, Itoh et al. reported a series of HDAC6-selective inhibitors, including compound **16b**, which showed growth inhibition in MCF-7 breast cancer cells after estrogen stimulation.[40] The structural motifs of such inhibitors, in particular the cycloalkylamino moiety and the *tert*-butoxycarbonyl portion at the CAP group attracted our attention. Thus, also considering the important biological effects shown by compound **1**,[38,39,41–43] we designed and synthesized a series of analogues of **1** belonging to both the hydroxamate (compounds **3a–g)** and 2-aminoanilide (compounds **4a–g)** series, in addition to the *N*-(2-aminophenyl)-3-(4-(3-(3-fluorophenyl)-3-oxoprop-1-enyl)-1-methyl-1*H*-pyrrol-2-yl)acrylamide **2**. In particular, herein we report the effects of the introduction of several bulky cycloalkylamino groups into the CAP moiety (at the benzene C4 position) of compounds **1** and **2**.



Lead compound **1** and derivatives **2**–**4** were tested at 5 μM against human recombinant (hr) HDAC1, HDAC4 and HDAC6 enzymes. These compounds were also subjected to functional tests at 5 μM to ascertain their effects on histone H3 and α-tubulin acetylation levels in human leukemia U937 cells. Finally, in the U937 cell line their effects on cell cycle and apoptosis induction at 5 μM were determined.

Compounds **3a–g** and **4a–g** were synthesized from the 3,4-difluoroacetophenone. The starting material was reacted with the corresponding cycloalkylamine in the presence of K_2CO_3 to obtain the intermediate **5a–g**. Further condensation of **5a–g** with the ethyl 3-(4-formyl-1-methyl-1*H*-pyrrol-2-yl)-2-propenoate[36] under basic conditions furnished the ethyl 3-(4-(3-(4-(cycloalkylamino)-3-fluorophenyl)-3-oxoprop-1-enyl)-1-methyl-1*H*-pyrrol-2-yl)acrylates **6a–g**, which underwent alkaline hydrolysis to yield the corresponding acrylic acids **7a–g**, key intermediate for the synthesis of the final compounds. Reaction of these key intermediates **7a–g** with ethyl chloroformate, followed by addition of *O*-(2-methoxy-2-propyl)hydroxylamine[44] and further cleavage in the presence of the Amberlyst 15 ion-exchange resin furnished the desired 3-(4-(3-(4-(cycloalkylamino)-3-fluorophenyl)-3-oxoprop-1-enyl)-1-methyl-1*H*-pyrrol-2-yl)-*N*-hydroxyacrylamides **3a–g**. The 2-aminoanilide derivatives **2** and **4a–g** were synthesized from the 3-(4-(3-(3-fluorophenyl)-3-oxoprop-1-enyl)-1-methyl-1*H*-pyrrol-2-yl)acrylic acid[36] and **7a–g**, respectively, by treatment with benzotriazole-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (Bop) and the *o*-phenylendiamine in the presence of triethylamine (Scheme 1).

Compounds **2**–**4** were tested at 5 μM against hr HDAC1, HDAC4 and HDAC6. Compound **1** and SAHA were used as reference drugs. The results showed that the hydroxamate derivatives **3**, in contrast to the lead compound **1**, showed moderate to high inhibition (22–80%) of HDAC1 (Table 1). Conversely, the same compounds were weakly active or inactive against HDAC4, with the exception of **3f** that showed 30% inhibition of HDAC4 at 5 μM. Against HDAC6, compound **1** displayed 35% of inhibition at 5 μM, and the hydroxamates **3** were equally or more active, with the exception of **3d** that was less effective against this isoform (21%). Compounds **3a** and **3b**, carrying a cyclopropylamino and a cyclobutylamino moiety at the C4 position of the benzene ring, respectively, displayed the highest inhibitory activity against both HDAC1 and HDAC6 (60–80%) among the tested derivatives. When compared with SAHA, they showed slightly lower or similar activities against HDAC1, and were more efficient against HDAC6. 2-Aminoanilides **2** and **4** failed to effectively inhibit HDAC1, with the exception of **4f**, that displayed appreciable (40%) HDAC1 inhibition at 5 μM. Additionally, compounds **2** and **4a–g** displayed no or weak activity against HDAC4, with the exception of **4e** that inhibited the enzyme by 38% at 5 μM. Conversely, compounds **2** and **4a–g** inhibited hrHDAC6 to varying extents. To clarify the isozyme selectivity of unique HDAC1/HDAC6 inhibitors, such as **3a**, **3b**, and **3g**, the IC_{50} values of these compounds against HDAC1 and HDAC6 were determined (Table 2). Compounds **3a** and **3b** showed nanomolar inhibition of HDAC1 and (sub)micromolar inhibition of HDAC6. The replacement of the cyclopropyl/cyclobutylamino moieties of **3a** and **3b** with the bulkier 1-adamantylamino substituent (**3g**) led to a decrease in both the HDAC1 and HDAC6 inhibitory activities, to different degrees (307–460 fold and 6–8 fold decrease for HDAC1 and HDAC6, respectively).

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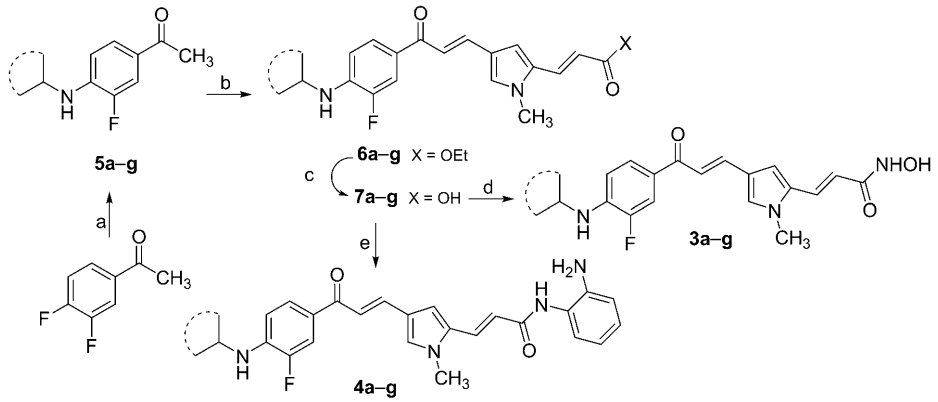

Scheme 1. *Reagents and conditions*: a) RNH_2, anhyd K_2CO_3, DMF, 90 °C, overnight, 59–78%; b) Na, C_2H_5OH, RT, 5 h, 62–76%; c) KOH, C_2H_5OH, 80 °C, 2 h, 77–87%; d) 1) $ClCOOC_2H_5$, $(C_2H_5)_3N$, anhyd THF, 0 °C; 2) $CH_3OC(CH_3)_2ONH_2$, anhyd THF, 0 °C; 3) Amberlyst 15, CH_3OH, RT, 1.5 h, 55–66%; e) 1) $(C_2H_5)_3N$, BOP, anhyd DMF, N_2 atmosphere, RT; 2) *o*-phenylendiamine, anhyd DMF, N_2 atmosphere, RT, 1 h, 64–74%.

Table 1. Human recombinant HDAC1, HDAC4, and HDAC6 inhibitory activities of compounds **1**–**4**.

Compd	Inhibition at 5 μM [%][a]		
	HDAC1	HDAC4	HDAC6
1	5	45	35
2	0	8	30
3a	80	0	62
3b	78	0	60
3c	30	17	30
3d	22	16	21
3e	32	0	32
3f	30	30	43
3g	38	0	40
4a	2	0	15
4b	5	0	20
4c	15	0	25
4d	0	13	25
4e	0	38	30
4f	40	19	25
4g	0	0	15
SAHA	88	49	53

[a] Data represent mean values of at least three separate experiments.

Table 2. IC_{50} values of selected derivatives **3a**, **3b**, and **3g** against human recombinant HDAC1 and HDAC6.

Compd	IC_{50} [μM][a]	
	HDAC1	HDAC6
3a	0.02	1.1
3b	0.03	0.8
3g	9.2	6.7

[a] Data represent mean values of at least three separate experiments.

The effects of compounds **2**–**4** (5 μM, 24 h) on histone H3 and α-tubulin acetylation levels, taken as markers of class I HDACs and HDAC6 inhibition, respectively, were determined in the human leukemia U937 cell line (Figure 1). SAHA, MS-275 and compound **1** were used as reference compounds. As seen by Western blot analysis, the hydroxamates **3a**–**g** typically increased the acetyl-H3 levels, **3a** and **3b** being the most potent in this test. Conversely, the 2-aminoanilides **4a**–**g** seem to be less efficient than the corresponding hydroxamates (Figure 1a). In the α-tubulin acetylation assay (Figure 1b), the majority of the tested hydroxamates **3**, as well as the 2-aminoanilides **4**, produced high acetyl-α-tubulin levels, with the exception of **3g**, which showed lower activity possibly due to poor cell permeability or differences in the kinetics of induction. These data confirmed the capability of such compounds to inhibit the HDAC6 activity.

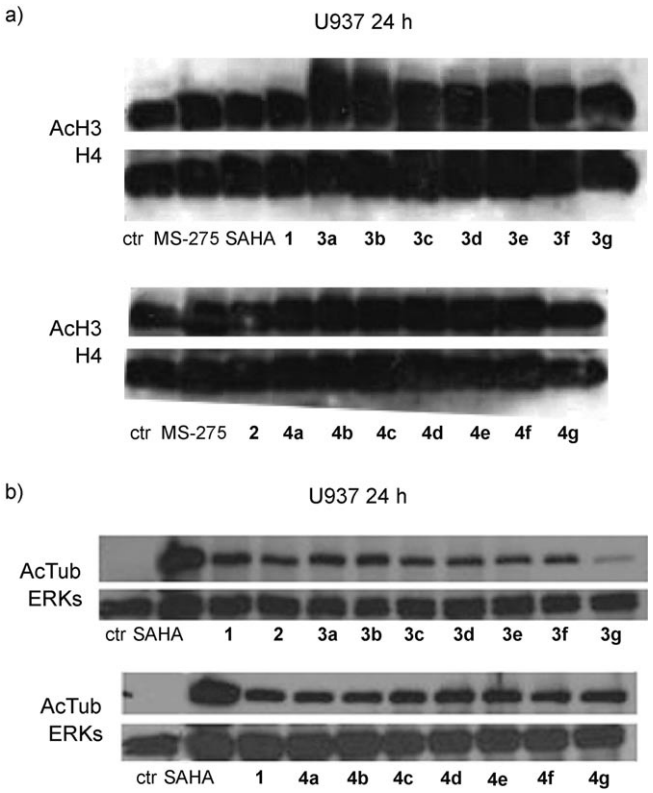

Figure 1. Western blot analysis on human leukemia U937 cells performed with derivatives **2**–**4** (at 5 μM) to determine their effects on a) histone H3 and b) α-tubulin acetylation. SAHA, MS-275 and compound **1** were tested for a direct comparison.

The pyrrole-based derivatives **2**–**4** were tested in the U937 cell line (5 or 1 μM, 30 h), to evaluate their effects on cell cycle and apoptosis induction. Compound **1** and SAHA were used as reference drugs. Among the tested compounds, the hydroxa-

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mates **3f** and **3g** blocked cell cycle in the S phase; this was more evident with the 2-aminoanilides **4c**–**e** (Figure 2). Derivatives **3a** and **3b** had to be tested at 1 μM because cell death at 5 μM was too great. The induction of apoptosis was measured using the caspase 3–7 method (Figure 3). The results revealed that **3a** and **3b**, tested at 1 μM, gave high apoptosis induction (28.8 and 28.3%, respectively) in the U937 cell line, higher than SAHA (16.3%), which was tested at 5 μM. Among the remaining derivatives, only the 2-aminoanilide **4a** gave appreciable apoptosis induction (14.5%) in our assay, though this effect seems to be unrelated to HDAC inhibition.

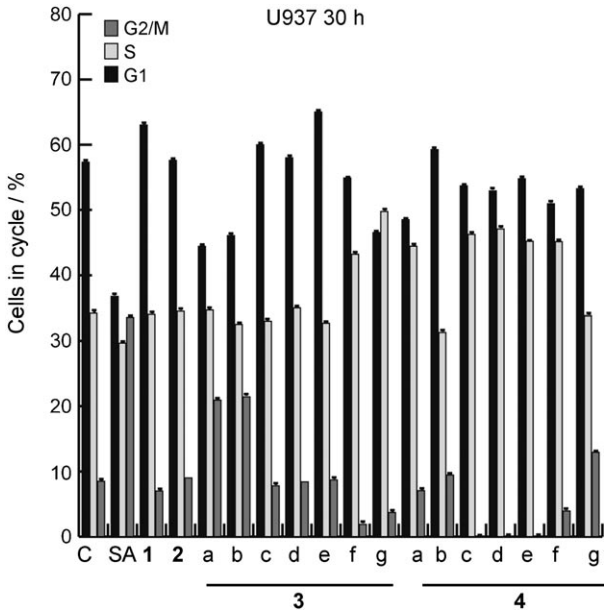

Figure 2. Effects of compounds **2**–**4** on cell-cycle phases in the human leukemia U937 cell line over 30 h. Compounds were used at 5 μM except for compounds **3a** and **3b**, which were tested at 1 μM.

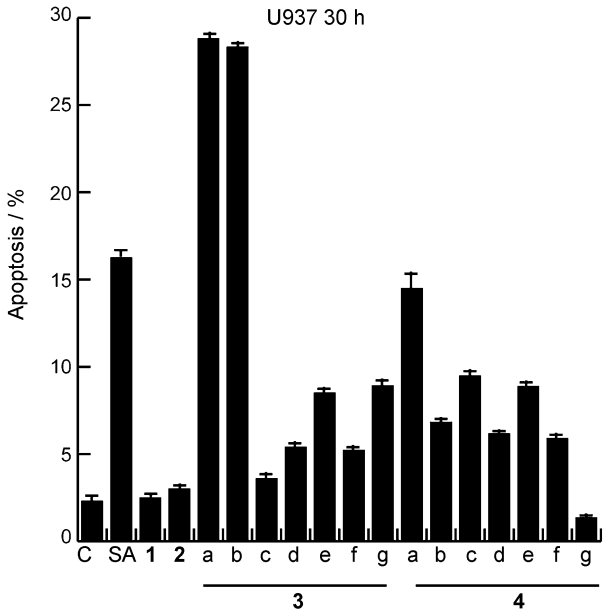

Figure 3. Effects of compounds **2**–**4** on apoptosis induction in the human leukemia U937 cell line over 30 h. Compounds were used at 5 μM except for compounds **3a** and **3b**, which were tested at 1 μM.

In conclusion, a series of pyrrole-containing HDACi **2**–**4**, based on the lead compound **1** (MC1568), bearing cycloalkylamino substituents of varying sizes at the C4 position of the benzene ring, and the hydroxamate or 2-aminoanilide moiety as the enzyme inhibiting group, have been reported. Generally, these compounds were more potent than **1** at inhibiting hrHDAC1 and hrHDAC6, whereas they displayed lower activities than **1** against hrHDAC4. In HDAC functional assays, the tested compounds increased the levels of both acetyl-H3 and acetyl-α-tubulin (compounds **3**) or only acetyl-α-tubulin (compounds **2**, **4**) in U937 cells. Notably, the cyclopropylamino and cyclobutylamino hydroxamates **3a** and **3b** showed the highest HDAC1/HDAC6 inhibitory activities, with IC_{50} values of 0.02 and 0.03 μM (against HDAC1) and 1.1 and 0.8 μM (HDAC6), respectively. When tested in U937 leukemia cells (caspase 3–7 method, 1 μM, 30 h), **3a** and **3b** induced apoptosis (28.8 and 28.3%, respectively) more effective than SAHA (16.3% at 5 μM) under the assay conditions. Further studies are in progress to optimize these pyrrole-based inhibitors.

Acknowledgements

This work was supported by grants from AIRC, PRIN, RETI FIRB, Fondazione Roma, and European Union (ATLAS project number 221952).

Keywords: apoptosis · epigenetics · histone deacetylases · inhibitors · aminoanilides · hydroxamates

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Received: March 2, 2009
Revised: May 11, 2009
Published online on June 5, 2009

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