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Class II (IIa)-Selective Histone Deacetylase Inhibitors. 1. Synthesis and Biological Evaluation of Novel (Aryloxopropenyl)pyrrolyl Hydroxyamides

Antonello Mai,*,§ Silvio Massa,# Riccardo Pezzi,§ Silvia Simeoni,§ Dante Rotili,§ Angela Nebbioso,⊥ Annamaria Scognamiglio,⊥ Lucia Altucci,*,⊥,‡ Peter Loidl,∥ and Gerald Brosch*,∥

Istituto Pasteur - Fondazione Cenci Bolognetti, Dipartimento di Studi Farmaceutici, Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, 00185 Roma, Italy, Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, via A. Moro, 53100 Siena, Italy, Dipartimento di Patologia Generale, Seconda Università degli Studi di Napoli, vico L. De Crecchio 7, 80138 Napoli, Italy, Centro di Oncogenomica AIRC, CEINGE Biotecnologia avanzata, Napoli, and Department of Molecular Biology, Innsbruck Medical University, Peter-Mayr-Strasse 4b, 6020 Innsbruck, Austria

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Chemical manipulations performed on aroyl-pyrrolyl-hydroxyamides (APHAs) led to (aryloxopropenyl)pyrrolyl hydroxamates **2a**−**w**, and their inhibition against maize HDACs and their class I or class II HDAC selectivity were determined. In particular, from these studies some benzene *meta*-substituted compounds emerged as highly class II (IIa)-selective HDAC inhibitors, the most selective being the 3-chloro- and 3-fluoro-substituted compounds **2c** (SI = 71.4) and **2f** (SI = 176.4). The replacement of benzene with a 1-naphthyl ring afforded **2s**, highly active against the class II homologue HD1-A (IC_{50} = 10 nM) but less class II-selective than **2c**,**f**. When tested against human HDAC1 and HDAC4, **2f** showed no inhibitory activity against HDAC1 but was able to inhibit HDAC4. Moreover, in human U937 acute myeloid leukaemia cells **2f** did not produce any effect on apoptosis, granulocytic differentiation, and the cell cycle, whereas **2s** (that retain class I HDAC inhibitory activity) was 2-fold less potent than SAHA used as reference.

Introduction

Histone acetylation and deacetylation play an essential role in modifying chromatin structure and regulating gene expression in eukaryotic cells. Hyperacetylated histones are generally found in transcriptionally active genes and hypoacetylated histones in transcriptionally silent regions of the genome.[1−4] Key enzymes, which modify histone proteins and thereby regulate gene expression, are histone acetyltransferases (HATs) and histone deacetylases (HDACs).[5−7] In mammals, both these acetylating/deacetylating enzymes are components of multiprotein complexes containing other proteins known to function in transcriptional activation/repression.[8−10] Such multiprotein complexes are recruited to specific regions in the mammalian genome and generate an unique spectrum of expressed and silenced genes. Known repressor multiprotein complexes contain DNA binding proteins (such as Rb and Rb-like proteins, N-CoR, SMRT, MEF, MeCP2, sin3A, etc.) that can recruit HDACs to repress transcription and to block the function of some tumor suppressor genes.[11−16] Compounds able to inhibit HDAC activity (HDAC inhibitors, such as trichostatin A (TSA), trapoxin (TPX), suberoylanilide hydroxamic acid (SAHA), sodium butyrate (NaB), sodium valproate (VPA), cyclic hydroxamic acid-containing peptides (CHAPs), depsipeptide FK-228, and MS-275) can de-repress these genes, resulting in antiproliferative effects in vitro and antitumor effects in vivo.[17−25]

To date, eighteen distinct human HDACs have been reported, grouped into three classes (I, II, and III) depending on their primary homology to three *Saccharomyces cerevisiae* HDACs (RPD3, HDA1, and SIR2, respectively).[26] Class I and II HDACs show some degree of homology in their catalytic domain, whereas class III HDACs (SIRT1−7, sirtuins) show no homology to class I/II enzymes. Class I HDACs, comprise HDAC1−3 and 8, are predominantly nuclear enzymes, and are ubiquitously expressed. HDAC11 is most closely related to the class I HDACs, but its overall sequence similarity is too low[27] for classifying it into class I, and a class IV of human HDACs (containing the sole HDAC11 until now) has been proposed.[28] Class II HDACs comprise HDAC4−7, 9, and 10 and are divided into two subclasses, IIa (HDAC4−7 and 9) and IIb (HDAC6 and 10, containing as a unique feature two deacetylase domains), according to their sequence homology and domain organization.[29−31] Class IIa HDACs shuttle between the nucleus and the cytoplasm, depending on their CaMK-mediated phosphorylation extent and subsequent binding of 14-3-3 proteins. In particular, phosphorylation-dependent binding of 14-3-3 proteins to the N-termini of class IIa HDACs masks the nuclear import signal and prevents nuclear import. Class IIb HDACs are mainly cytoplasmic, but show significant nuclear amounts in several cell lines. Differently from class I HDACs that are ubiquitarian, class II HDACs are expressed in a restricted number of cell types. Most class IIa HDACs (HDAC4, 5, and 9) are abundant in heart, skeletal

* To whom correspondence should be addressed: A.M., Tel.: +396-4991-3392; Fax:+396-491491; e-mail: antonello.mai@uniroma1.it. Biology: L.A., Tel.: +3981-566-7569; Fax: +3981450-169; e-mail: lucia.altucci@unina2.it. Biochemistry: G. B., Tel.: 0512-507-3608; Fax: 0512-507-9880; e-mail: gerald.brosch@uibk.ac.at.

§ Università degli Studi di Roma "La Sapienza".

Università degli Studi di Siena.

⊥ Seconda Università degli Studi di Napoli.

‡ Centro di Oncogenomica AIRC, CEINGE Biotecnologia avanzata, Napoli.

∥ Innsbruck Medical University.

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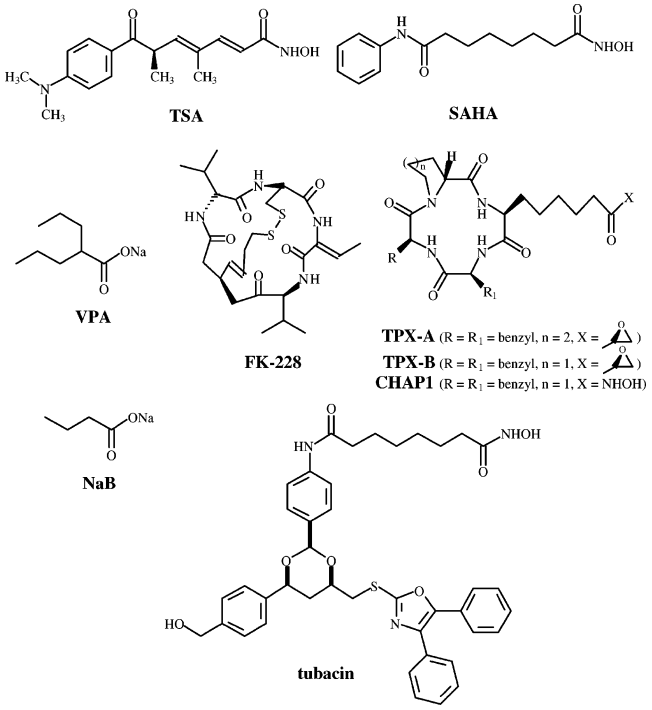
Chart 1. Known Unselective and Selective HDAC Inhibitors

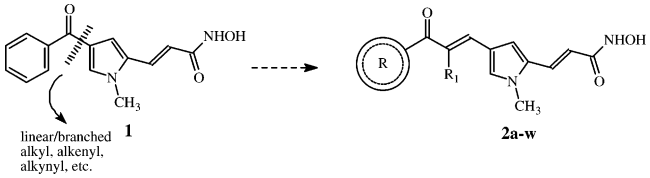


muscle, and brain,[32−36] and HDAC7 is highly expressed in CD4/CD8 double-positive thymocytes.[37] Class IIb HDAC6 is predominantly expressed in testis,[32] and HDAC10 is expressed in liver, spleen, and kidney.[38,39]

Class I HDACs are well-known transcriptional corepressors, acting through the block of the expression of some tumor suppressor genes. Class IIa HDACs have been reported to interact with one or more DNA-binding transcription factors, as well as with transcriptional corepressors.[29] Among these interactions, the class IIa HDACs−MEF2 interactions are the most studied.[40] MEF2 plays a critical role in cardiac and skeletal myogenesis, in negative selection of developing thymocytes, and in the transcriptional regulation of Epstein−Barr virus (EBV). Moreover, MEF2 has a role in neuronal resistance to excitotoxicity. By binding to MEF2 several promoters, class IIa HDACs can act as transcriptional repressors in a variety of biological functions, from myogenesis[41] to EBV latency.[42] Differently, class IIb HDAC6 takes part, together with the class III HDAC SIRT2, in the microtubule network and acts as specific α-tubulin deacetylase in vitro and in vivo.[43,44]

Most of the HDAC inhibitors described so far inhibit to the same extent class I as well as class II members of the HDAC family. The only exceptions are VPA, 5-fold more potent against HDAC1 than HDAC5 and HDAC6,[45] and FK-228, a class I-selective prodrug.[46] Class IIb HDAC6 and HDAC10 have been found to be resistant to the inhibitors TPX-A and -B, CHAPs, and NaB[47,48] and are selectively inhibited by tubacin, a 1,3-dioxane-containing hydroxamate discovered through a multidimensional, chemical genetic screen of 7392 molecules as a specific α-tubulin deacetylation inhibitor in mammalian cells (Chart 1).[49−52]

Chart 2. From Aroyl-pyrrolyl-hydroxamides (APHAs) to (Aryloxopropenyl)pyrrolyl Hydroxamates **2a**−**w**



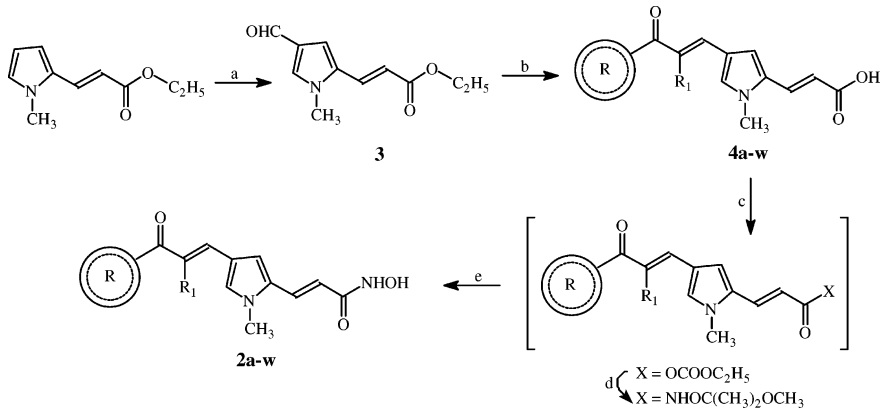
Further development of new small molecules with the specificity to selectively perturb only a (sub)class of the HDAC family is a very attractive goal to pursue, because such as compounds could be useful tools to dissect the role of a given HDAC in different protein complexes.[53]

Since 2001 a new class of HDAC inhibitors, namely aroyl-pyrrolyl-hydroxy-amides (APHAs), have been described by us.[54−60] In early studies, APHAs have been tested again HD2,[61] a maize deacetylase different from mammalian HDACs but being a good predictive model for the behavior of class I HDACs with various series of HDAC inhibitors.[55,56,58,62] Recently, we have undertaken a broad strategy[59,63,64] by testing the new derivatives against HD1-B[65,66] and HD1-A enzymes,[67,68] two maize deacetylases that are homologous of mammalian class I and class II (IIa) HDACs, respectively, to explore the potential selectivity of our compounds. Our maize system offers the advantage of completely separated enzyme forms, while human HDACs act as multiprotein- and, in some cases, multi-HDAC-containing complexes in their active forms.[69,70] First, anti-HD1-B/HD1-A data[59,63] showed that in APHA compounds just one type of chemical manipulation performed on the lead compound 3-(4-benzoyl-1-methyl-1*H*-pyrrol-2-yl)-*N*-hydroxy-2-propenamide **1** (Chart 2) was efficient to yield highly selective derivatives, that is the insertion of an alkyl/alkenyl spacer between the benzoyl moiety and the carbon atom at the pyrrole-C4 position in the **1** structure.[63]

Here we report in full details the synthesis and biochemical characterization of novel (aryloxopropenyl)-pyrrolyl hydroxyamides **2a**−**w**, structurally related to APHAs and highly selective against the class II (class IIa) histone deacetylase homologue HD1-A. Moreover, on selected compounds the inhibitory activities against human HDAC1 and HDAC4 as well as the cellular effects on apoptosis, granulocytic differentiation, and cell cycle phases in U937 acute myeloid leukemia (AML) cells are described.

Chemistry. Vilsmeier−Haack formylation of ethyl 3-(1-methyl-1*H*-pyrrol-2-yl)-2-propenoate[71] with oxalyl chloride/*N,N*-dimethylformamide afforded the ethyl 3-(4-formyl-1-methyl-1*H*-pyrrol-2-yl)-2-propenoate **3** as a sole product in high yield. Further condensation of **3** with the properly substituted alkyl aryl ketone in alkaline medium furnished the 3-[4-(3-aryl-3-oxopropen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-2-propenoic acids **4a**−**w**, key intermediate for the synthesis of the title compounds. By reaction of **4a**−**w** with ethyl chloroformate, followed by addition of *O*-(2-methoxy-2-propyl)hydroxylamine[72] and acidic treatment in the presence of the Amberlyst 15 ion-exchange resin, the desired 3-[4-(3-aryl-3-oxopropen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-*N*-hydroxy-2-propenamides **2a**−**w** have been obtained (Scheme 1).

Scheme 1[a]



[a] (a) $(COCl)_2$, DMF, dichloroethane, room temp; (b) aryl alkyl ketone, KOH, C_2H_5OH, H_2O, room temp; (c) $ClCOOC_2H_5$, $(C_2H_5)_3N$, THF, 0 °C; (d) $NH_2OC(CH_3)_2OCH_3$; (e) Amberlyst 15, MeOH, 45 °C.

Table 1. Chemical and Physical Data for Compounds **2a**−**w**

compd	R	R_1	mp, °C	recrystn solvent	% yield	formula	anal.[a]
2a	Ph	H	205−207	CH_3CN/benzene	54	$C_{17}H_{16}N_2O_3$	C, H, N
2b	2-Cl-Ph	H	224−226	CH_3CN/MeOH	58	$C_{17}H_{15}ClN_2O_3$	C, H, N, Cl
2c	3-Cl-Ph	H	198−200	MeOH	72	$C_{17}H_{15}ClN_2O_3$	C, H, N, Cl
2d	4-Cl-Ph	H	>260	CH_3CN/MeOH	80	$C_{17}H_{15}ClN_2O_3$	C, H, N, Cl
2e	2-F-Ph	H	220−222	CH_3CN/MeOH	65	$C_{17}H_{15}FN_2O_3$	C, H, N, F
2f	3-F-Ph	H	212−215	CH_3CN/MeOH	62	$C_{17}H_{15}FN_2O_3$	C, H, N, F
2g	4-F-Ph	H	>260	CH_3CN/MeOH	53	$C_{17}H_{15}FN_2O_3$	C, H, N, F
2h	2-Br-Ph	H	168−170	CH_3CN/MeOH	61	$C_{17}H_{15}BrN_2O_3$	C, H, N, Br
2i	3-Br-Ph	H	253−255	MeOH	56	$C_{17}H_{15}BrN_2O_3$	C, H, N, Br
2j	4-Br-Ph	H	250−252	MeOH	61	$C_{17}H_{15}BrN_2O_3$	C, H, N, Br
2k	2-Me-Ph	H	222−224	CH_3CN	45	$C_{18}H_{18}N_2O_3$	C, H, N
2l	3-Me-Ph	H	215−217	CH_3CN/MeOH	67	$C_{18}H_{18}N_2O_3$	C, H, N
2m	4-Me-Ph	H	246−248	CH_3CN/MeOH	61	$C_{18}H_{18}N_2O_3$	C, H, N
2n	2-MeO-Ph	H	165−166	MeOH	69	$C_{18}H_{18}N_2O_4$	C, H, N
2o	3-MeO-Ph	H	194−196	MeOH	65	$C_{18}H_{18}N_2O_4$	C, H, N
2p	4-MeO-Ph	H	>260	MeOH/DMF	52	$C_{18}H_{18}N_2O_4$	C, H, N
2q	3-CF_3-Ph	H	207−209	THF/MeOH	46	$C_{18}H_{15}F_3N_2O_3$	C, H, N, F
2r	3-CF_3O-Ph	H	>260	MeOH/DMF	45	$C_{17}H_{15}FN_2O_3$	C, H, N, F
2s	1-naphthyl	H	184−185	CH_3CN/MeOH	76	$C_{21}H_{18}N_2O_3$	C, H, N
2t	2-naphthyl	H	205−207	MeOH	68	$C_{21}H_{18}N_2O_3$	C, H, N
2u	4-biphenyl	H	184−185	MeOH	69	$C_{23}H_{20}N_2O_3$	C, H, N
2v	Ph	Me	174−175	CH_3CN/benzene	61	$C_{18}H_{18}N_2O_3$	C, H, N
2w	Ph	Et	190−192	CH_3CN/benzene	63	$C_{19}H_{20}N_2O_3$	C, H, N

[a] Analytical results were within ±0.4% of the theoretical values.

Chemical and physical data of compounds **2a**−**w** are listed in Table 1. Chemical and physical data of the intermediate compounds **3** and **4a**−**w** are listed in Table 2.

Results and Discussion

The novel (aryloxopropenyl)pyrrolyl hydroxyamides **2a**−**w** have been evaluated for their ability to inhibit maize HD2.[61,73−76] Two short-chain fatty acids (NaB[77] and VPA[37,78]), two hydroxamic acids (TSA[79] and SAHA[80]), two cyclic tetrapeptides (TPX[81] and HC-toxin[82]), and tubacin,[50−52] the sole class II (class IIb)-selective HDAC inhibitor reported to date, have been used as reference drugs. The results, expressed as the percent of inhibition at a fixed dose and IC_{50} (50% inhibitory concentration) values, are reported in Table 3. Compounds **2a**−**w** have been also tested against maize HD1-B[65,66] and HD1-A,[67,68] two mammalian class I and class II (IIa) HDAC homologues, respectively, and the resulting fold selectivity values (for class I HDACs: $IC_{50-HD1-A}$/$IC_{50-HD1-B}$ ratio; for class II HDACs: $IC_{50-HD1-B}$/$IC_{50-HD1-A}$ ratio) have been assessed. TSA, SAHA, and tubacin have been also tested for comparison purpose (Table 4).

HD2 Inhibitory Activity and Structure−Activity Relationships (SARs). (Aryloxopropenyl)pyrrolyl hydroxyamides **2a**−**w** have been first evaluated against maize HD2, in comparison with NaB, VPA, TSA, SAHA, TPX, HC-toxin, and tubacin (Table 3). From inhibitory data, it is clear that the type and the position of the substituent inserted at the benzene ring of the unsubstituted 3-[4-(3-phenyl-3-oxopropen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-*N*-hydroxy-2-propenamide **2a** play a crucial role in determining the inhibitory activity of the tested derivatives. Compound **2a** inhibited HD2 at submicromolar concentrations ($IC_{50} = 0.28\ \mu M$). The introduction of a halogen atom (chlorine, fluorine, or bromine) at each of the three positions of the **2a** benzene ring generally decreased or abated the potency of derivatives, the only exception being the 2-bromo analogue **2h** that retained almost the same activity as **2a**. The 2-chloro- and 2-fluoro derivatives **2b** and **2e** were 18- and 25-fold less active than **2a**, respectively; when the halogen was

Table 2. Chemical and Physical Data for Compounds **3** and **4a**−**w**

compd	R	R_1	mp, °C	recrystn solvent	% yield	formula	anal.[a]
3			102−104	cyclohexane	74	$C_{10}H_{13}NO_2$	C, H, N
4a	Ph	H	205−207	CH_3CN/MeOH	52	$C_{17}H_{15}NO_3$	C, H, N
4b	2-Cl-Ph	H	224−226	CH_3CN/MeOH	57	$C_{17}H_{14}ClNO_3$	C, H, N, Cl
4c	3-Cl-Ph	H	215−217	CH_3CN/MeOH	57	$C_{17}H_{14}ClNO_3$	C, H, N, Cl
4d	4-Cl-Ph	H	>260	CH_3CN/MeOH	75	$C_{17}H_{14}ClNO_3$	C, H, N, Cl
4e	2-F-Ph	H	220−222	CH_3CN/MeOH	56	$C_{17}H_{14}FNO_3$	C, H, N, F
4f	3-F-Ph	H	210−212	CH_3CN/MeOH	51	$C_{17}H_{14}FNO_3$	C, H, N, F
4g	4-F-Ph	H	>260	CH_3CN/MeOH	66	$C_{17}H_{14}FNO_3$	C, H, N, F
4h	2-Br-Ph	H	255−256	CH_3CN/MeOH	57	$C_{17}H_{14}BrNO_3$	C, H, N, Br
4i	3-Br-Ph	H	230−232	CH_3CN/MeOH	58	$C_{17}H_{14}BrNO_3$	C, H, N, Br
4j	4-Br-Ph	H	258−260	CH_3CN/MeOH	65	$C_{17}H_{14}BrNO_3$	C, H, N, Br
4k	2-Me-Ph	H	258−260	CH_3CN	41	$C_{18}H_{17}NO_3$	C, H, N
4l	3-Me-Ph	H	>260	CH_3CN/MeOH	47	$C_{18}H_{17}NO_3$	C, H, N
4m	4-Me-Ph	H	246−248	CH_3CN	45	$C_{18}H_{17}NO_3$	C, H, N
4n	2-MeO-Ph	H	205−207	CH_3CN/MeOH	50	$C_{18}H_{17}NO_4$	C, H, N
4o	3-MeO-Ph	H	193−195	CH_3CN/MeOH	66	$C_{18}H_{17}NO_4$	C, H, N
4p	4-MeO-Ph	H	203−204	MeOH	74	$C_{18}H_{17}NO_4$	C, H, N
4q	3-CF_3-Ph	H	232−234	THF/MeOH	86	$C_{18}H_{14}F_3NO_3$	C, H, N, F
4r	3-OCF_3-Ph	H	234−236	MeOH	84	$C_{17}H_{13}FNO_3$	C, H, N, F
4s	1-naphthyl	H	230−232	CH_3CN	71	$C_{21}H_{17}NO_3$	C, H, N
4t	2-naphthyl	H	261−263	CH_3CN	69	$C_{21}H_{17}NO_3$	C, H, N
4u	4-biphenyl	H	232−234	MeOH	70	$C_{23}H_{19}NO_3$	C, H, N
4v	Ph	Me	174−176	benzene	42	$C_{18}H_{17}NO_3$	C, H, N
4w	Ph	Et	190−192	benzene	37	$C_{19}H_{19}NO_3$	C, H, N

[a] Analytical results were within ±0.4% of the theoretical values.

Table 3. Maize HD2 Inhibitory Activity of Compounds **2a**−**w**[a]

compd	R	R_1	% inhbtn (at a fixed dose, μM)	IC_{50} ± SD (μM)
2a	Ph	H	92.3 (26)	0.28 ± 0.01
2b	2-Cl-Ph	H	79.1 (23.3)	5.2 ± 0.16
2c	3-Cl-Ph	H	49 (23.3)	24.2 ± 1.21
2d	4-Cl-Ph	H	45.5 (23.3)	25.2 ± 1.26
2e	2-F-Ph	H	73.9 (24.5)	7.1 ± 0.35
2f	3-F-Ph	H	56.5 (24.5)	22.0 ± 1.32
2g	4-F-Ph	H	33.2 (24.5)	35.3 ± 1.06
2h	2-Br-Ph	H	92.4 (20.5)	0.36 ± 0.02
2i	3-Br-Ph	H	76.8 (20.5)	2.1 ± 0.13
2j	4-Br-Ph	H	41.7 (20.5)	28.5 ± 1.71
2k	2-Me-Ph	H	96 (24.8)	0.27 ± 0.008
2l	3-Me-Ph	H	72 (24.8)	7.9 ± 0.24
2m	4-Me-Ph	H	65 (24.8)	10.2 ± 0.41
2n	2-MeO-Ph	H	95.7 (23.6)	0.25 ± 0.01
2o	3-MeO-Ph	H	78.2 (23.6)	0.54 ± 0.02
2p	4-MeO-Ph	H	74.2 (23.6)	0.57 ± 0.03
2q	3-CF_3-Ph	H	46.1 (21.1)	49.0 ± 2.0
2r	3-CF_3O-Ph	H	19.5 (20.2)	176.0 ± 5.3
2s	1-naphthyl	H	92 (22.2)	0.20 ± 0.006
2t	2-naphthyl	H	62.9 (25.2)	1.8 ± 0.09
2u	4-biphenyl	H	34 (20.6)	26.5 ± 1.06
2v	Ph	Me	89.9 (24.8)	0.68 ± 0.02
2w	Ph	Et	93 (23.7)	0.42 ± 0.02
NaB			35 (5000)	ND[b]
VPA				128.0 ± 3.8
TSA				0.007 ± 0.0002
SAHA				0.05 ± 0.001
TPX				0.01 ± 0.0003
HC-toxin				0.11 ± 0.004
tubacin			92.9 (40)	2.0 ± 0.1

[a] Data represent mean values of at least three separate experiments. [b] ND, not determined.

introduced at benzene C_3 as well as C_4 position, only substantially inactive compounds have been obtained, with the exception of the 3-bromo derivative **2i** (IC_{50} = 2.1 μM), that was 7.5 times less potent than **2a**. Methyl substitution at the benzene C_2 position furnished a compound (**2k**) showing the same activity as **2a**, while the 3- and 4-methyl counterparts (**2l** and **2m**) were from 28 to 36 times less potent than **2a** in inhibiting HD2. In comparison with the unsubstituted **2a**, the three methoxy derivatives **2n**−**p** were equally (**2n**) or slightly less (**2o**,**p**) active. Differently, 3-trifluoromethyl- and 3-trifluoromethoxy-substituted compounds **2q** and **2r** as well as 4-biphenyl derivative **2u** were substantially inactive. Replacement of the **2a** benzene ring with the bulky 1-naphthyl group led to **2s**, that was 1.4-fold more potent than **2a**, while compound **2t** bearing a 2-naphthyl moiety at the 3-propenyl position showed 6.4 times lower activity than **2a**. Finally, an alkyl (methyl or ethyl) substituent at the C_2 position of the 3-oxopropenyl chain was tolerated for HD2 inhibition, **2v** and **2w** being 2.4- and 1.5-fold less active than the unsubstituted **2a**, respectively.

HD1-B and HD1-A Inhibitory Assays: Assessment of Class Selectivity. The novel (aryloxopropenyl)pyrrolyl hydroxyamides **2a**−**w** have been tested against maize HD1-B and HD1-A, two mammalian class I and class II (IIa) HDAC homologues, and their class selectivity has been calculated. The unselective inhibitors TSA and SAHA and the class IIb-selective tubacin have been also tested as reference drugs. Data summarized in Table 4 show that the inhibitory trend of **2a**−**w** against HD1-B as well as against HD1-A is the same as that observed for **2a**−**w** against HD2, the main difference lying in the diverse degree of susceptibility of HD1-B and HD1-A to (aryloxopropenyl)pyrrolyl hydroxamate inhibitors. The inhibitory activity of the unsubstituted **2a** was at submicromolar concentrations ($IC_{50-HD1-B}$ = 0.26 μM; $IC_{50-HD1-A}$ = 0.19 μM), and the potency of substituted compounds against both the two maize enzymes varied according to the order of *ortho* > *meta* > *para*-substituted analogues, the last being in general the lowest active (or inactive) derivatives, while the *ortho*-substituted analogues were among the most potent compounds of the series. In anti-HD1-B assay, the chloro- and fluoro-substitution at the benzene C_2 position led to **2b** and **2e**, that were 6- and 13-fold less active than **2a**, respectively, while the corresponding C_3- and C_4-substituted **2c**,**d**,**f**,**g** were substantially inactive. Bromine and methyl substitutions generated more active compounds, the C_2-substituted analogues being

Table 4. Maize HD1-B and HD1-A Inhibitory Activities of Compounds **2a**−**w**[a]

			$IC_{50} \pm SD$ (μM)		SI[b]	
compd	R	R_1	HD1-B	HD1-A	class I	class II (IIa)
2a	Ph	H	0.26 ± 0.02	0.19 ± 0.01		
2b	2-Cl-Ph	H	1.6 ± 0.08	0.05 ± 0.003		32
2c	3-Cl-Ph	H	31.4 ± 1.26	0.44 ± 0.02		71.4
2d	4-Cl-Ph	H	29.4 ± 1.18	18.6 ± 1.12		
2e	2-F-Ph	H	3.4 ± 0.14	0.10 ± 0.004		34
2f	3-F-Ph	H	38.8 ± 1.16	0.22 ± 0.01		176.4
2g	4-F-Ph	H	24.9 ± 1.24	15.7 ± 0.94		
2h	2-Br-Ph	H	0.18 ± 0.007	0.21 ± 0.008		
2i	3-Br-Ph	H	1.1 ± 0.04	1.6 ± 0.06		
2j	4-Br-Ph	H	24.3 ± 0.97	20.3 ± 0.81		
2k	2-Me-Ph	H	0.39 ± 0.02	0.06 ± 0.002		6.5
2l	3-Me-Ph	H	2.5 ± 0.08	0.17 ± 0.007		14.7
2m	4-Me-Ph	H	4.8 ± 0.29	0.50 ± 0.02		9.6
2n	2-MeO-Ph	H	0.17 ± 0.008	0.16 ± 0.01		
2o	3-MeO-Ph	H	0.60 ± 0.03	0.39 ± 0.02		
2p	4-MeO-Ph	H	0.80 ± 0.05	0.79 ± 0.02		
2q	3-CF_3-Ph	H	159.0 ± 6.4	9.3 ± 0.46		17.1
2r	3-CF_3O-Ph	H	298.0 ± 14.9	90.0 ± 4.5		3.3
2s	1-naphthyl	H	0.33 ± 0.02	0.01 ± 0.0007		33
2t	2-naphthyl	H	1.8 ± 0.07	0.04 ± 0.002		45
2u	4-biphenyl	H	31.8 ± 1.27	2.2 ± 0.07		14.4
2v	Ph	Me	0.57 ± 0.02	0.78 ± 0.02		
2w	Ph	Et	4.5 ± 0.22	0.22 ± 0.01		20.4
TSA			0.0004 ± 0.00001	0.0008 ± 0.00003	2	
SAHA			0.03 ± 0.001	0.18 ± 0.009	6	
tubacin			0.98 ± 0.04	0.45 ± 0.02		2.2

[a] Data represent mean values of at least three separate experiments. [b]SI, Selectivity Index.

more potent (**2h**) than or as active (**2k**) as **2a**, and the C_3-substituted **2i** and **2l** were 4- and 10-fold less potent than **2a**. 2-Methoxy-substituted **2n** showed higher activity than **2a**, and the corresponding 3- and 4-isomers **2o** and **2p** were 2−4 times less active than **2a** in inhibiting HD1-B. As it has been observed in the anti-HD2 assay and also against HD1-B, the 3-trifluoromethyl (**2q**) and 3-trifluoromethoxy (**2r**) as well as the 4-biphenyl (**2u**) derivatives failed in inhibiting the enzyme. Compound **2s**, in which the **2a** benzene ring has been replaced with 1-naphthyl moiety, retained almost the same activity as **2a**, while the 2-naphthyl isomer **2t** was 7-fold less potent. At last, **2v** and **2w** carrying methyl and ethyl substituent at the C_2 position of the 3-oxopropenyl chain showed from 2 to 17 times lower activity than **2a** in inhibiting HD1-B.

In anti-HD1-A assay the order of potency of substituted compounds (*ortho* > *meta* > *para*) in comparison with the unsubstituted **2a** was fully maintained, but the susceptibility of the enzyme to the (aryloxopropenyl)-pyrrolyl hydroxamate inhibitors was very high. The 2-chloro- and 2-fluoro-substituted **2b** and **2e** were 4- and 2-fold more active than **2a** in inhibiting HD1-A, and the 3-substituted analogues **2c** and **2f** showed the same activity (**2f**) as or 2-fold lower potency (**2c**) than **2a**. Among bromo-substituted derivatives, the benzene-C_2 substituted **2h** retained the same activity than **2a**, the C_3 isomer **2i** being 8 times less potent than the unsubstituted reference. Methyl and methoxy substitutions gave highly active compounds, methyl- being more potent than the corresponding methoxy-containing derivatives. In particular, 2-methyl-, 3-methyl-, and 2-methoxyphenyloxopropenyl-pyrrolyl hydroxamates **2k**,**l**,**n** were up to 3 times more potent than **2a** in inhibiting HD1-A. Differently, the insertion of trifluoromethyl or trifluoromethoxy substituent at the C_3 position of the benzene ring led to inactive compounds (**2q** and **2r**). Both the naphthyl analogues **2s** and **2t** were more efficient (19- and 5-fold, respectively) than the phenyl counterpart in inhibiting HD1-A, and the 4-biphenyl compound **2u**, inactive against HD2 as well as HD1-B enzymes, showed $IC_{50} = 2.2$ μM against HD1-A. Finally, the insertion of a methyl or ethyl substituent at the oxopropenyl chain was well tolerated in anti-HD1-A assay, as phenyloxobutenyl and phenyloxopentenyl derivatives **2v** and **2w** showed slightly lower activity than the phenyloxopropenyl **2a**.

To determine the class selectivity for (aryloxopropenyl)pyrrolyl hydroxamates **2a**−**w**, the $IC_{50-HD1-A}/IC_{50-HD1-B}$ ratio for class I-selectivity and the $IC_{50-HD1-B}/IC_{50-HD1-A}$ ratio for class II (IIa)-selectivity have been calculated. Only selectivity values ≥2 have been reported. Table 4 shows that compounds substituted at C_2 or better at C_3 position of the benzene ring with chloro- (**2b**,**c**), fluoro- (**2e**,**f**), or methyl (**2k**,**l**) groups, as well as the naphthyl derivatives **2s**,**t**, were highly selective for the class II (IIa) HDAC homologue HD1-A, 3-[4-(3-(3-chlorophenyl)- and 3-[4-(3-(3-fluorophenyl)-3-oxopropen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-*N*-hydroxy-2-propenamides **2c** and **2f** being the most class II (IIa)-selective compounds, with a selectivity ratio of 71.4 and 176.4, respectively. Among the reference drugs, TSA and SAHA showed to be slightly selective (2 and 6 times) toward the class I HDAC homologue HD1-B. Tubacin, a highly "in cell" selective class IIb HDAC inhibitor, was 2.2 times more potent against HD1-A (class II homologue) than HD1-B (class I homologue). This result is as expected, because the tubacin selectivity is truly manifest in "in cell" functional assay, by comparing the induction of acetylated histone ($EC_{50} = 217 \pm 96$ μM) and acetylated tubulin ($EC_{50} = 2.9 \pm 0.9$ μM; selectivity ratio = 75) in mammalian cells,[50] while in enzyme (HDAC1, HDAC4, and HDAC6) assays tubacin showed

Table 5. Human HDAC1 and HDAC4 Inhibitory Activities of **2f** and SAHA[a]

	% inhibitory activity at 5 μM	
assay	**2f**	SAHA
HDAC1	0	52.9
HDAC4	54.9	37.2

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

about 4-fold selectivity for HDAC6 (J. C. Wong, personal communication).

Human HDAC1 and HDAC4 Inhibitory Assays. The most selective compound in the maize HD1-B/HD1-A system, i.e. the 3-[4-(3-(3-fluorophenyl)-3-oxopropen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-*N*-hydroxy-2-propenamide (**2f**) (class II fold selectivity = 176.4), was evaluated against human HDAC1 and HDAC4 in comparison with SAHA as reference drug. Human breast cancer ZR-75.1 cell lysates were immunoprecipitated with antibodies against HDAC1 and HDAC4, and inhibitory assays were performed on such immunoprecipitates (IPs) with **2f** (5 μM) and SAHA (5 μM). Data reported in Table 5 clearly show that **2f** lacked any inhibitory activity against human HDAC1 but was effective in inhibiting human HDAC4 enzyme.

Cellular Activities Evaluation: Apoptosis, Granulocytic Differentiation, and Cell Cycle Analysis. To investigate on cellular activities of selected (aryloxopropenyl)pyrrolyl hydroxamides, the effects of **2f** and **2s** on apoptosis, cytodifferentiation, and cell cycle phases in human U937 cell line were determined in comparison with SAHA.

It is well-known that class I HDACs induce the expression of some tumor suppressor genes, such as the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$,[17,83] while class II HDACs interact with one or more DNA-binding transcription factors as well as with transcriptional corepressors, such as MEF2.[29] A detailed study using small interfering double stranded RNA (siRNA) to selectively knockdown HDACs 1, 3, 4, and 7 in HeLa S3 cells showed that knockdowns of HDACs 1 and 3 produced a significant morphological phenotype similar to that observed with HDAC inhibitor treatment of these cells, giving an antiproliferative effect, whereas knockdowns of HDACs 4 and 7 produced no changes in cell morphology.[69,84] These studies established the critical role of class I HDACs in tumor cell proliferation/survival, while class II HDACs may not be involved in the process that controls proliferation and apoptosis in cancer cells, their involvement being important in diverse biological processes such as cardiac and skeletal myogenesis, negative selection of developing thymocytes, and other unknown processes.[29]

On these bases, the treatment of U937 AML cells with **2s**, that retain class I HDAC activity ($IC_{50-HD1-B} = 0.33$ μM; $IC_{50-HD1-A} = 0.01$ μM), was expected to induce apoptosis, cell differentiation, and cell cycle arrest, while treatment of U937 AML cells with **2f**, the selective class II (IIa)-inhibitor ($IC_{50-HD1-B} = 38.8$ μM; $IC_{50-HD1-A} = 0.22$ μM), was expected to have no effect. Indeed, as depicted in Figure 1, **2s** at 5 μM induced 4% (24 h) and 8% (48 h) apoptosis, 56% CD11c positive cells, and arrest of cell cycle in G1 phase in U937 cells. SAHA, used as reference, at 5 μM produced 9% (24 h) and 22% (48h) apoptosis, 73% CD11c positive cells, and cell cycle arrest in G2/M phase. In the same assays, **2f** at 5 μM showed % values (apoptosis, granulocytic differentiation, cell cycle phases) similar to those of controls.

Conclusion

A novel series of (aryloxopropenyl)pyrrolyl hydroxamates **2a**−**w** have been described as HDAC inhibitors, and their selectivity for maize HD1-A, a mammalian class II (IIa) HDAC homologue, has been determined. In general, the introduction of a substituent (from halogen atoms to methyl or methoxy groups) at the benzene ring of the (phenyloxopropenyl)pyrrolyl skeleton of the molecules did not improve the inhibitory activity, the order of potency of compounds (*ortho-* > *meta-* > *para*-substituted) being the same for all the three tested enzymes (HD2, HD1-B, and HD1-A). Nevertheless, in the anti-HD1-A assay some highly active derivatives have been obtained, in particular whit chloro-, fluoro-, methyl- and methoxy-substitutions at the benzene C_2 and, to a lesser extent, C_3 position. Moreover, since such derivatives (mainly the C_3-substituted compounds) showed little or no activity against HD1-B, a mammalian class I HDAC homologue, they were highly class II (IIa)-selective, the most selective being the 3-chloro- and 3-fluoro-substituted compounds **2c** and **2f**, with selectivity ratios of 71.4 (**2c**) and 176.4 (**2f**). Also the replacement of the benzene ring with the bulkier 1- and 2-naphthyl moieties furnished highly active derivatives against HD1-A (**2s**: $IC_{50} = 10$ nM; **2t**: $IC_{50} = 40$ nM), although less selective than **2c**,**f**. In comparison with TSA and SAHA, the inhibitory concentrations of (aryloxopropenyl)pyrrolyl hydroxyamides against HD1-A are substantially higher than that of TSA and in the range of SAHA. About selectivity, properly substituted (aryloxopropenyl)pyrrolyl hydroxyamides were class II (IIa) HDAC selective inhibitors, while TSA and SAHA showed a little selectivity against class I HDACs.

When tested against immunoprecipitated human HDAC1 and HDAC4, **2f** showed no inhibitory activity against HDAC1, while was able to inhibit HDAC4 (% inhibition at 5 μM = 54.9%). Moreover, in human U937 cancer cells **2f** did not produce any effect on apoptosis, granulocytic differentiation, and the cell cycle (class II HDAC-selective inhibitor behavior), whereas **2s** which retains class I HDAC inhibitory activity was 2-fold less potent than SAHA, used as reference to induce apoptosis and granulocytic differentiation, and blocked the cell cycle in G1 phase.

(Aryloxopropenyl)pyrrolyl hydroxamates **2a**−**w** are the first reported examples of class II (IIa)-selective compounds and together with the "in cell" class IIb-selective tubacin can be useful tools to probe the biology and to elucidate the functions, largely still unknown, of HDACs. Molecular modeling studies are in progress to rationalize the different behaviors of our class II (IIa)-selective molecules against HD1-B and HD1-A enzymes.

Experimental Section

Chemistry. Melting points were determined on a Büchi 530 melting point apparatus and are uncorrected. Infrared (IR) spectra (KBr) were recorded on a Perkin-Elmer Spectrum One instrument. ^{1}H NMR spectra were recorded at 200 MHz on a Bruker AC 200 spectrometer; chemical shifts are reported in

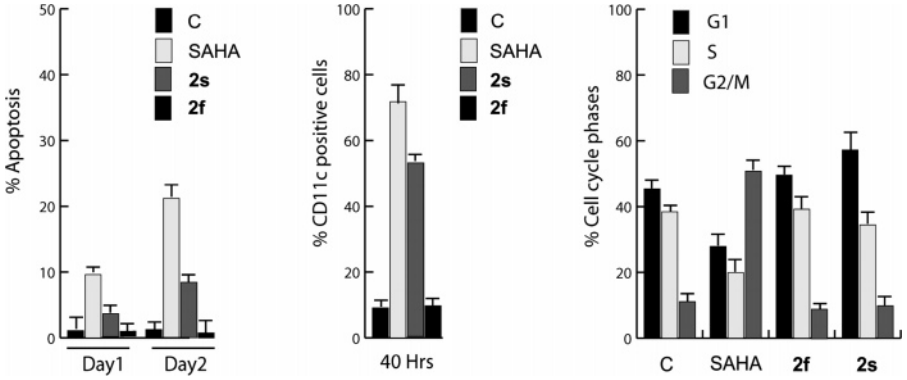


Figure 1. Effects of **2s** and **2f** on apoptosis, granulocytic differentiation, and cell cycle phases (U937 AML cell line) in comparison with SAHA.

δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). All compounds were routinely checked by TLC and ^1H NMR. TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. All solvents were reagent grade and, when necessary, were purified and dried by standards methods. Concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at a reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within $\pm$0.40% of the theoretical values. A SAHA sample for biological assays was prepared as previously reported by us.[85] Tubacin was kindly provided by Jason C. Wong (Harvard University, Cambridge, MA). All chemicals were purchased from Aldrich Chimica, Milan (Italy) or Lancaster Synthesis GmbH, Milan (Italy) and were of the highest purity.

Ethyl 3-(4-Formyl-1-methyl-1*H*-pyrrol-2-yl)-2-propenoate (3). A 50 mL 1,2-dichloroethane solution of oxalyl chloride (0.06 mol, 5.2 mL) was added to a cooled (0−5 °C) solution of *N,N*-dimethylformamide (0.06 mol, 4.6 mL) in 1,2-dichloroethane (50 mL) over a period of 5−10 min. After being stirred at room temperature for 15 min, the suspension was cooled (0−5 °C) again and treated with a solution of ethyl 3-(1-methyl-1*H*-pyrrol-2-yl)-2-propenoate[71] (0.06 mol, 10.7 g) in 1,2-dichloroethane (50 mL). The mixture was stirred at room temperature for 1 h and then was poured onto crushed ice (200 g) containing 50% NaOH (50 mL) and stirred for 10 min. The pH of the solution was adjusted to 4 with 37% HCl, the organic layer was separated, and the aqueous one was extracted with chloroform (2 × 50 mL). The combined organic solutions were washed with water, dried, and evaporated to dryness. The residual solid was purified by recrystallization. ^1H NMR (DMSO-d_6) δ 1.24 (t, 3 H, CH_2CH_3), 3.96 (s, 3 H, NCH_3), 4.20 (q, 2 H, CH_2CH_3), 6.60 (d, 1 H, CH=C*H*CO), 6.96 (d, 1 H, pyrrole β-proton), 7.03 (d, 1 H, pyrrole α-proton), 7.60 (d, 1 H, C*H*=CHCO), 9.58 (s, 1 H, CHO). Anal. ($C_{10}H_{13}NO_2$) C, H, N.

General Procedure for the Synthesis of 3-[4-(3-Aryl-3-oxopropen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-2-propenoic Acids 4a−w. Example: 3-[4-(3-(4-Chloro)phenyl-3-oxo-1-propen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-2-propenoic Acid 4d. A mixture of ethyl 3-(4-formyl-1-methyl-1*H*-pyrrol-2-yl)-2-propenoate **3** (6.0 mmol, 1.07 g), 4-chloroacetophenone (6.0 mmol, 0.93 g), and 2 N KOH (24.0 mmol, 12.4 mL) in ethanol (15 mL)/water (15 mL) was stirred at room temperature for 24 h. Afterward, the solution was poured into water (100 mL) and was made acid with 2 N HCl. The obtained precipitate was filtered and recrystallized to give the pure acid **4d**. ^1H NMR (DMSO-d_6) δ 3.75 (s, 3 H, NCH_3), 6.34 (d, 1 H, CH=C*H*COOH), 6.96 (d, 1 H, pyrrole β-proton), 7.21 (d, 1 H, pyrrole α-proton), 7.54 (d, 1 H, C*H*=CHCOOH), 7.65 (d, 2 H, benzene H-2,6), 7.73 (d, 1 H, COC*H*=CH), 7.77 (d, 1 H, COCH=C*H*), 8.12 (d, 2 H, benzene H-3,5), 12.22 (s, 1 H, OH). Anal. ($C_{17}H_{14}ClNO_3$) C, H, N, Cl.

General Procedure for the Synthesis of 3-[4-(3-Aryl-3-oxopropen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-*N*-hydroxy-2-propenamides 2a−w. Example: 3-[4-(3-Phenyl-3-oxo-1-propen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-*N*-hydroxy-2-propenamide (2a). Ethyl chloroformate (5.0 mmol, 0.5 mL) and triethylamine (5.4 mmol, 0.8 mL) were added to a cooled (0 °C) solution of 3-[4-(3-phenyl-3-oxopropen-1-yl)-1-methyl-1*H*-pyrrol-2-yl]-2-propenoic acid **4a** (4.2 mmol, 1.18 g) in dry THF (10 mL), and the mixture was stirred for 10 min. The solid was filtered off, and *O*-(2-methoxy-2-propyl)hydroxylamine (12.6 mmol, 1.1 mL)[72] was added to the filtrate. The solution was stirred for 15 min at 0 °C, then was evaporated under reduced pressure, and the residue was diluted in methanol (30 mL). Amberlyst 15 ion-exchange resin (0.6 g) was added to the solution of the *O*-protected hydroxamate, and the mixture was stirred at 45 °C for 1 h. Afterward, the reaction was filtered and the filtrate was concentrated in vacuo to give the crude **4a**, which was purified by crystallization. ^1H NMR (DMSO-d_6) δ 3.73 (s, 3 H, NCH_3), 6.30 (d, 1 H, CH=C*H*-CONHOH), 6.67 (s, 1 H, pyrrole β-proton), 7.17 (s, 1 H, pyrrole α-proton), 7.32 (d, 1 H, PhCOCH=CH), 7.53 (m, 3 H, benzene H-3,4,5), 7.62 m, 2 H, benzene H-2,6), 8.08 (d, 2 H, CH=CH−CONHOH and PhCOCH=CH). Anal. ($C_{17}H_{16}N_2O_3$) C, H, N.

In Vitro Maize HD2, HD1-B, and HD1-A Enzyme Inhibition. Radioactively labeled chicken core histones were used as the enzyme substrate according to established procedures.[73−76] The enzyme liberated tritiated acetic acid from the substrate, which was quantified by scintillation counting. IC_{50} values are results of triple determinations. A 50 μL sample of maize enzyme (at 30 °C) was incubated (30 min) with 10 μL of total [^3H]acetate-prelabeled chicken reticulocyte histones (2 mg/mL). Reaction was stopped by addition of 50 μL of 1 M HCl/0.4 M acetate and 800 μL of ethyl acetate. After centrifugation (10 000*g*, 5 min), an aliquot of 600 μL of the upper phase was counted for radioactivity in 3 mL of liquid scintillation cocktail. The compounds were tested at a starting concentration of 40 μM, and active substances were diluted further. NaB, VPA, TSA, SAHA,[85] TPX, HC-toxin, and tubacin were used as the reference compounds, and blank solvents were used as negative controls.

Human HDAC1 and HDAC4 Inhibition. Cell Culture and Ligands. Human breast cancer ZR-75.1 cells were propagated in DMEM medium supplemented with 10% FBS and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin-B). Before use, cells were plated at 60% confluence and treated the day after with 5 μM SAHA[85] or **2f**. All compounds were resuspended in DMSO (Sigma-Aldrich).

HDAC1 and HDAC4 Assays. A 1000 μg amount of total protein extracts (RIPA buffer for HDAC1 immunoprecipitation and NP-40 buffer for HDAC4 immunoprecipitation) was immunoprecipitated with 10 μg of HDAC1 antibody (Alexis) or HDAC4 antibody (Santa Cruz) overnight at 4 °C in slow rotation. As negative control the same amount of protein extracts was immunoprecipitated with purified IgG (Santa Cruz). One day later, the samples were incubated with 40 μL of protein A/G plus agarose (Santa Cruz) at 4 °C in slow rotation for 2−4 h. The samples were than washed six times with RIPA buffer or NP40-buffer respectively and twice in PBS

and resuspended in 20 μL of sterile PBS. The HDAC assays were carried out according to suppliers instructions (Upstate). Briefly, all samples immunoprecipitated with the HDAC1, HDAC4, or purified IgG were pooled respectively to homogenize all samples. A 10 μL amount of each HDAC IP was incubated with a previously labeled ^{3}H-Histone H4 peptide linked with streptavidin agarose beads (Upstate). In detail, 100.000 cpm of the H4-^{3}H-acetyl-peptide was used for each tube and incubated in 1X HDAC buffer with 10 μL of the sample in the presence or absence of the HDAC inhibitor with a final volume of 200 μL. Those samples were incubated overnight at 37 °C in slow rotation. One day later, 50 μL of a quenching solution was added and 100 μL of the samples was counted in duplicate after a brief centrifugation in a scintillation counter.

Determination of Apoptosis, Cell Differentiation, and Cell Cycle Effect on U937 AML Cells. Cell Lines and Cultures. U937 cell line was cultured in RPMI with 10% fetal calf serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 10 mM HEPES, and 2 mM glutamine.

Ligands. SAHA[85] was dissolved in DMSO and used at 5 μM. Compounds **2f** and **2s** were dissolved in DMSO and used at 5 μM.

Granulocytic Differentiation. U937 cells were harvested and resuspended in 10 μL of phycoerythrine-conjugated CD11c (CD11c-PE). Control samples were incubated with 10 μL of PE conjugated mouse IgG1, incubated for 30 min at 4 °C in the dark, washed in PBS, and resuspended in 500 μL of PBS-containing propidium iodide (0.25 μg/mL). Samples were analyzed by FACS with Cell Quest technology (Becton Dickinson) as previously reported.[86] Propidium iodide positive samples have been excluded from the analysis.

Cell Cycle Analysis. 2.5×10^5 Cells were collected and resuspended in 500 μL of an hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate, 50 μg/mL propidium iodide, RNAse A). Cells were incubated in the dark for 30 min. Samples were acquired on a FACS−Calibur flow cytometer using the Cell Quest software (Becton Dickinson) and analyzed with standard procedures using the Cell Quest software (Becton Dickinson) and the ModFit LT version 3 Software (Verity) as previously reported.[86] All the experiments were performed 3 times.

FACS Analysis of Apoptosis. Apoptosis was measured with Annexin V/Propidium iodide double staining detection (Roche) as recommended by the supplier; samples were analyzed by FACS with Cell Quest technology (Becton Dickinson) as previously reported.[87]

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Supporting Information Available: Elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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