Synthesis and Biological Properties of Novel, Uracil-Containing Histone Deacetylase Inhibitors

Antonello Mai,*[,†] Silvio Massa,[‡] Dante Rotili,[†] Silvia Simeoni,[†] Rino Ragno,[†] Giorgia Botta,[†] Angela Nebbioso,[§] Marco Miceli,[§] Lucia Altucci,*[,§,||] 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 Division of Molecular Biology, Biocenter, Innsbruck Medical University, Fritz-Preglstrasse 3, 6020 Innsbruck, Austria

Received May 11, 2006

A novel series of compounds containing a uracil moiety as the connection unit between a phenyl/phenylalkyl portion and a *N*-hydroxy-polymethylenealkanamide or -methylenecinnamylamide group (uracil-based hydroxamic acids, UBHAs) was tested against maize histone deacetylases (HDACs) and mouse HDAC1. Compounds with a phenyl/benzyl ring at the uracil-C6 position and bearing 4−5 carbon units as well as a *m*- or *p*-methylenecinnamyl moiety as a spacer were the most potent inhibitors. In cell-based human HDAC1 and HDAC4 assays, the two UBHAs tested inhibited the HDAC1 but not HDAC4 immunoprecipitate activity. When tested in human leukemia U937 cells, some UBHAs produced G1 phase arrest of the cell cycle. Moreover, **1j** showed high antiproliferative and dose-dependent granulocytic differentiation properties. The tested UBHAs displayed weak $p21^{WAF1/CIP1}$ induction in U937 cells, and **1d** and **1j** showed high histone H3 and α-tubulin acetylation effects.

Introduction

Histone deacetylase (HDAC) is a family of enzymes highly involved in chromatin remodeling and epigenetic regulation of gene expression.[1−3] HDACs catalyze the hydrolysis of ϵ-acetylamino groups of acetylated Lys residues in H3 and H4 histone tails, thus restoring the positive charges at the same residues and allowing their interactions with the phosphate groups of DNA. The result is a closed, hypoacetylated form of chromatin (heterochromatin) that is transcriptionally silent.[4−7] To date, four classes of mammalian HDACs are known according to their homology with the corresponding yeast transcriptional regulators. Class I (HDAC1-3,8), IIa (HDAC4,5,7,9), IIb (HDAC6,-10), and IV (HDAC11) HDACs are Zn^{2+}-dependent deacetylases, are components of multiprotein complexes containing other proteins known to function in transcriptional activation/repression, and differ for their subcellular localization and tissue expression.[8−10] Class III HDACs (SIRT1-7) have NAD^+ as cofactor for their catalytic activity, show no homology with the other classes of HDACs, and preferentially deacetylate nonhistone substrates.[11] Class I/IIa/IIb/IV HDACs can be recruited by known repressor multiprotein complexes (containing DNA binding proteins such as Rb and Rb-like proteins, N-CoR, SMRT, MEF, MeCP2, sin3A, etc.) to repress transcription and to block the function of some tumor suppressor genes.[12−17]

This scenario can be reverted by the use of HDAC inhibitors (HDACi), that activate the transcription of a small set of genes regulating cell proliferation and cell cycle progression.[18] In assays in vitro, HDACi induced growth arrest, differentiation, and/or apoptosis in cancer cells.[18−20] These findings have been confirmed in a xenograft tumor model in vivo, thus highlighting the therapeutic importance of HDACi as useful tools for cancer therapy.[21−25] To date, a number of HDACi have been entered in phase I to III clinical trials in hematological disorders as well as solid tumors, ranging from short-chain fatty acids (sodium valproate, sodium phenylbutyrate) to hydroxamates (suberoylanilide hydroxamic acid (SAHA) and LAQ824), benzamides (MS-275), and cyclic peptides (FK-228) (Chart 1).[26] Nevertheless, many of them suffer from low potency, low stability, or cardiovascular toxicity, and thus, the search for novel, potent HDACi is still a goal to pursue.[24,26]

Since the discovery that the anti-leukemia properties of trichostatin A (TSA) are due to the inhibition of HDAC enzymes,[27] a large number of structurally different HDACi have been reported as novel antiproliferative and cytodifferentiating agents. All of them share some common features able to interact with different portions of the catalytic tunnel of the enzyme, as elucidated by the reported X-ray crystal structures of TSA/and SAHA/histone deacetylase-like protein (HDLP) complexes.[28] These common features are summarized in a general pharmacophore model, which comprises a cap group (CAP) able to interact with the rim of the catalytic tunnel, often with a polar connection unit (CU) linking the cap to a hydrophobic spacer (HS), which allows the molecule to lie into the tunnel. Finally, the HS carries at the end a Zn-binding group (ZBG) that is able to complex the Zn^{2+} at the bottom of the cavity (Figure 1).[18,29]

Pursuing our searches in the anti-HDAC field,[30−40] we devoted our attention to the study of CU because very little information has been reported about it to date in comparison with that available for the other substructures of the pharmacophoric model. The most common CUs are ketone, amide, reverse amide, sulfonamide, sulfonanilide, or carbamate groups,[18] and in one report, oxazole and thiazole rings are described as heterocyclic examples of CUs.[41] Moreover, some of the most

* To whom correspondence should be addressed. Tel: +3906-4991-3392. Fax:+3906-491491. E-mail: antonello.mai@uniroma1.it (A.M.). Tel: +39081-566-7569. Fax: +39081-450-169. E-mail: lucia.altucci@unina2.it (L.A.). Tel: 0512-507-3608. Fax: 0512-507-9880. E-mail: gerald.brosch@uibk.ac.at (G.B.).

† Università degli Studi di Roma "La Sapienza".

‡ Università degli Studi di Siena.

§ Seconda Università degli Studi di Napoli.

|| Centro di Oncogenomica AIRC.

⊥ Innsbruck Medical University.

10.1021/jm0605536 CCC: $33.50
Published on Web 09/08/2006

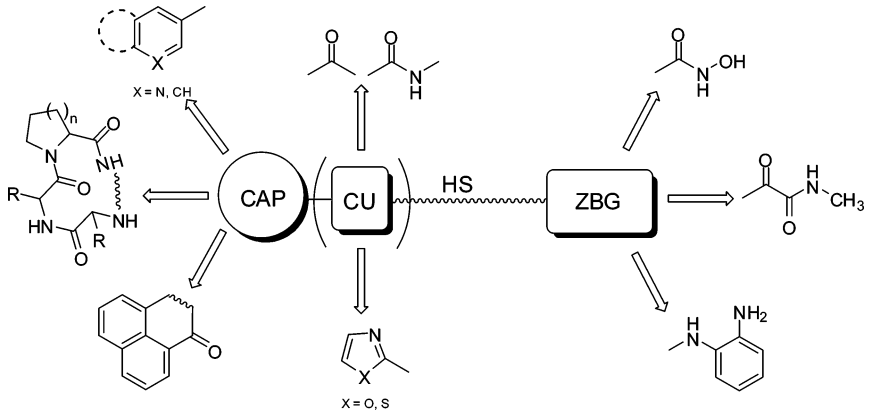
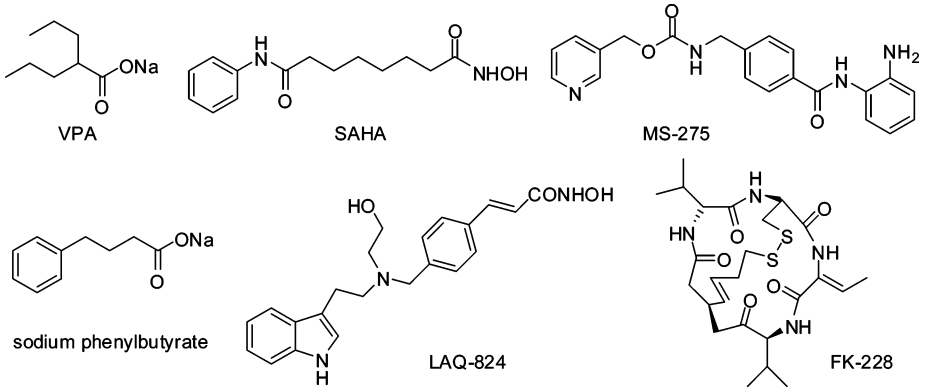


Figure 1. Pharmacophore model for HDACi design. Some examples for CAP, CU, and ZBG are depicted.

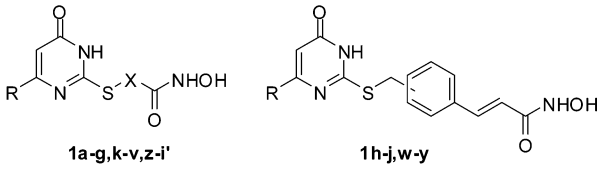
Chart 1. HDACi in Clinical Trials



potent HDACi lack any polar units between the CAP and the methylene linker chain;[42,43] therefore, it is not known whether the presence of a polar CU is a requirement for HDAC inhibition. From these bases, we designed a new series of HDACi[38] bearing aryl/arylalkyl groups as a CAP, a uracil moiety as a CU, connected through a sulfur atom with the HS (C2 to C7 carbon atom chains or methylenecinnamyl groups), and the hydroxamate function as a ZBG (compounds **1a**−**i′**) (Figure 2) with the aim to explore this issue. In particular, compounds **1a**−**g**,**k**−**v**,**z**−**i′** with linear HSs can be considered as SAHA analogues in which the anilide CU has been replaced by the uracil moiety.

The new uracil-based hydroxyamides (UBHAs) **1a**−**i′** have been tested against maize HD2,[44] HD1-B (class I HDAC),[45,46] and HD1-A (class II HDAC),[47,48] to determine the inhibitory activities and the putative class selectivity. TSA and SAHA[49] have been included in the tests as reference drugs. In a preliminary communication,[38] we reported the maize HDAC inhibitory data for compounds **1e**,**k**−**o**,**u**,**v**,**d′**,**g′**−**i′** bearing a straight polymethylene chain as the HS. In this article, we describe in full the synthesis of the title derivatives and report the anti-maize HDAC activity of further 23 analogues showing R = phenyl, substituted benzyl, or phenethyl at the C6 position of the pyrimidine ring (compounds **1a**−**d**,**f**,**g** (R = phenyl), **1p**−**t** (R = substituted benzyl), and **1z**−**c′**,**e′**,**f′** (R = phenethyl)) and a methylenecinnamyl spacer as X (**1h**−**j**,**w**−**y**) (Figure 2).

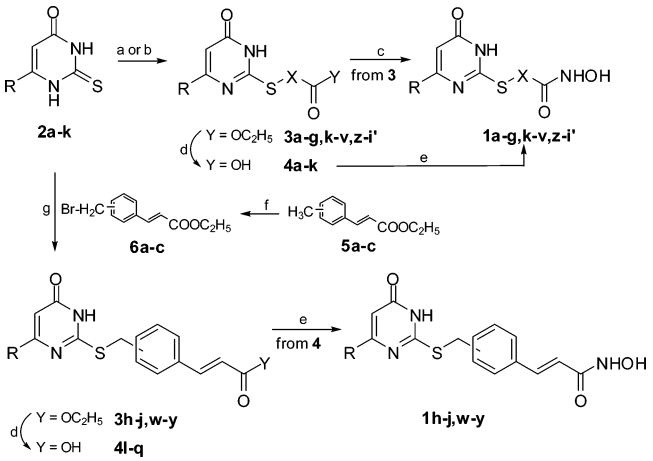
Mouse HDAC1 inhibitory assay and in vivo anti-HDAC1 and HDAC4 assays from human leukemia U937 and breast cancer ZR75.1 cells have been performed on selected title derivatives. In U937 cells, the effects of some UBHAs on cell cycle,



R = H, Me, *n*-Pr, Ph, $PhCH_2$, $PhCH(CH_3)$, $PhCH(C_2H_5)$, $PhCH(OCH_3)$, 1-Ph-1-*c*-Pr, PhCH(Ph), $PhCH_2CH_2$
X = CH=CH, $(CH_2)_n$; n = 2-7

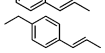
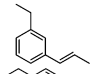
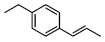
Figure 2. Novel uracil-based hydroxamic acid (UBHA) derivatives.

Scheme 1[a]



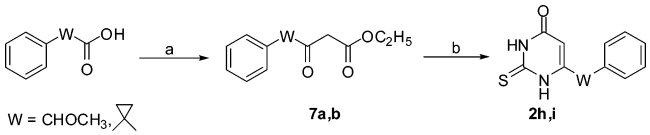
[a] (a) Br-X-COOEt, K_2CO_3, DMF, rt; (b) ethyl propiolate, TBAF, THF, 70 °C; (c) NH_2OH·HCl, KOH, EtOH, rt; (d) 2 N KOH, EtOH/H_2O, rt; (e) (i) ClCOOEt, $(C_2H_5)_3N$, THF, 0 °C, (ii) $NH_2OC(CH_3)_2OCH_3$, rt, (iii) Amberlyst 15, MeOH, rt. (f) NBS, benzoyl peroxide, CCl_4, reflux; (g) K_2CO_3, DMF, rt.

Table 1. Physical and Chemical Data for Compounds **1a**−**i′**

cpd	R	X	mp, °C	recrystn solvent[a]	% yield	formula	anal.[b]
1a	Ph	CH=CH	144-146	a	55	$C_{13}H_{11}N_3O_3S$	C, H, N, S
1b	Ph	$(CH_2)_2$	206-208	a	60	$C_{13}H_{13}N_3O_3S$	C, H, N, S
1c	Ph	$(CH_2)_3$	194-196	b	57	$C_{14}H_{15}N_3O_3S$	C, H, N, S
1d	Ph	$(CH_2)_4$	194-196	b	54	$C_{15}H_{17}N_3O_3S$	C, H, N, S
1e	Ph	$(CH_2)_5$	144-146	c	57	$C_{16}H_{19}N_3O_3S$	C, H, N, S
1f	Ph	$(CH_2)_6$	157-160	a	40	$C_{17}H_{21}N_3O_3S$	C, H, N, S
1g	Ph	$(CH_2)_7$	132-135	a	33	$C_{18}H_{23}N_3O_3S$	C, H, N, S
1h	Ph		230-232	a	59	$C_{20}H_{17}N_3O_3S$	C, H, N, S
1i	Ph		212-214	a	52	$C_{20}H_{17}N_3O_3S$	C, H, N, S
1j	Ph		240-242	a	58	$C_{20}H_{17}N_3O_3S$	C, H, N, S
1k	$PhCH_2$	CH=CH	156-158	c	62	$C_{14}H_{13}N_3O_3S$	C, H, N, S
1l	$PhCH_2$	$(CH_2)_2$	170-171	c	55	$C_{14}H_{15}N_3O_3S$	C, H, N, S
1m	$PhCH_2$	$(CH_2)_3$	129-130	c	60	$C_{15}H_{17}N_3O_3S$	C, H, N, S
1n	$PhCH_2$	$(CH_2)_4$	104-106	c	49	$C_{16}H_{19}N_3O_3S$	C, H, N, S
1o	$PhCH_2$	$(CH_2)_5$	179-181	b	57	$C_{17}H_{21}N_3O_3S$	C, H, N, S
1p	$PhCH(CH_3)$	$(CH_2)_5$	94-96	c	63	$C_{18}H_{23}N_3O_3S$	C, H, N, S
1q	$PhCH(C_2H_5)$	$(CH_2)_5$	85-87	d	55	$C_{19}H_{25}N_3O_3S$	C, H, N, S
1r	$PhCH(OCH_3)$	$(CH_2)_5$	112-114	c	60	$C_{18}H_{23}N_3O_4S$	C, H, N, S
1s		$(CH_2)_5$	142-145	b	54	$C_{19}H_{23}N_3O_3S$	C, H, N, S
1t	PhCH(Ph)	$(CH_2)_5$	131-134	b	47	$C_{23}H_{25}N_3O_3S$	C, H, N, S
1u	$PhCH_2$	$(CH_2)_6$	158-162	c	38	$C_{18}H_{23}N_3O_3S$	C, H, N, S
1v	$PhCH_2$	$(CH_2)_7$	139-141	c	35	$C_{19}H_{25}N_3O_3S$	C, H, N, S
1w	$PhCH_2$		211-213	a	57	$C_{21}H_{19}N_3O_3S$	C, H, N, S
1x	$PhCH_2$		198-200	a	55	$C_{21}H_{19}N_3O_3S$	C, H, N, S
1y	$PhCH_2$		224-226	e	61	$C_{21}H_{19}N_3O_3S$	C, H, N, S
1z	$PhCH_2CH_2$	CH=CH	196-198	a	63	$C_{14}H_{15}N_3O_3S$	C, H, N, S
1a'	$PhCH_2CH_2$	$(CH_2)_2$	170-172	f	56	$C_{15}H_{17}N_3O_3S$	C, H, N, S
1b'	$PhCH_2CH_2$	$(CH_2)_3$	125-127	g	53	$C_{16}H_{19}N_3O_3S$	C, H, N, S
1c'	$PhCH_2CH_2$	$(CH_2)_4$	98-100	f	43	$C_{17}H_{21}N_3O_3S$	C, H, N, S
1d'	$PhCH_2CH_2$	$(CH_2)_5$	118-120	g	56	$C_{18}H_{23}N_3O_3S$	C, H, N, S
1e'	$PhCH_2CH_2$	$(CH_2)_6$	76-78	f	30	$C_{19}H_{25}N_3O_3S$	C, H, N, S
1f'	$PhCH_2CH_2$	$(CH_2)_7$	58-60	g	22	$C_{20}H_{27}N_3O_3S$	C, H, N, S
1g'	H	$(CH_2)_5$	218-220	a	52	$C_{10}H_{15}N_3O_3S$	C, H, N, S
1h'	Me	$(CH_2)_5$	143-146	c	46	$C_{11}H_{17}N_3O_3S$	C, H, N, S
1i'	*n*-Pr	$(CH_2)_5$	120-122	h	60	$C_{13}H_{21}N_3O_3S$	C, H, N, S

[a] a: methanol; b: acetonitrile/methanol; c: acetonitrile; d: benzene; e: ethanol; f: diethyl ether; g: dichloromethane/cyclohexane; h: ethyl acetate/diethyl ether. [b] Analytic results were within ±0.40% of the theoretical values.

Scheme 2[a]

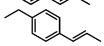
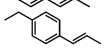
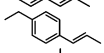
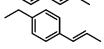


[a] (a) (1) CDI, MeCN, rt, (2) $EtOCOCH_2COOK$, $MgCl_2$, $(C_2H_5)_3N$, MeCN, rt, (3) 13% HCl; (b) (1) EtONa, NH_2CSNH_2, EtOH, reflux, (2) 2 N HCl.

apoptosis induction, proliferation, and cytodifferentiation have been determined. Moreover, the increased expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (p21)[50] and the acetylation extents of both H3 histones and α-tubulin[51] in U937 cells have been determined as functional tests for HDACi.

Chemistry. For the synthesis of the UBHA derivatives bearing linear HSs, the properly 6-substituted 2-thiouracils **2a**−**k** (R = H (a), Me (b), *n*-Pr (c), Ph (d),[52] $PhCH_2$ (e),[53] $PhCH(CH_3)$ (f),[54] $PhCH(C_2H_5)$ (g),[54] $PhCH(OCH_3)$ (h), 1-Ph-1-*c*-Pr (i), PhCH(Ph) (j),[55] and $PhCH_2CH_2$ (k)[52]) were treated with various sizes of ethyl ω-bromoalkanoates in the presence of anhydrous potassium carbonate to afford the ethyl esters **3a**−**g**,**k**−**v**,**z**−**i′**, which were, in part, converted into the corresponding hydroxamates **1b**−**g**,**l**−**o**,**u**,**v**,**a′**−**f′** with hydroxylamine hydrochloride and potassium hydroxyde in ethanol. Alternatively, the ethyl esters **3a**,**k**,**p**−**t**,**z**,**g′**−**i′** were hydrolyzed to the related carboxylic acids **4a**−**k** and then treated with (i) ethyl chloroformate and triethylamine, (ii) *O*-(2-methoxy-2-propyl)-hydroxylamine,[56] and (iii) Amberlyst 15 ion-exchange resin in methanol to give hydroxamates **1a**,**k**,**p**−**t**,**z**,**g′**−**i′** (Scheme 1). Selective *S*-alkylation of **2d**,**e** with ethyl 2-, 3-, and 4-bromomethylcinnamates **6a**−**c**, previously prepared from the reaction between the appropriate ethyl 2-, 3-, and 4-methylcinnamates **5a**−**c**[57,58] and *N*-bromosuccinimide,[59] furnished the methylenecinnamyl esters **3h**−**j**,**w**−**y**, which were hydrolyzed in alkaline medium to carboxylic acids **4l**−**q** and converted into related hydroxamates **1h**−**j**,**w**−**y** through the one-pot, three-step procedure reported above (Scheme 1). The synthesis of the 2-thiouracils **2h**,**i**, key intermediate for the preparation of derivatives **1r**,**s**, has been accomplished by condensation of thiourea in alkaline medium with the appropriate ethyl 4-phenyl-3-oxobutanoates **7a**,**b**, previously prepared by reacting potassium monoethylmalonate with 2-methoxyphenylacetyl- or 1-phenyl-1-cyclopropanecarbonylimidazolide in the presence of the magnesium dichloride/triethylamine system[54] (Scheme 2).

Table 2. Physical and Chemical Data for Compounds **2h**,**i**, **3a**−**i′**, **4a**−**q**, and **7a**,**b**

compd	R	X	mp, °C	recryst solvent[a]	yield, %	formula	anal.[b]
2h			242-245	a	68	$C_{12}H_{12}N_2O_2S$	C, H, N,S
2i			178-180	a	84	$C_{13}H_{12}N_2OS$	C, H, N,S
3a	Ph	CH=CH	195-197	b	63	$C_{15}H_{14}N_2O_3S$	C, H, N, S
3b	Ph	$(CH_2)_2$	196-198	b	88	$C_{15}H_{16}N_2O_3S$	C, H, N, S
3c	Ph	$(CH_2)_3$	120-122	c	56	$C_{16}H_{18}N_2O_3S$	C, H, N, S
3d	Ph	$(CH_2)_4$	116-118	b	49	$C_{17}H_{20}N_2O_3S$	C, H, N, S
3e	Ph	$(CH_2)_5$	108-110	d	45	$C_{18}H_{22}N_2O_3S$	C, H, N, S
3f	Ph	$(CH_2)_6$	125-128	d	75	$C_{19}H_{24}N_2O_3S$	C, H, N, S
3g	Ph	$(CH_2)_7$	99-101	c	69	$C_{20}H_{26}N_2O_3S$	C, H, N, S
3h	Ph		204-206	b	28	$C_{22}H_{20}N_2O_3S$	C, H, N, S
3i	Ph		201-203	b	25	$C_{22}H_{20}N_2O_3S$	C, H, N, S
3j	Ph		224-226	b	34	$C_{22}H_{20}N_2O_3S$	C, H, N, S
3k	$PhCH_2$	CH=CH	160-162	d	47	$C_{16}H_{16}N_2O_3S$	C, H, N, S
3l	$PhCH_2$	$(CH_2)_2$	134-137	c	52	$C_{16}H_{18}N_2O_3S$	C, H, N, S
3m	$PhCH_2$	$(CH_2)_3$	124-126	c	66	$C_{17}H_{20}N_2O_3S$	C, H, N, S
3n	$PhCH_2$	$(CH_2)_4$	100-102	b	44	$C_{18}H_{22}N_2O_3S$	C, H, N, S
3o	$PhCH_2$	$(CH_2)_5$	104-106	b	49	$C_{19}H_{24}N_2O_3S$	C, H, N, S
3p	$PhCH(CH_3)$	$(CH_2)_5$	82-84	d	48	$C_{20}H_{26}N_2O_3S$	C, H, N, S
3q	$PhCH(C_2H_5)$	$(CH_2)_5$	oil		57	$C_{21}H_{28}N_2O_3S$	C, H, N, S
3r	$PhCH(OCH_3)$	$(CH_2)_5$	73-75	d	63	$C_{20}H_{26}N_2O_4S$	C, H, N, S
3s		$(CH_2)_5$	138-141	b	43	$C_{21}H_{26}N_2O_3S$	C, H, N, S
3t	PhCH(Ph)	$(CH_2)_5$	109-111	b	53	$C_{25}H_{28}N_2O_3S$	C, H, N, S
3u	$PhCH_2$	$(CH_2)_6$	125-129	c	64	$C_{20}H_{26}N_2O_3S$	C, H, N, S
3v	$PhCH_2$	$(CH_2)_7$	80-83	c	56	$C_{21}H_{28}N_2O_3S$	C, H, N, S
3w	$PhCH_2$		175-177	b	39	$C_{23}H_{22}N_2O_3S$	C, H, N, S
3x	$PhCH_2$		138-141	b	42	$C_{23}H_{22}N_2O_3S$	C, H, N, S
3y	$PhCH_2$		173-175	b	35	$C_{23}H_{22}N_2O_3S$	C, H, N, S
3z	$PhCH_2CH_2$	CH=CH	136-138	c	49	$C_{17}H_{18}N_2O_3S$	C, H, N, S
3a'	$PhCH_2CH_2$	$(CH_2)_2$	104-106	c	52	$C_{17}H_{20}N_2O_3S$	C, H, N, S
3b'	$PhCH_2CH_2$	$(CH_2)_3$	97-99	c	45	$C_{18}H_{22}N_2O_3S$	C, H, N, S
3c'	$PhCH_2CH_2$	$(CH_2)_4$	92-94	c	56	$C_{19}H_{24}N_2O_3S$	C, H, N, S
3d'	$PhCH_2CH_2$	$(CH_2)_5$	52-54	c	60	$C_{20}H_{26}N_2O_3S$	C, H, N, S
3e'	$PhCH_2CH_2$	$(CH_2)_6$	63-65	c	63	$C_{21}H_{28}N_2O_3S$	C, H, N, S
3f'	$PhCH_2CH_2$	$(CH_2)_7$	40-42	e	50	$C_{22}H_{30}N_2O_3S$	C, H, N, S
3g'	H	$(CH_2)_5$	84-86	e	52	$C_{12}H_{18}N_2O_3S$	C, H, N, S
3h'	Me	$(CH_2)_5$	63-65	e	40	$C_{13}H_{20}N_2O_3S$	C, H, N, S
3i'	*n*-Pr	$(CH_2)_5$	75-77	e	63	$C_{15}H_{24}N_2O_3S$	C, H, N, S
4a	Ph	CH=CH	200-202	b	93	$C_{13}H_{20}N_2O_3S$	C, H, N, S
4b	$PhCH_2$	CH=CH	170-172	c	95	$C_{14}H_{12}N_2O_3S$	C, H, N, S
4c	$PhCH(CH_3)$	$(CH_2)_5$	131-133	b	96	$C_{18}H_{22}N_2O_3S$	C, H, N, S
4d	$PhCH(C_2H_5)$	$(CH_2)_5$	136-139	b	92	$C_{19}H_{24}N_2O_3S$	C, H, N, S
4e	$PhCH(OCH_3)$	$(CH_2)_5$	105-107	d	94	$C_{18}H_{22}N_2O_4S$	C, H, N, S
4f		$(CH_2)_5$	173-175	b	97	$C_{19}H_{22}N_2O_3S$	C, H, N, S
4g	PhCH(Ph)	$(CH_2)_5$	149-151	b	87	$C_{23}H_{24}N_2O_3S$	C, H, N, S
4h	$PhCH_2CH_2$	CH=CH	186-190	b	91	$C_{15}H_{14}N_2O_3S$	C, H, N, S
4i	H	$(CH_2)_5$	160-162	b	87	$C_{10}H_{14}N_2O_3S$	C, H, N, S
4j	Me	$(CH_2)_5$	148-150	f	67	$C_{11}H_{16}N_2O_3S$	C, H, N, S
4k	*n*-Pr	$(CH_2)_5$	85-87	e	77	$C_{13}H_{20}N_2O_3S$	C, H, N, S
4l	Ph		270-273	a	79	$C_{20}H_{16}N_2O_3S$	C, H, N, S
4m	Ph		235-237	b	88	$C_{20}H_{16}N_2O_3S$	C, H, N, S
4n	Ph		262-265	b	93	$C_{20}H_{16}N_2O_3S$	C, H, N, S
4o	$PhCH_2$		190-192	b	89	$C_{21}H_{18}N_2O_3S$	C, H, N, S
4p	$PhCH_2$		202-204	b	97	$C_{21}H_{18}N_2O_3S$	C, H, N, S
4q	$PhCH_2$		220-222	b	85	$C_{21}H_{18}N_2O_3S$	C, H, N, S
7a			oil		88	$C_{13}H_{16}O_4$	C, H
7b			oil		89	$C_{14}H_{16}O_3$	C, H

[a] a: ethanol; b: methanol; c: acetonitrile; d: acetonitrile/methanol; e: cyclohexane; f: toluene/acetonitrile. [b] Analytic results were within ±0.40% of the theoretical values.

Table 3. Maize HD2, HD1-B, and HD1-A Inhibitory Activities of Compounds **1a**−**i′**[a]

compd	R	X	IC_{50} ± SD (nM)		
			HD2	HD1-B	HD1-A
1a	Ph	CH=CH	NI[b]	NI[b]	NI[b]
1b	Ph	$(CH_2)_2$	822 ± 41.1	890 ± 35.6	1020 ± 51.0
1c	Ph	$(CH_2)_3$	27 ± 1.1	31 ± 1.5	56 ± 2.2
1d	Ph	$(CH_2)_4$	8 ± 0.3	10 ± 0.3	10 ± 0.3
1e[c]	Ph	$(CH_2)_5$	12 ± 0.5	19 ± 0.8	6 ± 0.3
1f	Ph	$(CH_2)_6$	38 ± 1.9	48 ± 1.4	11 ± 0.7
1g	Ph	$(CH_2)_7$	42 ± 1.7	89 ± 2.7	28 ± 1.4
1h	Ph		367 ± 14.7	780 ± 31.2	458 ± 22.9
1i	Ph		17 ± 0.7	64 ± 1.9	77 ± 3.8
1j	Ph		23 ± 0.9	112 ± 3.4	122 ± 4.9
1k[c]	$PhCH_2$	CH=CH	9000 ± 360	13300 ± 339	7400 ± 222
1l[c]	$PhCH_2$	$(CH_2)_2$	38 ± 1.5	41 ± 1.6	126 ± 6.3
1m[c]	$PhCH_2$	$(CH_2)_3$	229 ± 11.4	251 ± 10.0	885 ± 26.5
1n[c]	$PhCH_2$	$(CH_2)_4$	125 ± 6.2	130 ± 3.9	181 ± 10.9
1o[c]	$PhCH_2$	$(CH_2)_5$	18 ± 0.5	18 ± 0.5	29 ± 1.2
1p	$PhCH(CH_3)$	$(CH_2)_5$	9 ± 0.4	92 ± 3.7	80 ± 1.6
1q	$PhCH(C_2H_5)$	$(CH_2)_5$	32 ± 1.0	31 ± 0.9	105 ± 3.1
1r	$PhCH(OCH_3)$	$(CH_2)_5$	82 ± 3.3	79 ± 3.9	130 ± 6.5
1s		$(CH_2)_5$	62 ± 2.5	47 ± 1.9	30 ± 1.5
1t	PhCH(Ph)	$(CH_2)_5$	52 ± 3.1	58 ± 2.3	43 ± 2.6
1u[c]	$PhCH_2$	$(CH_2)_6$	37 ± 1.1	76 ± 3.8	53 ± 2.1
1v[c]	$PhCH_2$	$(CH_2)_7$	61 ± 3.0	193 ± 9.6	64 ± 1.9
1w	$PhCH_2$		760 ± 38.0	1060 ± 53.0	970 ± 29.1
1x	$PhCH_2$		41 ± 1.2	42 ± 1.7	17 ± 0.7
1y	$PhCH_2$		80 ± 4.0	73 ± 2.9	52 ± 2.6
1z	$PhCH_2CH_2$	CH=CH	41000 ± 2050	96000 ± 3840	52000 ± 1040
1a'	$PhCH_2CH_2$	$(CH_2)_2$	37 ± 1.8	60 ± 2.4	23 ± 0.7
1b'	$PhCH_2CH_2$	$(CH_2)_3$	205 ± 10.2	170 ± 6.8	215 ± 6.4
1c'	$PhCH_2CH_2$	$(CH_2)_4$	40 ± 1.2	46 ± 1.8	11 ± 0.4
1d'[c]	$PhCH_2CH_2$	$(CH_2)_5$	35 ± 1.0	48 ± 1.9	25 ± 1.0
1e'	$PhCH_2CH_2$	$(CH_2)_6$	83 ± 3.3	62 ± 2.5	40 ± 2.0
1f'	$PhCH_2CH_2$	$(CH_2)_7$	90 ± 3.6	62 ± 2.5	52 ± 3.1
1g'[c]	H	$(CH_2)_5$	213 ± 10.6	239 ± 7.2	132 ± 6.6
1h'[c]	Me	$(CH_2)_5$	110 ± 5.5	78 ± 2.3	112 ± 4.5
1i'[c]	*n*-Pr	$(CH_2)_5$	135 ± 5.4	116 ± 4.6	66 ± 3.3
TSA			7 ± 0.2	0.4 ± 0.01	0.8 ± 0.03
SAHA			50 ± 1.0	28 ± 1.0	180 ± 9.0

[a] Data represent the mean values of at least three separate experiments. [b] NI, no inhibition (0%) at 20.9 μM. [c] Ref 38. The duplication of data is only aimed to make the SAR more easily understandable.

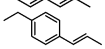
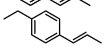
Chemical and physical data of tested compounds **1a**−**i′** and intermediate **2h**,**i**, **3a**−**i′**, **4a**−**q**, and **7a**,**b** are reported in Table 1 and Table 2, respectively.

Results and Discussion

Enzyme Assays. The novel derivatives **1a**−**i′** were routinely tested against three maize deacetylases, HD2, HD1-B (class I), and HD1-A (class II). Data in Table 3 report IC_{50} (50% inhibitory concentration) values for all tested derivatives in comparison with those for TSA and SAHA used as reference drugs. From the analysis of such data, we can draw the following structure−activity relationship (SAR). CAP group: In the UBHA general structure, the introduction of phenyl, benzyl, and, to a lesser extent, the 2-phenylethyl moiety at the C6 position of the uracil group furnished highly active derivatives endowed with IC_{50} values at submicromolar to single-digit nanomolar concentrations, depending on either the size of the linear spacer or the methylenecinnamyl regioisomer connecting the uracil with the hydroxamate group. Exceptions to this rule were the three *N*-hydroxypropenamides **1a**,**k**,**z**, which showed very low (**1k**,**z**) or no (**1a**) inhibitory activity against the three maize enzymes. In the C6-benzyl series, the introduction of various substituents at the benzylic position (compounds **1p**−**t**) generally decreased the anti-HDAC activity of the derivatives in comparison with the unsubstituted counterpart **1o**, with the exception of the methyl-substituted **1p**, which was 2-fold more potent than **1o** against HD2. The deletion of the substituent at the pyrimidine C6 position (compound **1g′**) as well as the replacement of the phenyl/benzyl CAP group of **1e**,**o** with the smaller methyl or *n*-propyl group (compounds **1h′**,**i′**) gave up to a 22-fold reduction of the anti-HDAC activity. Hydrophobic spacer: Compounds with a linear, polymethylene spacer between the uracil and the hydroxamate showed the highest activity with the insertion of 4 to 5 carbon units (see **1d**,**e** in the 6-phenyl, **1o** in the 6-benzyl, and **1c′**,**d′** in the 6-phenethyl series). By increasing this number to 7 carbon units as well as by introducing shorter, linear HSs in the UBHA general formula, a decrease of inhibiting activity was recorded. Exceptions to this SAR were **1l** and **1a′**, bearing a short *N*-hydroxypropanamide moiety, which was endowed with high maize HDAC inhibiting activity. Particularly, the corresponding unsaturated analogues **1k**,**z** failed in inhibiting HDACs. Between the methylenecinnamyl derivatives **1h**−**j** and **1w**−**y**, the ortho-substituted analogues **1h**,**w** were scarcely active in inhibiting the maize deacetylases, whereas the *m*- and, to a lesser extent, the *p*-methylenecinnamyl compounds **1i**,**x** and **1j**,**y** were highly efficient inhibitors.

From the comparison of HD1-B (class I) and HD1-A (class II) inhibitory data (Table 3), UBHAs proved to be devoid of class selectivity in their HDAC inhibiting action; among the

Table 4. Mouse HDAC1 Inhibitory Activity of Selected UBHAs **1d**,**e**,**i**,**j**,**o**,**p**,**x**,**y**[a]

compd	R	X	mHDAC1 IC_{50} ± SD (nM)
1d	Ph	$(CH_2)_4$	40 ± 2.4
1e	Ph	$(CH_2)_5$	39 ± 1.6
1i	Ph		57 ± 1.7
1j	Ph		34 ± 1.7
1o	$PhCH_2$	$(CH_2)_5$	103 ± 4.1
1p	$PhCH(CH_3)$	$(CH_2)_5$	121 ± 4.8
1x	$PhCH_2$		90 ± 4.5
1y	$PhCH_2$		110 ± 5.5
TSA			1.8 ± 0.07
SAHA			112 ± 4.0

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

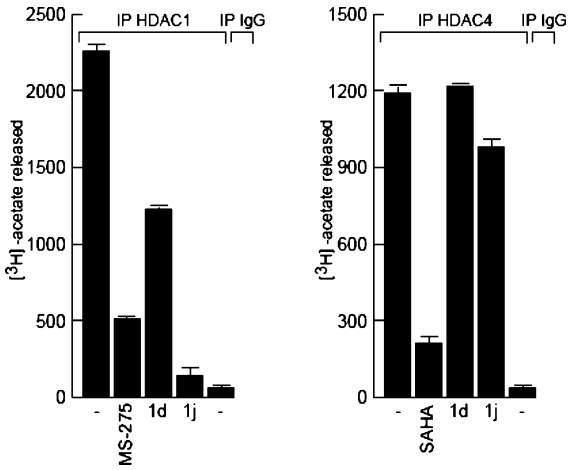


Figure 3. Cell-based human HDAC1 and HDAC4 assays.

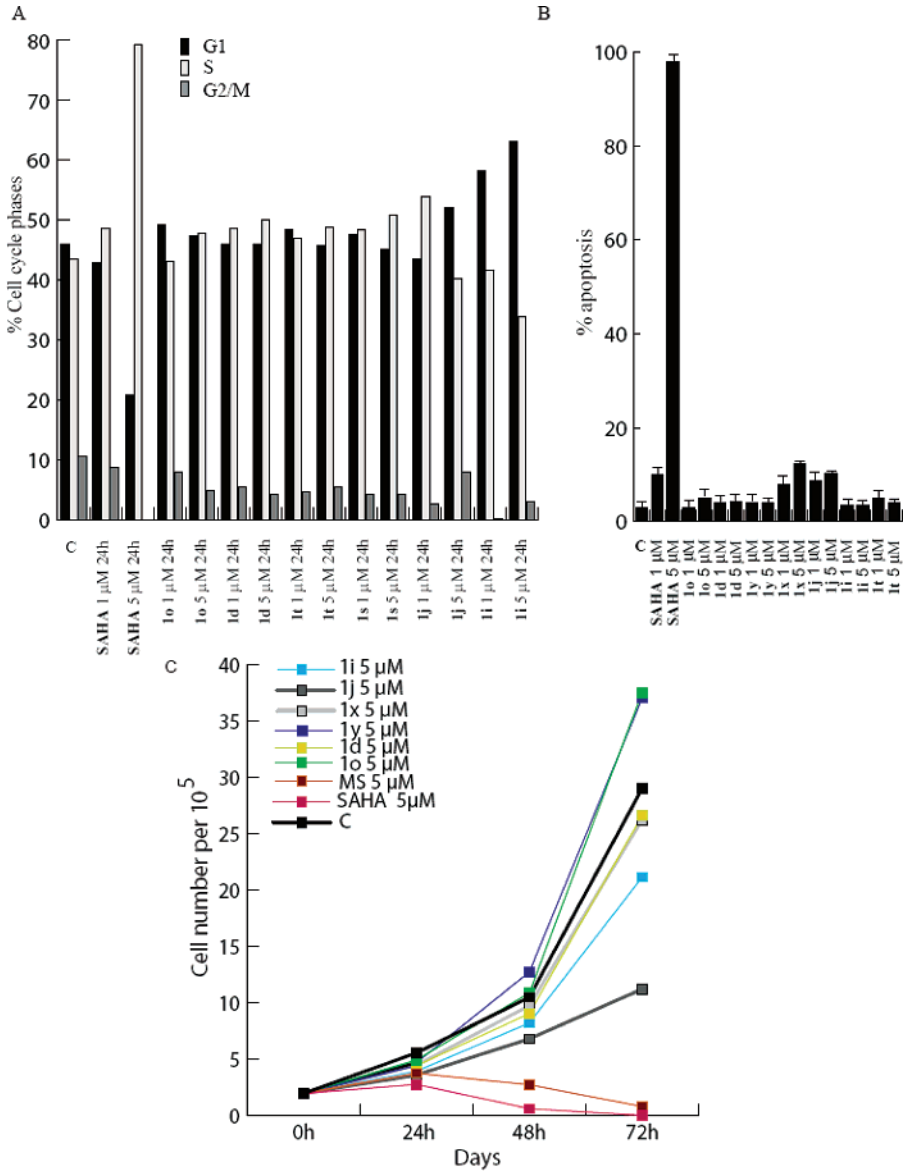


Figure 4. Cell cycle effects (A), apoptosis induction (B), and antiproliferative activity (C) exerted by selected UBHA derivatives in human U937 cells. The concentrations used are indicated.

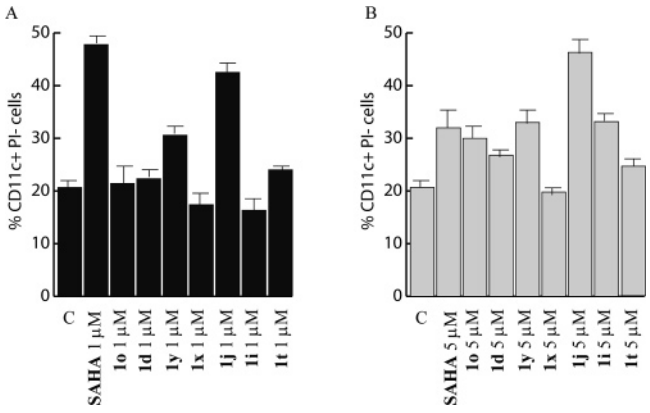


Figure 5. Granulocytic differentiation showed by selected UBHAs at 1 (A) and 5 (B) μM on U937 cells.

30 compounds synthesized and tested, 5 of them (**1e**−**g**,**v**,**c′**) showed a class II selectivity ratio >3, whereas 3 (**1l**,**m**,**q**) were slightly class I-selective.

The mouse HDAC1 inhibitory assay was performed on selected UBHA derivatives (Table 4). The IC_{50} values fully agree with those determined against maize deacetylases, the main discrepancies showing up to a 6-fold of difference between mammalian/maize activities. Against mouse HDAC1, the 6-phenyl derivatives **1d**,**e**,**i**,**j** were 2-fold more potent than the 6-benzyl analogues **1o**,**p**,**x**,**y**, and the compounds with linear HS (**1d**,**e** and **1o**,**p**) showed the same potency as that of the methylene-cinnamyl derivatives **1i**,**j** and **1x**,**y**, respectively. In comparison with the references, in the anti-mouse HDAC1 assay, UBHAs were 20- to 50-fold less active than TSA and up to 3-fold more potent than SAHA (Table 4).

Cell-Based Assays. Human HDAC1 and HDAC4 Inhibitory Activity in Vivo. Human leukemia U937 and breast cancer ZR-75.1 cell lysates were immunoprecipitated with antibodies against HDAC1 (Abcam) and HDAC4 (Sigma), respectively, and inhibitory assays were performed on these immunoprecipitates (IPs) using **1d** and **1j** as UBHA prototypes in comparison with MS-275 and SAHA as reference drugs. All compounds were tested at 5 μM. The data reported in Figure 3 clearly show that **1j** and, to a lesser extent, **1d** were able to inhibit class I HDAC (HDAC1) IP activity (**1j** = 94% of inhibition, **1d** = 46% of inhibition), whereas they were ineffective in inhibiting

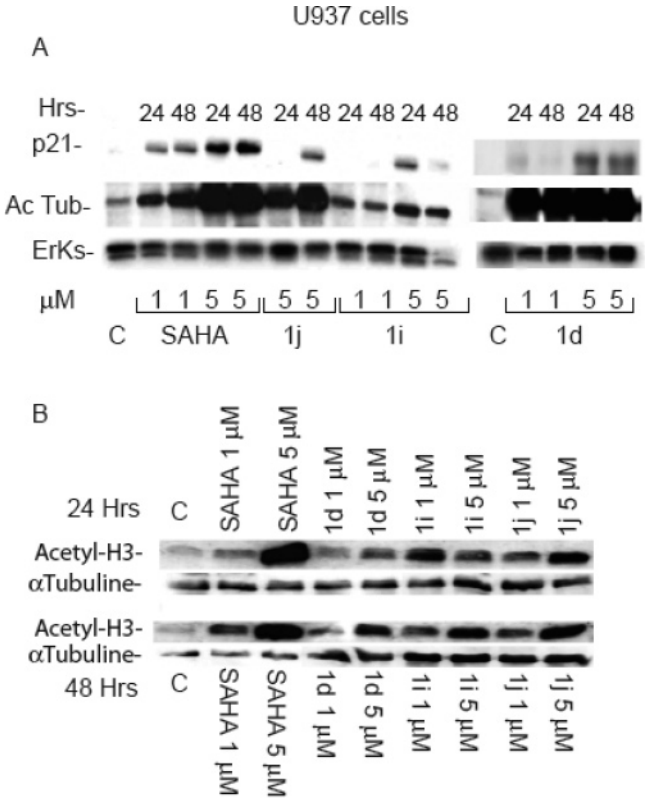


Figure 6. p21 induction, and histone H3 and α-tubulin acetylation assays by selected UBHAs on U937 cells.

the class IIa HDAC4 IP (**1j** = 14% of inhibition, **1d** = 0% of inhibition), thus displaying, for this aspect, a MS-275-like behavior on HDAC1 and 4.[60]

Effects on Human Leukemia U937 Cells. Cell cycle, Apoptosis, and Granulocytic Differentiation. To evaluate the effects of UBHA derivatives on cell cycle and apoptosis in the human leukemia U937 cell line, cell cultures were treated with 1 and 5 μM concentration of selected UBHA derivatives in comparison with SAHA (1 and 5 μM), and after 24 h, the cell cycle analysis was determined. Apoptosis, measured as caspase 3 cleavage (data not shown) and AnnexinV/propidium iodide (PI) double staining by FACS analyses, was checked after 48 h of treatment with selected UBHAs used at 1 and 5 μM. While SAHA was inducing a S/G2/M block, when used at the concentration of 5 μM, UBHA compounds showed a different tendency. Indeed, at 24 h after induction, only **1j** (this only at the concentration of 5 μM) and **1i** induced a G1 arrest in the cell cycle, whereas all of the other compounds just weakly altered the cell cycle (Figure 4A).

Of all of the UBHAs tested at 1 μM, the *N*-hydroxy-4-[3,4-dihydro-4-oxo-6-phenyl- and the *N*-hydroxy-3-[3,4-dihydro-4-oxo-6-benzylpyrimidin-2-ylthio]methyl cinnamylamides **1j** and **1x** showed low apoptosis induction in U937 cells (Figure 4B), being in all cases less powerful than SAHA. At 5 μM, SAHA showed massive apoptosis (98%), whereas UBHAs did not display an increase.

Finally, the capacity to inhibit the proliferation of selected UBHA compounds used at 5 μM has been tested in U937 cells (Figure 4C). Clearly, UBHAs **1j** and **1i** exerted inhibitory activities with **1j** being the stronger one. Note, nevertheless, that both SAHA and MS-275 (used as references) showed higher proliferation inhibition.

Granulocytic differentiation of human leukemia U937 cells was determined by CD11c expression level upon 48 h of stimulation with selected UBHAs. To this end, cell cultures were treated with UBHA compounds at both 1 μM and 5 μM concentrations together with SAHA as the reference drug, and after 48 h, the percent values of CD11c positive PI negative cells were determined (Figure 5). In this assay, at 1 μM, the two *p*-methylenecinnamyl derivatives **1j** and **1y** gave the highest differentiating effect among UBHA compounds, **1j** (42.5% CD11c positive cells) being more potent than **1y** (30%) and slightly less potent than SAHA (48%). At 5 μM, **1j** was the most effective compound in inducing granulocytic differentiation (46% CD11c positive cells), whereas **1i** (33%), **1o** (30%), and **1y** (33%) showed an effect similar to that of SAHA (32%) in U937 cells. Note that in this condition, SAHA-treated cells showed massive apoptosis.

p21 Induction and H3 Histones and α-Tubulin Acetylation Assays. Taking the cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$ (p21) induction as well as the acetylation extents of histone H3 and α-tubulin as markers of HDACi activity in U937 cells, we performed Western blot analyses to test the activity of selected UBHA derivatives. Figure 6 shows that **1j**, **1i**, and **1d** weakly increased the p21 expression. On histone H3 and α-tubulin acetylation assays, **1j**, **1i**, and **1d** showed a hyperacetylation of histone H3 comparable to or slightly weaker (5 μM) than that of SAHA (1 μM), whereas in the α-tubulin acetylation assay, **1d** influenced α-tubulin acetylation to a major extent than SAHA, **1j** and **1i** being respectively comparable to and much weaker than SAHA. The high α-tubulin acetylation activity showed by **1d** is not in contrast with the inefficiency of the same compound to inhibit HDAC4 (see cell-based anti-HDAC1 and -HDAC4 assay) because α-tubulin acetylation is a functional test for the evaluation of HDAC6 activity,[51] a class IIb HDAC, whereas HDAC4 is a member of class IIa HDAC. Thus, **1d** is able to inhibit class I and IIb but not IIa HDAC enzymes.

Conclusion

Starting from the pharmacophore model for the design of novel HDACi, we prepared a new series of uracil-containing hydroxamates (uracil-based hydroxamic acids, UBHAs) **1a**−**i′** bearing a phenyl/phenylalkyl group as the CAP, a uracil moiety as the CU, either a polymethylene or a methylenecinnamyl as the hydrophobic spacer (HS), and the hydroxamate function to bind the Zn ion, crucial for the catalytic activity of the enzyme. Compounds **1a**−**i′** were tested against three maize histone deacetylases, HD2, HD1-B (class I), and HD1-A (class II), and selected derivatives were assayed against the mouse HDAC1 enzyme. From inhibitory data (Tables 3 and 4), UBHAs with 4 to 5 methylene units showed the highest inhibitory potency, and among the cinnamyl derivatives, the meta- and para-substituted **1i**,**j** and **1x**,**y** were highly effective in inhibiting the maize HDACs. In comparison with TSA and SAHA, the most active UBHAs were less potent than TSA and up to 30-fold more potent than SAHA in inhibiting these enzymes. Mouse HDAC1 inhibitory data fully matched with the related anti-maize enzymes data.

Molecular modeling studies confirmed the crucial role of the hydroxamate and the properly sized HS for the inhibiting activity of UBHAs, highlighting the functionality of the uracil ring in making positive hydrogen-bonding interactions with two residues (Glu90 and Asp91) at the rim of the catalytic pocket of the enzyme (Supporting Information).

Cell-based human HDAC1 and HDAC4 inhibitory assays were performed on two UBHA prototypes, **1d** and **1j**. The tested compounds were able to inhibit HDAC1 (class I) but not HDAC4 (class IIa) IP activity, thus showing a MS-275-like

behavior for this aspect. Selected UBHAs were tested on human myeloid leukemic U937 cells to determine the effect on cell cycle, apoptosis, proliferation, and granulocyte differentiation. The majority of tested compounds after 48 h at 1 μM showed an arrest of the cell cycle in G1 phase (data not shown), whereas only compounds **1j** and **1i** were able to induce G1 arrest at 24 h. The two cinnamyl-*N*-hydroxyamides **1j** and **1x** induced apoptosis (8.8% and 8.0%, respectively) slightly lower than that of SAHA (10%) when used at the concentration of 1 μM. At 5 μM, SAHA-treated cells underwent massive apoptosis (98%), whereas with UBHAs, no increase of apoptosis induction was recorded. In antiproliferative assays, at 5 μM, **1j** showed the strongest effect among the UBHAs tested on U937 cells, and it was, however, less potent than SAHA and MS-275.

Tested as cytodifferentiating agents, at 1 μM, only **1j** showed high CD11c expression level (42.5%), again being less efficient than SAHA. By increasing the dose (5 μM), **1i**, **1o**, and **1y** were able to induce granulocytic differentiation with the same potency as that of SAHA, whereas **1j** was more effective.

In U937 cells, the capability of selected UBHAs to induce p21 expression as well as acetylate histone H3 and α-tubulin was also evaluated. The distinct capabilities of UBHA compounds to influence the cell cycle, differentiation, proliferation, and apoptosis as well as the capacity of p21 induction and histone H3 and α-tubulin acetylation might be interpreted as an advancement in the correlation between chemical modeling and biological functions.

Experimental Section

Chemistry. Melting points were determined on a Buchi 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 400 MHz on a Bruker AC 400 spectrometer; chemical shifts are reported in δ (ppm) units relative to the internal reference, tetramethylsilane (Me_4Si). All compounds were routinely checked by TLC and ^{1}H 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 standard methods. The concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within ±0.40% of the theoretical values. A SAHA sample for biological assays was prepared as previously reported by us.[61] All chemicals were purchased from Aldrich Chimica, Milan (Italy) or from Lancaster Synthesis GmbH, Milan (Italy) and were of the highest purity.

General Procedure for the Synthesis of Ethyl ω-(3,4-Dihydro-4-oxo-6-(un)substituted-2-pyrimidinylthio)alkanoates (3b−g,l−q,v−d′). Example: Ethyl 6-(6-Benzyl-3,4-dihydro-4-oxopyrimidin-2-ylthio)hexanoate (3o). A mixture of 6-benzyl-4-hydroxy-2-mercaptopyrimidine (**2e**) (9.16 mmol, 2.0 g), ethyl 6-bromohexanoate (10 mmol, 1.8 mL), and anhydrous potassium carbonate (10 mmol, 1.4 g) in 3 mL of anhydrous DMF was stirred at room temperature for 1 h. After treatment with cold water (100 mL), the obtained precipitate was filtered and washed to furnish **3o** (1.6 g), which was purified by crystallization. ^{1}H NMR ($CDCl_3$) δ 1.25 (t, 3H, CH_2CH_3), 1.40 (m, 2H, $CH_2CH_2CH_2CO$), 1.63 (m, 4H, CH_2CH_2-CO and CH_2CH_2S), 2.28 (t, 2H, CH_2CO), 3.13 (t, 2H, CH_2S), 3.80 (s, 2H, $PhCH_2$), 4.13 (q, 2H, CH_2CH_3), 5.96 (s, 1H, C_5−H), 7.28 (m, 5H, benzene ring), 12.87 (s, 1H, NH). Anal. C, H, N, S.

General Procedure for the Synthesis of Ethyl 3-(3,4-Dihydro-4-oxo-6-substituted-2-pyrimidinylthio)-2-propenoates (3a,k,z). Example: Ethyl 3-(6-Benzyl-3,4-dihydro-4-oxopyrimidin-2-ylthio)-2-propenoate (3k). To a solution of 6-benzyl-4-hydroxy-2-mercaptopyrimidine (**2e**) (2.29 mmol, 0.50 g) and ethyl propiolate (2.75 mmol, 0.28 mL) in anhydrous THF (10 mL), tetrabutylammonium fluoride trihydrate (2.75 mmol, 0.87 g) was added, and the resulting mixture was stirred at 70 °C for 8 h. After treatment with cold water (100 mL), the resulting precipitate was filtered and washed to furnish **3k** (0.34 g), which was purified by crystallization. ^{1}H NMR ($CDCl_3$) δ 1.32 (t, 3H, CH_2CH_3), 3.87 (s, 2H, $PhCH_2$), 4.24 (q, 2H, CH_2CH_3), 6.02 (s, 1H, C_5−H), 6.14 (d, 1 H, CH=CHCO), 7.29 (m, 5H, benzene ring), 8.29 (d, 1H, CH=CHCO), 12.56 (s, 1H, NH). Anal. C, H, N, S.

General Procedure for the Synthesis of Ethyl Esters of 2-, 3-, and 4-(3,4-Dihydro-4-oxo-6-substituted-2-pyrimidinylthio)-methylcinnamic Acids (3h−j,w−y). Example: Ethyl Ester of 3-(3,4-Dihydro-4-oxo-6-benzyl-2-pyrimidinylthio)methylcinnamic Acid (3x). A mixture of 6-benzyl-4-hydroxy-2-mercaptopyrimidine (**2e**) (6.87 mmol, 1.5 g), crude ethyl 3-bromomethylcinnamate (**6b**)[59] (7.56 mmol, 2.2 g), and anhydrous potassium carbonate (7.56 mmol, 1.0 g) in 3 mL of anhydrous DMF was stirred at room temperature for 1 h. After treatment with cold water (100 mL), the aqueous phase was extracted with ethyl acetate (3 × 40 mL). The organic phase was washed with brine (3 × 40 mL), dried, and evaporated to dryness to furnish crude **3x**, which was purified by chromatography on a silica gel column, eluting with a mixture ethyl acetate/hexane (1:1) to give the desired product as a white solid (1.2 g). ^{1}H NMR ($CDCl_3$) δ 1.33 (t, 3H, CH_2CH_3), 3.83 (s, 2H, $PhCH_2$), 4.26 (q, 2H, CH_2CH_3), 4.38 (s, 2H, CH_2S), 5.98 (s, 1H, C_5−H), 6.40 (d, 1H, CH=CHCO), 7.33 (m, 9H, two benzene rings), 7.61 (d, 1H, CH=CHCO), 13.20 (s, 1H, NH). Anal. C, H, N, S.

General Procedure for the Synthesis of *N*-Hydroxy-ω-(3,4-dihydro-4-oxo-6-substituted-2-pyrimidinylthio)alkanamides (1b−g,l−o,p,q,a′−f′). Example: *N*-Hydroxy-6-(6-benzyl-3,4-dihydro-4-oxopyrimidin-2-ylthio)hexanamide (1o). To hydroxylamine hydrochloride (39 mmol, 2.7 g) solution in dry ethanol (5 mL), potassium hydroxide (39 mmol, 2.2 g) solution in dry ethanol (5 mL) was added at 40 °C. The mixture was cooled at 0 °C and then filtered, and to the clear solution, **3o** (4.2 mmol, 1.5 g) and well-crushed potassium hydroxide (7.3 mmol, 0.4 g) were added. After 1 h, the mixture was diluted with water (50 mL), made neutral with 2 N HCl, and filtered under vacuum. The solid **1o** was collected, dried, and recrystallized by acetonitrile/methanol. ^{1}H NMR (DMSO-d_6) δ 1.27 (m, 2H, $CH_2CH_2CH_2S$), 1.44 (m, 2H, CH_2CH_2CO), 1.50 (m, 2H, CH_2CH_2S), 1.92 (t, 2H, CH_2CO), 3.01 (t, 2H, CH_2S), 3.72 (s, 2H, $PhCH_2$), 5.92 (s, 1H, C_5−H), 7.24 (m, 5H, benzene ring), 8.66 (s, 1H, NHOH), 10.33 (s, 1H, NHOH), 12.45 (s, 1H, NH uracil ring). Anal. C, H, N, S.

General Procedure for the Synthesis of 3-(3,4-Dihydro-4-oxo-6-substituted-2-pyrimidinylthio)-2-propenoic Acids (4a,b,h), 6-(3,4-Dihydro-4-oxo-6-(un)substituted-2-pyrimidinylthio)-hexanoic Acids (4c−g,i−k), and 2-, 3-, and 4-(3,4-Dihydro-6-substituted-4-oxopyrimidin-2-ylthio)methylcinnamic Acids (4l−q). Example: 6-(3,4-Dihydro-4-oxopyrimidin-2-ylthio)hexanoic Acid (4i). A mixture of **3g′** (1.1 mmol, 0.3 g), 2 N KOH (8.8 mmol, 0.49 g), and EtOH (5 mL) was stirred at room temperature for 18 h. The solution was poured into water (50 mL) and extracted with ethyl acetate (2 × 20 mL). HCl (2 N) was added to the aqueous layer until the pH 5, and the precipitate was filtered and recrystallized to yield the title compound **4i** (0.23 g) as a pure solid. ^{1}H NMR (DMSO-d_6) δ 1.32 (m, 2H, $CH_2CH_2CH_2S$), 1.49 (m, 2H, CH_2CH_2CO), 1.61 (m, 2H, CH_2CH_2S), 1.93 (t, 2H, CH_2CO), 3.06 (t, 2H, CH_2S), 6.07 (s, 1H, C_5−H), 7.83 (s, 1H, C_6−H), 12.2 (s, 1H, COOH). Anal. C, H, N, S.

General Procedure for the Synthesis of *N*-Hydroxy-3-(3,4-dihydro-4-oxo-6-substituted-2-pyrimidinylthio)-2-propenamides (1a,k,z), *N*-Hydroxy-6-(3,4-dihydro-4-oxo-6-(un)substituted-2-pyrimidinylthio)hexanamides (1p−t,g′−i′), and *N*-Hydroxy-2-, -3-, and -4-(3,4-dihydro-6-substituted-4-oxopyrimidin-2-ylthio)-methylcinnamylamides (1h−j,w−y). Example: *N*-Hydroxy-6-(3,4-dihydro-4-oxopyrimidin-2-ylthio)hexanamide (1g′). To a 0 °C cooled solution of **4i** (0.9 mmol, 0.22 g) in dry tetrahydrofuran (5 mL), ethyl chloroformate (2.2 mmol, 0.21 mL) and triethylamine (2.3 mmol, 0.33 mL) were added, and the mixture was stirred for 10 min. The solid was filtered off, and to the filtrate was added *O*-(2-methoxy-2-propyl)hydroxylamine[56] (5.4

mmol, 0.4 mL). The resulting mixture was stirred at room temperature for 1 h, then it was evaporated under reduced pressure, and the residue was diluted in MeOH (5 mL). Amberlyst 15 ion-exchange resin (0.18 g) was added to the solution of the *O*-protected hydroxamate, and the mixture was stirred at room temperature for 1 h. Afterward, the reaction was filtered, and the filtrate was concentrated in a vacuum to give crude **1g′**, which was purified by crystallization. ^{1}H NMR (DMSO-d_6) δ 1.30 (m, 2H, CH_2CH$_2$CH$_2$S), 1.46 (m, 2H, CH_2CH$_2$CO), 1.60 (m, 2H, CH_2CH$_2$S), 1.90 (t, 2H, CH$_2$CO), 3.02 (t, 2H, CH$_2$S), 6.10 (s, 1H, C$_5$−H), 7.85 (s, 1H, C$_6$−H), 8.66 (s, 1H, N*H*OH), 10.33 (s, 1H, NHO*H*), 12.5 (s, 1H, uracil N*H*). Anal. C, H, N, S.

General Procedure for the Synthesis of the β-Oxoesters 7a,b. Example: Ethyl 4-Phenyl-4-methoxy-3-oxobutanoate (7a). Triethylamine (6.7 mL, 48.0 mmol) and magnesium chloride (3.6 g, 37.5 mmol) were added to a stirred suspension of potassium ethyl malonate (5.4 g, 31.5 mmol) in acetonitrile (50 mL), and stirring was continued at room temperature for 2 h. Then, a solution of α-methoxyphenylacetyl imidazolide, prepared from α-methoxyphenylacetic acid (2.5 g, 15.0 mmol) and *N*,*N*′-carbonyldiimidazole (CDI, 2.9 g, 18.0 mmol) in acetonitrile (15 mL), was added, and the reaction mixture was stirred overnight at room temperature. Then, 13% HCl (90 mL) was cautiously added while keeping the temperature below 25 °C, and the resulting clear mixture was stirred for a further 15 min. The organic layer was separated from the aqueous mixture and evaporated; then, the residue was treated with ethyl acetate (30 mL). The aqueous layer was extracted with ethyl acetate (3 × 30 mL), and the organic phases were combined, washed with saturated sodium bicarbonate solution (3 × 30 mL) and brine (3 × 30 mL), dried, and concentrated to give **7a** as a yellow oil. Yield: 88%; ^{1}H NMR (CDCl$_3$) δ 1.22 (t, 3H, OCH$_2$C*H*$_3$), 3.39 (s, 3H, OCH$_3$), 3.50 (m, 2H, COCH$_2$CO), 4.13 (q, 2H, OC*H*$_2$CH$_3$), 4.77 (s, 1H, C*H*OCH$_3$), 7.35 (m, 5H, benzene ring). Anal. C, H.

General Procedure for the Synthesis of the 2-Thiouracils 2h,i. Example: 3,4-dihydro-6-(1-phenyl-1-cyclopropyl)-2-thioxopyrimidin-4(*3H*)-one (2i). Sodium metal (0.67 g, 29.2 g-atom) was dissolved in 26 mL of absolute ethanol, and thiourea (1.56 g, 20.5 mmol) and **7b** (3.40 g, 14.6 mmol) were added to the clear solution. The mixture was heated at reflux for 12 h. After the completion of the reaction, the mixture was cooled, the solvent was distilled in vacuo at 40−50 °C until dry, and the residue was dissolved in a little water (15 mL) and made acidic with 2 N HCl. The resulting precipitate was filtered under reduced pressure, washed with diethyl ether, and vacuum dried at 80 °C for 12 h to give title compound **2i** as a pure white solid, which was further purified by crystallization from ethanol. ^{1}H NMR (DMSO) δ 1.18 (m, 2H, c-Pr−CH$_2$), 1.32 (m, 2H, c-Pr−C*H*$_2$), 5.92 (s, 1H, C$_5$−H), 7.32 (m, 5H, benzene ring), 12.36 (s, 1H, NH), 12.41 (s, 1H, NH). Anal. C, H, N, S.

Maize HD2, HD1-B, and HD1-A Enzyme Inhibition in Vitro. Radioactively labeled chicken core histones were used as the enzyme substrate according to established procedures.[62−64] The enzyme liberated tritiated acetic acid from the substrate, which was quantified by scintillation counting. The IC$_{50}$ values are the results of triple determinations. A 50 μL sample of maize enzyme (at 30 °C) was incubated (30 min) with 10 μL of total [^{3}H]acetate-prelabeled chicken reticulocyte histones (2 mg/mL). The reaction was stopped by the addition of 36 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. TSA and SAHA were used as the reference compounds, and blank solvents were used as negative controls.

Mouse HDAC1 Enzyme Assay. For the inhibition assay, partially purified HDAC1 from mouse liver (anion exchange chromatography) was used as the enzyme source. HDAC activity was determined as described[65] using [^{3}H]acetate-prelabeled chicken reticulocyte histones as the substrate. Mouse HDAC1 (50 μL) was incubated with different concentrations of compounds for 15 min on ice, and 10 μL of total [^{3}H]acetate-prelabeled chicken reticulocyte histones (2 mg/mL) were added, resulting in a concentration of 41 μM. The mixture was incubated at 37 °C for 1 h. The reaction was stopped by the addition of 50 μL of 1 M HCl/0.4 M acetylacetate and 1 mL ethyl acetate. After centrifugation at 10 000*g* for 5 min, an aliquot of 600 μL of the upper phase was counted for radioactivity in a 3 mL liquid scintillation cocktail.

Cellular Assays. Cell Lines and Cultures. The U937 cell line was cultured in RPMI with 10% fetal calf serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin-B, 10 mM HEPES and 2 mM glutamine. U937 cells were kept at the constant concentration of 200 000 cells per milliliter of culture medium. Human breast cancer ZR-75.1 cells were propagated in DMEM medium supplemented with 10% fetal calf serum and antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin-B).

Ligands and Materials. SAHA was dissolved in DMSO and used at 1 or 5 μM. MS-275 (kind gift from Schering AG) was dissolved in ethanol and used at 5 μM. UBHA compounds **1d** and **1j** were dissolved in DMSO and used at 1 or 5 μM.

Cell-Based Human HDAC1 and HDAC4 Assays. Cells (U937 cells for the HDAC1 assay and ZR75.1 cells for the HDAC4 assay) were lysed in IP buffer (50 mM Tris-HCl at pH 7.0, 180 mM NaCl, 0.15% NP-40, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM $NaMO_4$, and 0.5 mM NaF) with a protease inhibitor cocktail (Sigma), 1 mM DTT, and 0.2 mM PMSF for 10 min in ice and centrifuged at 13 000 rpm for 30 min. Then, 1000 μg of extracts were diluted in IP buffer up to 1 mL and pre-cleared by incubating with 20 μL of A/G plus Agarose (Santa Cruz) for 30 min to 1 h on a rocking table at 4 °C. Supernatants were transferred into a new tube, and the antibodies (around 3 to 4 μg) were added and IP was allowed to proceed overnight at 4 °C on a rocking table. The antibodies used were HDAC1 (Abcam) and HDAC4 (Sigma). As the negative control, the same amount of protein extracts were immunoprecipitated with the corresponding purified IgG (Santa Cruz). On the next day, 20 μL of A/G and Agarose (Santa Cruz) were added to each IP, and incubation was continued for 2 h. The beads were recovered by brief centrifugation and washed with cold IP buffer several times. The samples were than washed twice in PBS and re-suspended in 20 μL of sterile PBS. The HDAC assay was carried out according to the supplier's instructions (Upstate). Briefly, samples immunoprecipitated with the HDAC4 and HDAC1 or with purified IgG were pooled separately to homogenize all samples. Then, 10 μL of the IP was incubated with a previously labeled ^{3}H-Histone H4 peptide linked with streptavidine agarose beads (Upstate). In detail, 120 000 CPM of the H4-^{3}H-acetyl-peptide was used for each tube and incubated in 1× HDAC buffer with 10 μL of the sample in the presence or absence of HDAC inhibitors with a final volume of 200 μL. Those samples were incubated overnight at 37 °C in slow rotation. On the next day, 50 μL of a quenching solution was added, and 100 μL of the samples were counted in duplicate after brief centrifugation in a scintillation counter. Experiments have been carried out in quadruplicate.

Cell Cycle Analysis on U937 Cells. Cells (2.5 × 10^5) were collected and resuspended in 500 μL of hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate, 50 μg/mL of propidium iodide (PI), and 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.[60] All of the experiments were performed three times.

FACS Analysis of Apoptosis on U937 Cells. Apoptosis was measured with Annexin V/PI double staining detection (Roche and Sigma-Aldrich, respectively) as recommended by the suppliers; samples were analyzed by FACS with Cell Quest technology (Becton Dickinson) as previously reported.[66] As second assays, the caspase 3 detection (B-Bridge) was performed and quantified by FACS (data not shown; Becton Dickinson).

Proliferation Assay on U937 Cells. U937 cells have been cultured in 24 multiwells (Corning) at the initial dilution of 200 000

cells/mL with vehicle or with HDAC inhibitors used at the indicated concentrations. Every 24 h, living U937 cells have been counted using the Trypan Blue dye (Sigma) for dead cells staining. The graph shows data plotted after three days. The experiment has been carried out in triplicate. In parallel, an MTT colorimetric proliferation assay (Promega) has been carried out in duplicate (data not shown) following manufacturer's instructions.

Granulocytic Differentiation on U937 Cells. Granulocytic differentiation was carried out as previously described.[66] Briefly, 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 PI (0.25 μg/mL). Samples were analyzed by FACS with Cell Quest technology (Becton Dickinson). PI positive cells have been excluded from the analysis.

Determination of p21$^{WAF1/CIP1}$ Induction in U937 Cells. Total protein extracts (100 μg) were separated on a 15% polyacrylamide gel and blotted as previously described.[60,66] Western blots were shown for p21 (Transduction Laboratories, dilution 1:500), and total ERKs (Santa Cruz) were used to normalize for equal loading.

Histone H3 and α-Tubulin Acetylation in U937 Cells. For determination of α-tubulin acetylation, 25 μg of total protein extracts were separated on a 10% polyacrylamide gel and blotted. Western blots were shown for acetylated α-tubulin (Sigma, dilution 1:500), and total ERKs (Santa Cruz, dilution 1:1000) were used to normalize for equal loading. For the quantification of histone H3 acetylation, 100 μg of total protein extracts were separated on a 15% polyacrylamide gel and blotted. Western blots were shown for acetylated histone H3 (Upstate), and total tubulin (Sigma) was used to normalize for equal loading.

Acknowledgment. This work was partially supported by grants from AIRC 2005 (to A.M.), PRIN 2004 (to A.M.), European Union (LSHC-CT2005-518417) (to L.A.), and PRIN 2004 (to L.A.). A.N. and M.M. have been financed within the LSHC-CT2005-518417 EU contract.

Supporting Information Available: Molecular modeling studies, ^{1}H NMR data of final compounds **1a**−**i′**, and elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Cheung, W. L.; Briggs, S. D.; Allis, C. D. Acetylation and chromosomal functions. *Curr. Opin. Cell Biol.* **2000**, *12*, 326−333.
(2) Grunstein, M. Histone acetylation in chromatin structure and transcription. *Nature (London)* **1997**, *389*, 349−352.
(3) Kouzarides, T. Histone acetylases and deacetylases in cell proliferation. *Curr. Opin. Genet. Dev.* **1999**, *9*, 40−48.
(4) Hassig, C. A.; Schreiber, S. L. Nuclear histone acetylases and deacetylases and transcriptional regulation: HATs off to HDACs. *Curr. Opin. Chem. Biol.* **1997**, *1*, 300−308.
(5) Strahl, B. D.; Allis, C. D. The language of covalent histone modifications. *Nature (London)* **2000**, *403*, 41−45.
(6) Wu, J.; Grunstein, M. 25 Years after the nucleosome model: chromatin modifications. *Trends Biochem. Sci.* **2000**, *25*, 619−623.
(7) Wolffe, A. P.; Guschin, D. Chromatin structural features and targets that regulate transcription. *J. Struct. Biol.* **2000**, *129*, 102−122.
(8) Grozinger, C. M.; Schreiber, S. L. Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. *Chem. Biol.* **2002**, *9*, 3−16.
(9) Gregoretti, I. V.; Lee, Y.-M.; Goodson, H. V. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J. Mol. Biol.* **2004**, *338*, 17−31.
(10) Verdin, E.; Dequiedt, F.; Kasler, H. G. Class II histone deacetylases: versatile regulators. *Trends Genet.* **2003**, *19*, 286−293.
(11) Blander, G.; Guarente, L. The Sir2 family of protein deacetylases. *Annu. Rev. Biochem.* **2004**, *73*, 417−435.
(12) Ng, H. H.; Bird, A. Histone deacetylases: silencers for hire. *Trends Biochem. Sci.* **2000**, *25*, 121−126.
(13) Ferreira, R.; Magnaghi-Jaulin, L.; Robin, P.; Harel-Bellan, A.; Trouche, D. The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10493−10498.
(14) Stiegler, P.; De Luca, A.; Bagella, L.; Giordano, A. The COOH-terminal region of pRb2/p130 binds to histone deacetylase 1 (HDAC1), enhancing transcriptional repression of the E2F-dependent cyclin A promoter. *Cancer Res.* **1998**, *58*, 5049−5052.
(15) Nagy, L.; Kao, H.-Y.; Chakravarti, D.; Lin, R. J.; Hassig, C. A.; Ayer, D. E.; Schreiber, S. L.; Evans, R. M. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **1997**, *89*, 373−380.
(16) Alland, L.; Muhle, R.; Hou, H.; Potes, J.; Chin, L.; Schreiber-Agus, N.; DePinho, R. A. Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature (London)* **1997**, *387*, 49−55.
(17) Heinzel, T.; Lavinsky, R. M.; Mullen, T.-M.; Soderstrom, M.; Laherty, C. D.; Torchia, J.; Yang, W.-M.; Brard, G.; Ngo, S. D.; Davie, J. R.; Seto, E.; Eisenman, R. N.; Rose, D. W.; Glass, C. K.; Rosenfeld, M. G. A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **1997**, *387*, 43−48
(18) Mai, A.; Massa, S.; Rotili, D.; Cerbara, I.; Valente, S.; Pezzi, R.; Simeoni, S.; Ragno, R. Histone deacetylation in epigenetics: an attractive target for anticancer therapy. *Med. Res. Rev.* **2005**, *25*, 261−309.
(19) Weidle, U. H.; Grossmann, A. Inhibition of histone deacetylases: a new strategy to target epigenetic modifications for anticancer treatment. *Anticancer Res.* **2000**, *20*, 1471−1486.
(20) Kramer, O. H.; Göttlicher, M. G.; Heinzel, T. Histone deacetylase as a therapeutic target. *Trends Endocrinol. Metab.* **2001**, *12*, 294−300.
(21) Marks, P. A.; Richon, V. M.; Breslow, R.; Rifkind, R. A. Histone deacetylase inhibitors as new cancer drugs. *Curr. Opin. Oncol.* **2001**, *13*, 477−483.
(22) Marks, P. A.; Rifkind, R. A.; Richon, V. M.; Breslow, R.; Miller, T.; Kelly, W. K. Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer* **2001**, *1*, 194−202.
(23) Jung, M. Inhibitors of histone deacetylase as new anticancer agents. *Curr. Med. Chem.* **2001**, *8*, 1505−1511.
(24) Vigushin, D. M.; Coombes, R. C. Histone deacetylase inhibitors in cancer treatment. *Anti-Cancer Drugs* **2002**, *13*, 1−13.
(25) Johnstone, R. W. Histone deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat. Rev. Drug Discov*ery **2002**, *1*, 287−299.
(26) Kelly, W. K.; O'Connor, O. A.; Marks, P. A. Histone deacetylase inhibitors: from target to clinical trials. *Expert Opin. Invest. Drugs* **2002**, *11*, 1695−1713.
(27) Yoshida, M.; Kijima, M.; Akita, M.; Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J. Biol. Chem.* **1990**, *265*, 17174−17179.
(28) Finnin, M. S.; Donigian, J. R.; Cohen, A.; Richon, V. M.; Rifkind, R. A.; Marks, P. A.; Breslow, R.; Pavletich, N. P. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* **1999**, *401*, 188−193.
(29) Miller, T. A.; Witter, D. J.; Belvedere, S. Histone deacetylase inhibitors. *J. Med. Chem.* **2003**, *46*, 5097−5116.
(30) Massa, S.; Mai, A.; Sbardella, G.; Esposito, M.; Ragno, R.; Loidl, P.; Brosch, G. 3-(4-Aroyl-1*H*-pyrrol-2-yl)-*N*-hydroxy-2-propenamides, a new class of synthetic histone deacetylase inhibitors. *J. Med. Chem.* **2001**, *44*, 2069−2072.
(31) Mai, A.; Massa, S.; Ragno, R.; Esposito, M.; Sbardella, G.; Nocca, G.; Scatena, R.; Jesacher, F.; Loidl, P.; Brosch, G. Binding mode analysis of 3-(4-benzoyl-1-methyl-1*H*-2-pyrrolyl)-*N*-hydroxy-2-propenamide: a new synthetic histone deacetylase inhibitor inducing histone hyperacetylation, growth inhibition, and terminal cell differentiation. *J. Med. Chem.* **2002**, *45*, 1778−1784.
(32) Mai, A.; Massa, S.; Ragno, R.; Cerbara, I.; Jesacher, F.; Loidl, P.; Brosch, G. 3-(4-Aroyl-1-methyl-1*H*-2-pyrrolyl)-*N*-hydroxy-2-alkylamides as a new class of synthetic histone deacetylase inhibitors. 1. Design, synthesis, biological evaluation, and binding mode studies performed through three different docking procedures. *J. Med. Chem.* **2003**, *46*, 512−524.
(33) Mai, A.; Massa, S.; Pezzi, R.; Rotili, D.; Loidl, P.; Brosch, G. Discovery of (aryloxopropenyl)pyrrolyl hydroxamides as selective inhibitors of class IIa histone deacetylase homologue HD1-A. *J. Med. Chem.* **2003**, *46*, 4826−4829.
(34) Mai, A.; Massa, S.; Cerbara, I.; Valente, S.; Ragno, R.; Bottoni, P.; Scatena, R.; Loidl, P.; Brosch, G. 3-(4-Aroyl-1-methyl-1*H*-2-pyrrolyl)-*N*-hydroxy-2-propenamides as a new class of synthetic histone deacetylase inhibitors. 2. Effect of pyrrole C2 and/or C4 substitutions on biological activity. *J. Med. Chem.* **2004**, *47*, 1098−1109.

(35) Ragno, R.; Mai, A.; Massa, S.; Cerbara, I.; Valente, S.; Bottoni, P.; Scatena, R.; Jesacher, F.; Loidl, P.; Brosch, G. 3-(4-Aroyl-1-methyl-1*H*-pyrrol-2-yl)-*N*-hydroxy-2-propenamides as a new class of synthetic histone deacetylase inhibitors. 3. Discovery of novel lead compounds through structure-based drug design and docking studies. *J. Med. Chem.* **2004**, *47*, 1351−1359.

(36) Mai, A.; Massa, S.; Pezzi, R.; Simeoni, S.; Rotili, D.; Nebbioso, A.; Scognamiglio, A.; Altucci, L.; Loidl, P.; Brosch, G. Class II (IIa)-selective histone deacetylase inhibitors. 1. Synthesis and biological evaluation of novel (aryloxopropenyl)pyrrolyl hydroxamides. *J. Med. Chem.* **2005**, *48*, 3344−3353.

(37) Mai, A.; Massa, S.; Lavu, S.; Pezzi, R.; Simeoni, S.; Ragno, R.; Mariotti, F. R.; Chiani, F.; Camilloni, G.; Sinclair, D. A. Design, Synthesis, and biological evaluation of sirtinol analogues as class III histone/protein deacetylase (sirtuin) inhibitors. *J. Med. Chem.* **2005**, *48*, 7789−7795.

(38) Mai, A.; Massa, S.; Rotili, D.; Pezzi, R.; Bottoni, P.; Scatena, R.; Meraner, J.; Brosch, G. Exploring the connection unit in the HDAC inhibitor pharmacophore model: Novel uracil-based hydroxamates. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4656−4661.

(39) Mai, A.; Massa, S.; Pezzi, R.; Valente, S.; Loidl, P.; Brosch, G. Synthesis and biological evaluation of 2-, 3-, and 4-acylaminocinnamyl-*N*-hydroxyamides as novel synthetic HDAC inhibitors. *Med. Chem. (Academic Press)* **2005**, *1*, 245−254.

(40) Mai, A.; Massa, S.; Valente, S.; Simeoni, S.; Ragno, R.; Bottoni, P.; Scatena, R.; Brosch, G. Aroyl-pyrrolyl hydroxyamides: influence of pyrrole C4-phenylacetyl substitution on histone deacetylase inhibition. *ChemMedChem* **2006**, *1*, 225−237.

(41) Dai, Y.; Guo, Y.; Curtin, M. L.; Li, J.; Pease, L. J.; Guo, J.; Marcotte, P. A.; Glaser, K. B.; Davidsen, S. K.; Michaelides, M. R. A novel series of histone deacetylase inhibitors incorporating heteroaromatic ring systems as connection units. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3817−3820.

(42) Woo, S. H.; Frechette, S.; Khalil, E. A.; Bouchain, G.; Vaisburg, A.; Bernstein, N.; Moradei, O.; Leit, S.; Allan, M.; Fournel, M.; Trachy-Bourget, M.-C.; Li, Z.; Besterman, J. M.; Delorme, D. Structurally simple trichostatin A-like straight chain hydroxamates as potent histone deacetylase inhibitors. *J. Med. Chem.* **2002**, *45*, 2877−2885.

(43) Curtin, M.; Glaser, K. Histone deacetylase inhibitors: The Abbott experience. *Curr. Med. Chem.* **2003**, *10*, 2373−2392.

(44) Lusser, A.; Brosch, G.; Loidl, A.; Haas, H.; Loidl, P. Identification of maize histone deacetylase HD2 as an acidic nucleolar phosphoprotein. *Science* **1997**, *277*, 88−91.

(45) Kölle, D.; Brosch, G.; Lechner, T.; Pipal, A.; Helliger, W.; Taplick, J.; Loidl, P. Different types of maize histone deacetylases are distinguished by a highly complex substrate and site specificity. *Biochemistry* **1999**, *38*, 6769−6773.

(46) Lechner, T.; Lusser, A.; Pipal, A.; Brosch, G.; Loidl, A.; Goralik-Schramel, M.; Sendra, R.; Wegener, S.; Walton, J. D.; Loidl, P. RPD3-Type histone deacetylases in maize embryos. *Biochemistry* **2000**, *39*, 1683−1692.

(47) Brosch, G.; Goralik-Schramel, M.; Loidl, P. Purification of histone deacetylase HD1-A of germinating maize embryos. *FEBS Lett.* **1996**, *393*, 287−291.

(48) Brosch, G.; Georgieva, E.; Lopez-Rodas, G.; Lindner, H.; Loidl, P. Specificity of Zea mays histone deacetylase is regulated by phosphorylation. *J. Biol. Chem.* **1992**, *267*, 20561−20564.

(49) Richon, V. M.; Emiliani, S.; Verdin, E.; Webb, Y.; Breslow, R.; Rifkind, R. A.; Marks, P. A. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3003−3007.

(50) Sambucetti, L. C.; Fischer, D. D.; Zabludoff, S.; Kwon, P. O.; Chamberlin, H.; Trogani, N.; Xu, H.; Cohen, D. Histone deacetylase inhibition selectively alters the activity and expression of cell cycle proteins leading to specific chromatin acetylation and antiproliferative effects. *J. Biol. Chem.* **1999**, *274*, 34940−34947.

(51) Haggarty, S. J.; Koeller, K. M.; Wong, J. C.; Grozinger, C. M.; Schreiber, S. L. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4389−4394.

(52) Mai, A.; Artico, M.; Sbardella, G.; Quartarone, S.; Massa, S.; Loi, A. G.; De Montis, A.; Scintu, F.; Putzolu, M.; La Colla, P. Dihydro-(alkylthio)(naphthylmethyl) oxopyrimidines: novel non-nucleoside reverse transcriptase inhibitors of the *S*-DABO series. *J. Med. Chem.* **1997**, *40*, 1447−1454.

(53) Andersen, G. W.; Halverstadt, I. F.; Miller, W. H.; Roblin, R. O., Jr. Studies in chemotherapy. X. Antithyroids compounds. Synthesis of 5- and 6-substituted 2 thiouracils from β-oxoesters and thiourea. *J. Am. Chem. Soc.* **1945**, *67*, 2197−2200.

(54) Mai, A.; Sbardella, G.; Artico, M.; Ragno, R.; Massa, S.; Novellino, E.; Greco, G.; Lavecchia, A.; Musiu, C.; La Colla, M.; Murgioni, C.; La Colla, P.; Loddo, R. Structure-based design, synthesis, and biological evaluation of conformationally restricted novel 2-alkylthio-6-[1-(2,6-difluorophenyl)alkyl]-3,4-dihydro-5-alkylpyrimidin-4(*3H*)-ones as nonnucleoside inhibitors of HIV-1 reverse transcriptase. *J. Med. Chem.* **2001**, *44*, 2544−2554.

(55) Libermann, D.; Hengl, L. Antithyroid substances. III. Ethyl γ,γ-diphenyl γ,γ-dibenzyl, and γ-phenyl-γ-benzylacetoacetates and the corresponding thiouracils. *Bull. Soc. Chim. Fr.* **1951**, 974−976.

(56) Mori, K.; Koseki, K. Synthesis of trichostatin A, a potent differentiation inducer of Friend leukemic cells, and its antipode. *Tetrahedron* **1988**, *44*, 6013−6020.

(57) Takeuchi, R.; Ue, N.; Tanabe, K.; Yamashita, K.; Shiga, N. Iridium complex-catalyzed allylic amination of allylic esters. *J. Am. Chem. Soc.* **2001**, *123*, 9525−9534.

(58) Tani, M.; Sakaguchi, S.; Ishii, Y. $Pd(OAc)_2$-catalyzed oxidative coupling reaction of benzenes with olefins in the presence of molybdovanadophosphoric acid under atmospheric dioxygen and air. *J. Org. Chem.* **2004**, *69*, 1221−1226.

(59) Ohnishi, H.; Miyakoshi, M.; Isozaki, M.; Fujitake, M.; Mikami, N.; Yanoshita, R.; Akasofu, H.; Sugizaki, K.; Nakata, N. Preparation of N-(3-Pyridylalkyl)sulphonamide Derivatives as Drugs. Eur. Pat. Appl. EP 501876, 1992.

(60) Nebbioso, A.; Clarke, N.; Voltz, E.; Germain, E.; Ambrosino, C.; Bontempo, P.; Alvarez, R.; Schiavone, E. M.; Ferrara, F.; Bresciani, F.; Weisz, A.; de Lera, A. R.; Gronemeyer, H.; Altucci, L. Tumor-selective action of HDAC inhibitors involves TRAIL induction in acute myeloid leukemia cells. *Nat. Med.* **2005**, *11*, 77−84.

(61) Mai, A.; Esposito, M.; Sbardella, G.; Massa, S. A new facile and expeditious synthesis of *N*-hydroxy-*N*′-phenyloctanediamide, a potent inducer of terminal cytodifferentiation. *Org. Prep. Proced. Int.* **2001**, *33*, 391−394.

(62) Lechner, T.; Lusser, A.; Brosch, G.; Eberharter, A.; Goralik-Schramel, M.; Loidl, P. A comparative study of histone deacetylases of plant, fungal and vertebrate cells. *Biochim. Biophys. Acta* **1996**, *1296*, 181−188.

(63) Brosch, G.; Lusser, A.; Goralik-Schramel, M.; Loidl, P. Purification and characterization of a high molecular weight histone deacetylase complex (HD2) of maize embryos. *Biochemistry* **1996**, *35*, 15907−15914.

(64) Kölle, D.; Brosch, G.; Lechner, T.; Lusser, A.; Loidl, P. Biochemical methods for analysis of histone deacetylases. *Methods* **1998**, *15*, 323−331.

(65) Sendra, R.; Rodrigo, I.; Salvador, M. L.; Franco, L. Characterization of pea histone deacetylases. *Plant Mol. Biol.* **1988**, *11*, 857−866.

(66) Altucci, L.; Rossin, A.; Raffelsberger, W.; Reitmair, A.; Chomienne, C.; Gronemeyer, H. Retinoic acid-induced apoptosis in leukemia cells is mediated by paracrine action of tumor-selective death ligand TRAIL. *Nat. Med.* **2001**, *7*, 680−686.

JM0605536