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Antiproliferative and Differentiating Activities of a Novel Series of Histone Deacetylase Inhibitors

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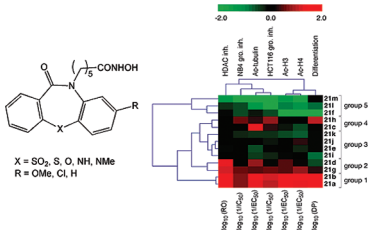
ABSTRACT Histone deacetylases are promising molecular targets for the development of antitumor agents. A novel series of histone deacetylase inhibitors of the hydroxamic acid type were synthesized for structure−activity studies. Thirteen tricyclic dibenzo-diazepine, -oxazepine, and -thiazepine analogues were studied and shown to induce variable degrees of histone H3/H4 and tubulin acetylation in a cellular model of myeloid leukemia sensitive to all-*trans* retinoic acid (ATRA). Multiparametric correlations between acetylation of the three substrates, tumor cell growth inhibition, and ATRA-dependent cytodifferentiation were performed, providing information on the chemical functionalities governing these activities. For two analogues, antitumor activity in the animal was demonstrated.

KEYWORDS HDAC inhibitors, hydroxamic acid, retinoic acid, antiproliferative effect, differentiation



Histone deacetylase (HDAC) proteins are classified in four groups (class I−IV) based on function and sequence similarity. A common observation in neoplastic cells is high level expression of class I and II HDACs with corresponding hypoacetylation of histones.[1] Increased HDAC activity may play a critical role in the pathogenesis of leukemia.[2] HDACs of significance for cancer cell biology reside not only in the nucleus but also in the cytoplasm, where they act on substrates other than histones. Within class I, HDACs 1, 2, and 8 are primarily found in the nucleus. Class II HDACs (HDAC 4, 5, 6, 7, 9, and 10) are able to shuttle in and out of the nucleus depending on different signals.[3] HDAC 6 is primarily a cytoplasmic enzyme, deacetylating proteins like tubulin, Hsp90, and cortactin.[4]

Transformed cells are generally more sensitive to HDAC inhibitor-induced growth inhibition and apoptosis than their normal counterparts.[5] Hence, anticancer therapeutic strategies based on HDAC inhibitors have raised significant interest. Some of the most powerful natural and synthetic HDAC inhibitors[6] are derivatives of hydroxamic acid.

Here, we describe the chemical synthesis and the pharmacologic characterization of a novel series of hydroxamic acid derivatives characterized by tricyclic dibenzo-diazepine, -oxazepine, and -thiazepine rings. As the catalytic domain of all HDAC isoforms is highly conserved,[7] our approach was to design novel molecules targeting the variable external part of the channel leading to the catalytic center. We studied the effects of a limited number of systematic modifications to the tricyclic core, keeping the hydroxamic group and the linker chain of our molecules constant. The tricyclic core was selected, as structural modifications affecting hydrogen bonding, electronic features, and angles between the two aromatic rings can be easily obtained by accessible chemical modifications. Test compounds (0.01, 0.1, 1.0, 10, and 50 μM) were initially screened using an enzymatic assay measuring the total HDAC activity in HeLa cell extracts, which resulted in the selection of 13 molecules. Scheme 1 illustrates the synthesis and structures of the most promising HDAC inhibitors identified. All of the selected molecules were characterized by combinations of different functionalities in the central heptameric ring (compounds **21a**−**c** = SO_2; compounds **21d**−**f** = S; compounds **21g**−**i** = O; and compounds **21j**−**m** = NH or NCH_3) and three types of bulky residues in the aromatic ring closer to the hydroxamic chain (compounds **21c**,**f**,**i**,**l**,**m** = H; compounds **21b**,**e**,**h**,**j** = Cl; and compounds **21a**,**d**,**g**,**k** = OCH_3) and were endowed with detectable HDAC inhibitory activity (Table 1, column 2). Four compounds (**21a**, **21b**, **21d**, and **21g**) were already active at 0.01 μM, causing 21−47% inhibition of HDAC activity, and were more powerful than the prototypic HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA; 54% inhibition at 0.1 μM). The remaining members of the series had a variable degree of activity (20−75% inhibition) at 0.1 μM. Using a semiquantitative index (RO, rank order) taking into account the lowest concentration of the test molecule endowed with significant HDAC inhibitory activity and the size of the effect observed, the compounds were ranked as follows: **21a**, **21b** > **21d**, **21g** ≥ **21i**, **21k**, **21c**, **21j**, **21h**, **21f**, **21e**, **21l** > **21m**.

To determine whether HDAC inhibition in vitro (RO) was paralleled by similar effects in cultured cells, the acetylation

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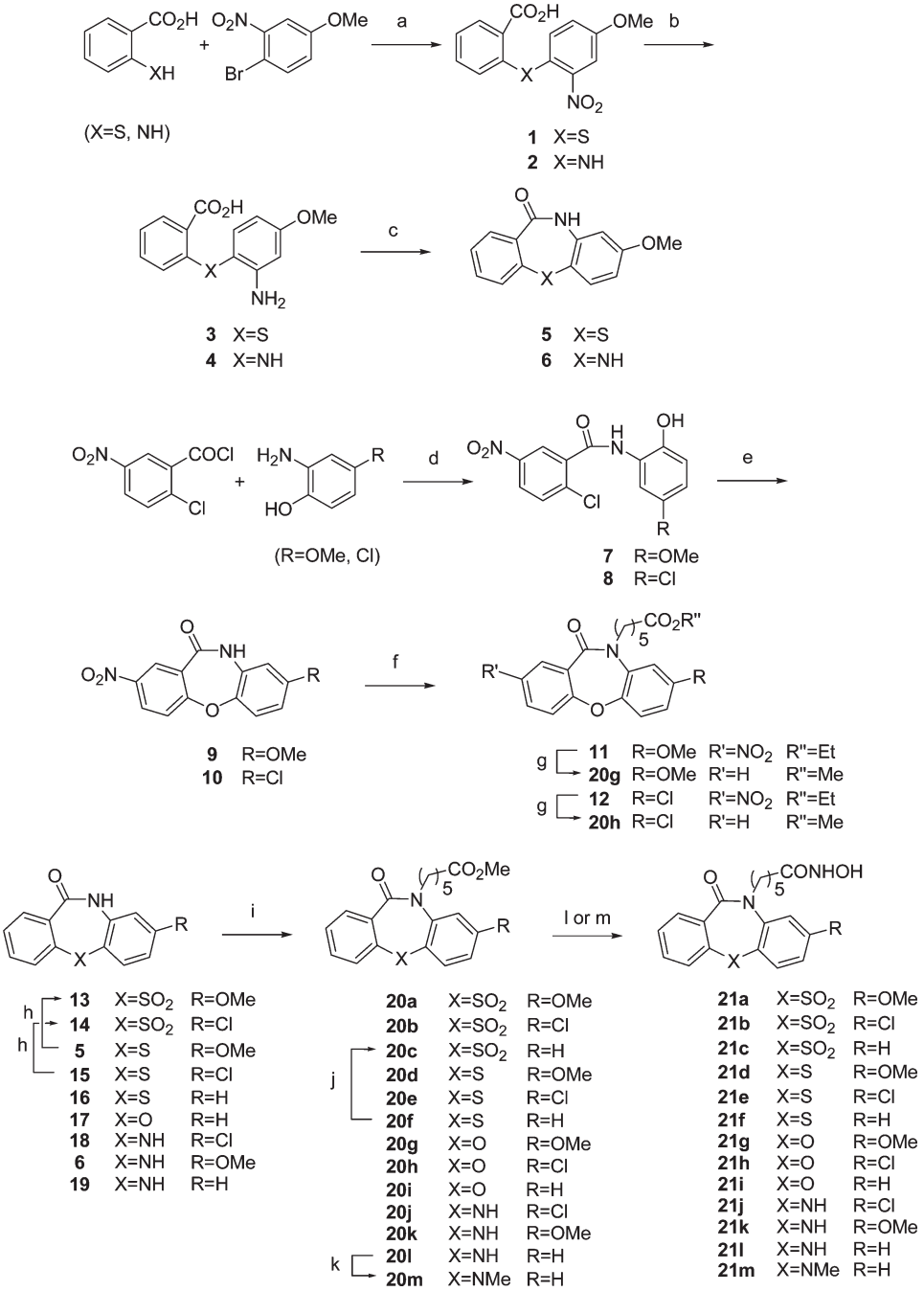
Scheme 1. Chemical Structure and Synthesis of HDAC Inhibitors[a]



[a] Reagents and conditions: (a) Cu(0), K_2CO_3, 1-pentanol, reflux, 4 h. (b) For X = NH, $Na_2S_2O_4$, EtOH, H_2O, reflux, 2 h; for X = S, $Na_2S \cdot 9H_2O$, $NaHCO_3$, EtOH, reflux, 6 h. (c) EDC·HCl, HOBt, DMF, room temperature, 3−18 h. (d) DIPEA, THF, 0 °C to room temperature, 48 h. (e) NaOH 2 M, H_2O, reflux, 5 h. (f) NaH, $Br(CH_2)_5CO_2Et$, DMF, room temperature, 6 h. (g) (i) Fe, $FeCl_3$, EtOH, reflux, 3 h; (ii) *t*BuONO, DMF, 50 °C, 1 h; (iii) 4 M HCl/dioxane, MeOH, room temperature, 16 h. (h) $RuCl_3$, $NaIO_4$, $C_2H_2Cl_4$, H_2O, room temperature, 16 h. (i) NaH, $Br(CH_2)_5CO_2Me$, DMF, room temperature. (j) Oxone, MeOH, H_2O, 50 °C, 3 days. (k) NaH, MeI, DMF, 50 °C, 14 h. (l) $NH_2OH \cdot HCl$, NaOMe, MeOH, room temperature. (m) NH_2OH(aq), MeOH, room temperature.

levels of histone H3 and H4, two nuclear substrates, as well as tubulin, a cytosolic substrate, were evaluated in the NB4 myeloid leukemia cell line challenged with increasing concentrations of our test molecules (Supporting Information, Figure 1, for a representative experiment). Two inversely correlated parameters (Supporting Information, Table 1) were used to determine the HDAC inhibitory activity in cultured cells: EC_{50}, the concentration causing 50% of the maximal effect, and maximal activity (MA), the maximal inhibitory effect observed at the EC_{50}. All molecules enhanced acetylation of H3, H4, and tubulin (Table 1, columns 3−8). RO correlated with the EC_{50} but not with the MA values calculated for acetylated H3, H4, and tubulin (Supporting Information, Figure 2A and Table 1).

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Table 1. Pharmacological Activity of HDAC Inhibitors[a]

	HDACi	AcH3		AcH4		AcTu		HCT116	NB4	
compd	RO	EC_{50}	MA	EC_{50}	MA	EC_{50}	MA	IC_{50}	IC_{50}	DP
21a	4700	0.03 (0.02−0.04)	4737	0.09 (0.05−1.50)	1462	0.05 (0.04−0.05)	30550	0.04	0.02 (0.01−0.02)	79
21b	4400	0.03 (0.02−0.04)	2111	0.06 (0.04−0.09)	795	0.04 (0.01−0.13)	8847	0.03	0.02 (0.01−0.02)	56
21g	2500	0.20 (0.15−0.27)	61	0.32 (0.19−0.53)	206	0.09 (0.03−0.27)	1780	0.31	0.14 (0.13−0.15)	7
21d	2100	0.19 (0.09−0.40)	12	0.15 (0.01−5.81)	28	0.13 (0.05−0.34)	47	0.09	0.03 (0.01−0.05)	16
21f	750	0.46 (0.18−1.39)	190	0.43 (0.10−1.00)	89	0.37 (0.13−1.01)	1998	0.39	0.19 (0.16−0.25)	5
21j	740	0.84 (0.53−1.31)	31	0.86 (0.54−1.38)	78	0.32 (0.20−0.49)	413	0.45	0.29 (0.25−0.35)	1
21c	730	0.50 (0.20−1.22)	342	0.72 (0.47−1.08)	336	0.05 (0.02−0.15)	10681	0.15	0.12 (0.10−0.13)	14
21k	700	0.39 (0.08−2.00)	7	0.28 (0.15−0.52)	244	0.25 (0.19−0.35)	199	0.36	0.14 (0.10−0.19)	1
21e	680	0.34 (0.19−0.61)	200	0.36 (0.18−0.73)	2326	0.15 (0.13−0.18)	13206	0.06	0.03 (0.01−0.05)	31
21i	660	6.24 (2.9−13.4)	15	3.27 (1.43−7.48)	54	0.29 (0.10−0.60)	2626	0.64	0.61 (0.59−0.63)	2
21h	650	0.38 (0.23−0.63)	3	0.53 (0.31−0.89)	9	0.44 (0.26−0.75)	932	0.20	0.16 (0.15−0.17)	1
21l	530	1.30 (0.67−2.52)	188	1.53 (1.11−2.12)	126	1.17 (0.96−1.45)	402	> 10	0.73 (0.65−0.83)	6
21m	240	2.65 (1.63−4.30)	10	2.95 (2.24−3.87)	14	2.74 (2.09−3.60)	40	8.10	2.46 (1.87−3.23)	13

[a] Column 2: HDACi = HDAC inhibitory activity, as determined by the in vitro assay used for the screening procedure. RO = (% inhibition of HDAC enzymatic activity) × (μM concentration of the test compound). The higher the RO value, the more active is the HDAC inhibitor. Columns 3−8: Acetylation of H3/H4 and tubulin, following quantitative Western blot analysis of NB4 cells (24 h) challenged with the test compound. AcH3 = acetylated histone H3; AcH4 = acetylated histone H4; and AcTu = acetylated tubulin. The results are expressed as EC_{50} or as MA (maximal induction of acetylation) values. MA = induction ratio (AcH3 treated/AcH3 control; AcH4 treated/AcH4 control; and AcTu treated/AcTu control) in NB4 cells treated with the EC_{50} of the test compound and vehicle (DMSO). Columns 9 and 10: Inhibition of NB4 or HCT116 cell growth (4 days). Results are expressed as IC_{50} values (mean, $N = 3$). The interval of confidence is shown in parentheses. Column 11: Effect of HDAC inhibitors on ATRA-dependent differentiation of NB4 cells (72 h; ATRA, 0.01 μM) prior to determination of the NBT-reductase activity. DP = Max F.I./[C], where Max F.I. (maximal fold induction at the most active concentration of HDAC inhibitor) is the ratio between the NBT-reductase activity values determined after treatment with ATRA + HDAC inhibitor and the HDAC inhibitor alone, while [C] is the concentration of the HDAC inhibitor at which Max F.I. was calculated. When we observed no induction of the NBT-reductase activity at all of the concentrations of HDAC inhibitor considered, a DP value of 1 was assigned (compounds **21h**, **21k**, and **21j**).

For each compound, a direct correlation between the EC_{50} values for the acetylation of H3 and H4 was evident (Supporting Information, Figure 2B and Table 1). A weaker, albeit significant, correlation between H3 or H4 and tubulin acetylation was also observed (Supporting Information, Figure 2C and Table 1). Relative to the other members of the series, compounds **21c** and **21i** induced acetylation of tubulin more efficiently than acetylation of H3/H4. The observation suggested that compounds **21c** and **21i** have a preference for cytosolic HDACs or limited access to the nuclear compartment. Thus, we compared the in vitro inhibition profile of **21c**, **21i**, **21a**, **21b**, **21g**, **21k**, and trycostatin A (TSA) on some of the known class I and class II HDACs (Supporting Information, Table 2), focusing on strictly nuclear and cytosolic HDACs, that is, HDAC2 and HDAC6. The IC_{50} values of **21c** and **21i** for HDAC2 were substantially higher than the corresponding values for HDAC6, with HDAC2/HDAC6 ratios of 48.0 and 61.0, respectively. The HDAC2/HDAC6 ratios calculated for **21a**, **21b**, **21g**, **21k**, and TSA, molecules devoid of selectivity for tubulin acetylation, were much lower. These data suggested that HDAC-isoform selectivity contributes to the differential effect exerted by **21c** and **21i** on tubulin and histone acetylation. At present, the structural determinants underlying the selectivity of **21c** and **21i** for HDAC 6 are unknown, although it is interesting to notice that both compounds are devoid of a bulky residue in the aromatic ring closer to the hydroxamic chain. This is similar to what is observed in the active **21f** and the two inactive **21l** and **21m** congeners that differ from **21c** and **21i** for the bridging group present in the central ring.

HDAC inhibitors are endowed with antiproliferative, apoptotic, and differentiating properties, each concurring to the overall antineoplastic activity of these compounds. The antiproliferative activity of our compounds was determined in NB4 and HCT116 colon carcinoma cells (Table 1, columns 9 and 10). The results obtained indicated a strict correlation between the IC_{50} values calculated in the two cell lines (Supporting Information, Table 1), suggesting a spectrum of antiproliferative activity largely independent of the cellular context considered. In terms of growth inhibition, our HDAC inhibitors could be grouped in three different classes according to the IC_{50} value calculated: high (**21b**, **21a**, **21h**, and **21g**), intermediate (**21d**, **21i**, **21k**, **21c**, **21j**, **21e**, and **21f**), and low (**21l** and **21m**) activity. The IC_{50} values of the first class of molecules fell in the low nanomolar range and were lower than the corresponding value calculated for the prototypical HDAC inhibitor, SAHA (0.2 μM).

The antiproliferative activity of all our compounds in both HCT116 and NB4 cells was correlated to the corresponding HDAC inhibitory effects in vitro (Supporting Information, Figure 2D and Table 1). In NB4 cells, a linear correlation between the IC_{50} values calculated for the growth inhibition and hyperacetylation of histones H3/H4 (Supporting Information, Figure 2E and Table 1) or tubulin (Supporting Information, Figure 2F and Table 1) was also observed. The R value for the linear correlation between growth inhibition and tubulin acetylation was lower and less significant than the corresponding values calculated for H3/H4 acetylation. The abnormal behavior of compounds **21c** and **21i**, which are characterized by the already noticed dissociation between tubulin and H3/H4 acetylation (Supporting Information, Figure 2C), concurred to the phenomenon. Thus, increased acetylation of nuclear substrates may be more important than increased acetylation of the cytosolic counterparts for the growth inhibitory effect of our HDAC inhibitors.

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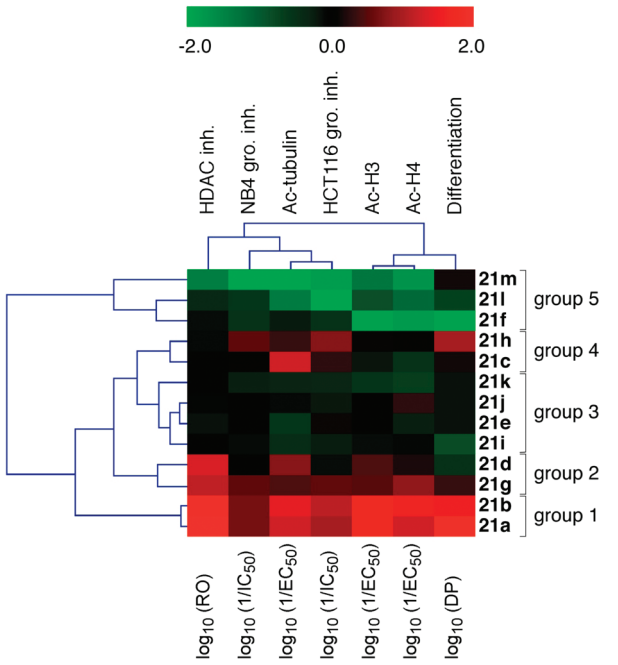

Figure 1. Cluster analysis. Correlations between the structures of HDAC inhibitors (rows) and the biological activities considered (columns): Scale units for each parameter are shown below the heat map.

An important function of HDAC inhibitors is the ability to enhance the activity of an established cytodifferentiating agents, like all-*trans* retinoic acid (ATRA).[8−10] Differentiation is an emerging modality for the management of certain types of leukemia,[11,12] and ATRA is used clinically in the treatment of acute promyelocytic leukemia (APL). Hence, we assessed the ability of our HDAC inhibitors, alone and in combination with a subeffective concentration of ATRA, to induce myeloid differentiation of APL-derived NB4 cells[13−15] (Supporting Information, Figure 3), measuring an established marker like NBT-reductase activity.[13−15] No HDAC inhibitor increased NBT-reductase activity on its own, regardless of the concentration considered. However, for many of our HDAC inhibitors, one or more concentrations induced the differentiation marker in the presence of ATRA. Using a simple index (DP = differentiation potency) taking into account the induction of the NBT-reductase activity observed in the presence of ATRA and the minimal concentration of HDAC inhibitor causing this induction, our compounds were ranked as follows: **21a** > **21b** > **21e** > **21d**, **21c**, **21m** > **21g**, **21l**, **21f** ≥ **21i**, **21h**, **21j**, **21k**. Acetylation of H3 and inhibition of NB4 cell growth showed the most significant correlations with the ability of our HDAC inhibitors to enhance the differentiating activity of ATRA (Supporting Information, Table 1), suggesting that these effects are linked.[3]

To group the members of our series in discrete classes according to the chemical structure and the effects exerted on the biochemical and cellular parameters studied, we performed an unsupervised cluster analysis (Figure 1). On the basis of this multiparametric approach, our compounds were clustered in five discrete groups. The first cluster (group 1) consisted of compounds **21a** and **21b**, the most powerful members of the chemical series in terms of all of the parameters considered. Although the limited number of derivatives analyzed does not allow definitive conclusions on structure−activity relationships, our analysis suggests the following considerations. The SO_2 group in the heptameric central ring seems to provide an optimal structure for HDAC inhibition not only in vitro but also in intact cells. Efficient HDAC inhibition is accompanied by a strong and cell context-independent growth inhibitory action, as well as a significant differentiating effect in combination with ATRA. The presence of the $-SO_2$ functionality is necessary but not sufficient for effective HDAC inhibition, as demonstrated by compound **21c**, which clustered with analogue **21h** in a group (group 4) characterized by poor overall activity. Compound **21c** lacks the −Cl or $-OCH_3$ group present in congeners **21b** and **21a**, suggesting the importance of a bulky group in the adjacent aromatic ring for powerful HDAC activity. As already noticed, the presence of a bulky group in the aromatic ring diminished H3/H4 acetylation although it had no impact on tubulin acetylation. Indeed, compound **21c** was equally effective as **21b** and **21a** in causing acetylation of this last substrate. The observation suggested that substitution of a hydrogen with a −Cl or an $-OCH_3$ group decreases the selectivity of our compounds for certain subtypes of HDACs, that is, HDAC 6, potentially involved in the acetylation of cytosolic substrates like tubulin (see Supporting Information, Table 2).

In terms of HDAC inhibitory activity in vitro, compounds **21d** and **21g** (group 2) were slightly less effective than **21b** and **21a**. This indicated that substituting $-SO_2$ with an −S or −O group in the context of a structure characterized by an optimal bulky substituent on the aromatic ring does not have a significant effect on the parameter. However, these structural modifications' impact on the HDAC inhibitory activity is measurable in whole cells. In fact, group 2 compounds have diminished capacity to induce H3/H4 and tubulin acetylation than group 2 molecules.

Group 3 (compounds **21i**, **21e**, **21j**, and **21k**), group 4 (compounds **21c** and **21h**), and group 5 (compounds **21f**, **21l**, and **21m**) consist of molecules with a progressively decreasing HDAC inhibitory activity in vitro and in whole cells. This is generally accompanied by inefficient growth inhibition and enhancement of ATRA-induced differentiation, indicating that substitution of $-SO_2$ with a secondary or ternary amino functionality is deleterious for the overall pharmacological activity of our compounds. In addition, the presence of compounds **21c**, **21f**, **21i**, and **21l** in this subset supports the idea that the absence of a bulky −Cl or $-OCH_3$ group on the aromatic ring has a major negative effect, which is not complemented by the structure of the central heptameric ring.

To provide insights into the in vivo activity of some of our molecules, we selected two compounds of group 1 (**21a** and **21b**) and one molecule each for group 2 (**21d**) and group 3 (**21e**) and tested their activity on the growth of human HCT116 xeno-transplants . The in vivo antitumor activities of these molecules and SAHA were compared, using nude mice transplanted subcutaneously with HCT116 cells. Eight days following transplant, animals were treated with vehicle, SAHA, or two doses of **21a**, **21b**, **21d**, and **21e** intravenously. Dose-dependent inhibition of tumor growth (Figure 2A,B) was observed after administration of **21d** (12.5 and 25 mg/kg). At 6.2 mg/kg, administration of **21e** was associated with limited

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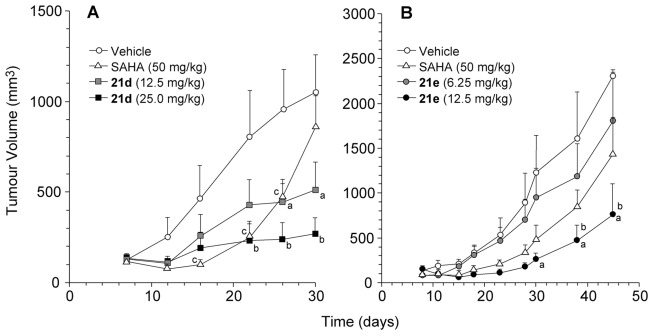

Figure 2. In vivo antitumor activity of compounds **21d** and **21e**. Nude mice were transplanted with HCT116 cells and treated with vehicle or compounds, **21d** (A), **21e** (B), and SAHA as a control. Each experimental point indicates the average volume of four tumors ± SD. *a* = significantly different: **21d** (12.5 mg/kg) vs vehicle; and **21e** (12.5 mg/kg) vs vehicle ($p < 0.01$). *b* = significantly different: **21d** (25 mg/kg) vs vehicle; and **21e** (12.5 mg/kg) vs SAHA ($p < 0.05$) 50 mg/kg. *c* = significantly different: SAHA vs vehicle ($p < 0.05$).

growth inhibition of the tumor, while at 12.5 mg/kg, the compound showed significant antitumor activity. For both molecules, the highest dose considered was more effective than an optimal dose of SAHA (50 mg/kg). In these conditions, the two compounds belonging to group 1, which were the most active molecules in all of the in vitro tests considered, showed no (**21a**) or mild (**21b**) antitumor activity (Supporting Information, Figure 4A,B). This is not surprising, as it may be the consequence of metabolic/distribution issues or other undefined causes. Nevertheless, our data provide proof of principle that members of our series of HDAC inhibitors selected on the basis of optimal on-target in vitro activity are endowed with significant in vivo antitumor potential.

SUPPORTING INFORMATION AVAILABLE

Experimental procedures, chemical synthesis details, analytical data for all compounds, Table 1, and Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions: A.B., M.G., C.V., and M.T. carried out all of the biological experiments with NB4 cells; M.F. performed all of the biocomputing analyses; M.B., M.B., and M.P. designed and supervised the in vivo experiments and the experiments involving HDAC isoform inhibition; A.G. and C.A.M. modeled and supervised the structural rationale of the compounds; M.G. synthesized and characterized the compounds described in the paper; and E.G. designed and supervised the entire work.

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ABBREVIATIONS

HDAC, histone deacetylase; ATRA, all-*trans* retinoic acid; RO, rank order; SAHA, suberoylanilide hydroxamic acid; TSA, trycostatin A; DP, differentiation potency; IC_{50}, inhibitory concentration 50, the concentration causing 50% of the maximal inhibitory effect.

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