European Journal of Medicinal Chemistry 44 (2009) 1900–1912

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design, synthesis, and evaluation of biphenyl-4-yl-acrylohydroxamic acid derivatives as histone deacetylase (HDAC) inhibitors

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ARTICLE INFO

Article history:
Received 5 August 2008
Received in revised form
30 September 2008
Accepted 6 November 2008
Available online 19 November 2008

Keywords:
Histone deacetylase
Antiproliferative activity
Synthesis

ABSTRACT

A series of hydroxamic acid-based histone deacetylase (HDAC) inhibitors were designed on the basis of a model of the HDAC2 binding site and synthesized. They are characterized by a cinnamic spacer, capped with a substituted phenyl group. Modifications of the spacer are also reported. In an *in vitro* assay with the isoenzyme HDAC2, a good correlation of the activity with the docking energy was found. In human ovarian carcinoma IGROV-1 cells, selected compounds produced significant acetylation of p53 and α-tubulin. Most compounds showed an antiproliferative activity comparable to that of SAHA. At equitoxic concentrations, the tested compounds were more effective than SAHA in inducing apoptotic cell death. Compounds selected for *in vivo* evaluation exhibited a significant antitumor activity on three tumor models at well tolerated doses, thus suggesting a good therapeutic index.

1. Introduction

Inappropriate epigenetic modifications of gene expression are associated with malignant phenotype and tumor progression. Regulation of gene expression is mediated by several mechanisms such as DNA methylation, ATP-dependent chromatin remodeling, and post-translational modifications of histones. The latter mechanism includes dynamic acetylation and deacetylation of ε-amino groups of lysine residues present in the tail of the core histones. Enzymes responsible for the reversible acetylation/deacetylation processes are histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively [1,2]. Inhibitors of histone deacetylase (HDAC) enzymes have recently gained prominence as an emerging class of anticancer agents [1].

When HDACs are inhibited, histone hyperacetylation occurs. The disruption of the chromatin structure by histone hyperacetylation leads to the transcriptional activation of a number of genes [3,4]. In addition to modulation of histone acetylation, HDAC inhibitors are also involved in acetylation status of non-histone proteins implicated in regulatory processes (e.g., transcription factors and tubulins) [4,5]. The response to HDAC inhibitors indicates a pleiotropic effect on critical pathways involved in cell proliferation, apoptosis, microtubule function and DNA repair [4,5]. Indeed, HDAC inhibitors have been reported to inhibit cell growth, induce terminal differentiation in tumor cells and prevent the formation of malignant tumors in mice [6].

In addition to the antitumor effects seen with HDAC inhibitors alone, these compounds may also potentiate cytotoxic agents or synergize with other targeted anticancer agents [7]. The exact mechanism by which HDAC inhibitors cause cell death is still unclear and the specific roles of individual HDAC isoenzymes as therapeutic targets have not been established.

However, *in vivo* efficacy in tumor xenograft models has further substantiated HDACs as a valid target for developing novel anticancer agents [1a,8]. Natural and synthetic HDAC inhibitors have been studied extensively. While suberoylanilide hydroxamic acid (SAHA; Zolinza™) [9] (Chart 1) has been approved by the FDA for once-daily oral treatment of advanced cutaneous T-cell lymphoma (CTCL) [10] many other small molecules that inhibit HDAC proteins are currently in clinical trials for cancer treatment, e.g. MS-275 and NVP-LAQ824 [1c,d] (Chart 1).

Several biological studies have indicated that HDACs are heterogeneous, consisting of 18 isozymes, which can be categorized into four classes, namely class I (HDAC 1, 2, 3 and 8), class II (HDAC 4, 5, 6, 7, 9 and 10) and classes III and IV, based on their sequence homology. Class I, II and IV HDACs are zinc-containing amide

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doi:10.1016/j.ejmech.2008.11.005

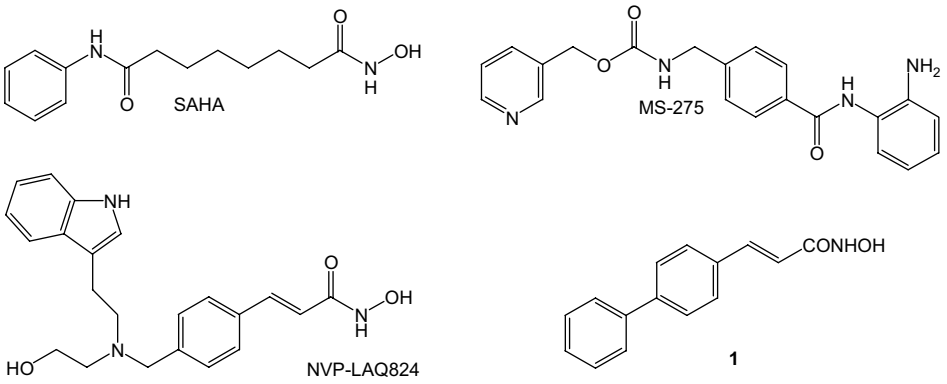


Chart 1. Structures of representative HDAC inhibitors.

hydrolases, with a conserved catalytic core but differing in size, domain structure and tissue expression pattern. Class III consists of NAD-dependent amide hydrolases, unrelated in sequences and mechanism to classes I and II [11]. Zinc-dependent HDACs have received much attention as anticancer drug targets.

A bacterial HDAC homologue (histone deacetylase-like protein, HDLP) [12], a human HDAC8 [13] and a bacterial class II histone deacetylase homologue [14] have been co-crystallized with the hydroxamic acid-based inhibitor SAHA (Chart 1). The alkyl chain of SAHA inserts into a tubular pocket, and the hydroxamic group serves as a bidentate chelator of the catalytic Zn^{++} ion bound in the enzyme active site. Based on these crystallographic analyses, several human class I HDAC inhibitors have been designed. In all of these designs the common structural motif consists of a metal-binding head group, which interacts with the Zn^{++} ion at the bottom of the active site, a linker domain, which occupies the narrow tube-like channel, and a *cap* group, which interacts with the residues on the rim of the active site. The linker serves to appropriately position the capping and metal-binding groups for high-affinity interactions with the target proteins.

In the present study, we have designed novel structurally simple HDAC inhibitors with a hydroxamic acid as the zinc chelating head group attached to a cinnamic linker domain and an aromatic group as a *cap* structure. The prototype of these new HDAC inhibitors (**1**) is shown in Chart 1. Here we report the synthesis, the HDAC2 isoenzyme and cancer cell growth inhibition of this novel class of compounds. HDAC2 was chosen as a representative isoenzyme of class I HDACs, which are known to have an almost exclusive nuclear localization and to be essential to cell proliferation [15].

2. Molecular modelling and design

Compound **1** was designed on the basis of the hypothesis that the proximal phenyl ring, extended from the hydroxamic acid via a double bond, could be suitable to occupy the narrow tube-like pocket of the HDAC class I active site. A hydrophobic phenyl ring was selected because crystallographic analysis of HDAC class I proteins indicates that the active site residues surrounding the linker chain are hydrophobic. During the course of this work, other examples of cinnamyl linkers were reported [1d], but none directly connected to an aromatic ring.

To understand the binding mode in the catalytic site and derive structure–activity relationships (SAR) for compound **1** and analogues, we developed a model of the active site of HDAC2 based on the homology model derived and validated by Wang et al. [16] using HDLP as a template. We chose HDAC2 because this isoenzyme was used for *in vitro* assays.

Compound **1** was inserted in the obtained HDAC2 site (the hydroxamic acid moiety was overlapped to that of SAHA previously inserted in the HDAC2 model, then SAHA was removed), and the new complex energy minimized. The binding affinity was evaluated by calculating the intermolecular interaction energy between the appropriate compound and the HDAC2 model (Docking module in Insight II). The intermolecular interaction energy of compound **1** docked in HDAC2 appeared more favourable than that of SAHA. In the crystal structure of HDAC8 complexed with SAHA, the amino acids lining the binding pocket are hydrophobic residues, G151, F152, F208, M274 and Y306 and they present multiple favourable contacts with the aliphatic chain of SAHA. Four of them (G151, F152, F208 and Y306) are conserved across the class I HDACs, while M274 becomes a leucine in the other members of the family. These amino acids form the tunnel that the acetyllysine substrate penetrates to access the catalytic machinery. Our modelling indicated that the proximal phenyl ring of compound **1** exhibits a π–π stacking interaction with the side chain benzyl group of Phe151, Phe206 and Tyr304 in our HDAC2 model (corresponding to F152, F208 and Y306 in HDAC8). The distal phenyl ring of 1 (*cap* structure) appears to accommodate in a large cavity, without a significant steric clash. However, its environment contains some important amino acid residues which may be involved in electrostatic interactions, such as Glu99 and Glu147 (corresponding to Tyr100 and Glu148 in HDAC8).

Therefore the distal phenyl ring of 1 could be used as a scaffold to place a variety of substituent groups at appropriate distances for such interactions (e.g. an OH group, Fig. 1).

3. Chemistry

The prototype 4-phenylcinnamohydroxamic acid **1** was produced by condensing commercially available 4-phenylcinnamic acid with hydroxylamine in the presence of HATU and DIPEA.

The substituted aryl or heteroaryl derivatives **3b**,**e**–**g**,**i**–**l** were obtained by a Suzuki coupling reaction between methyl 4-bromocinnamate and the appropriate aromatic or heteroaromatic boronic acids. Conversion of the esters to the corresponding hydroxamic acids was achieved either by condensing the acids obtained after hydrolysis with hydroxylamine in the presence of HATU or HBTU or by coupling the ester with *O*-THP-protected hydroxylamine, followed by acidic deprotection. Compounds **3c**,**d**,**h** were obtained by a Suzuki coupling reaction between *O*-THP-protected 4-bromocinnamohydroxamic acid **5** and the appropriate aromatic or heteroaromatic boronic acids, followed by acid hydrolysis (Scheme 1).

Compound **3a** was obtained by Heck condensation of 4-(4′-bromophenyl)phenol with methyl acrylate, followed by basic hydrolysis. The conversion to hydroxamic acid was accomplished using WSC and HOBT as coupling reagents. A Suzuki one-pot reaction between 2-chloro-4-bromophenol and methyl 4-bromocinnamate,

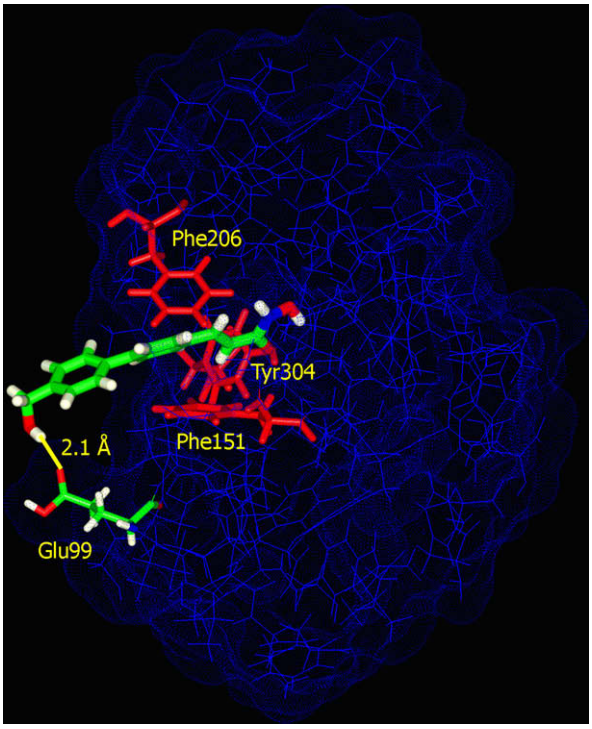


Fig. 1. Model of compound **3b** in the putative HDAC2 binding site.

followed by hydrolysis with LiOH and condensation with hydroxylamine using HATU and DIPEA, gave compound **3m**. Compound **4** was obtained from the corresponding cinnamyl ester by catalytic hydrogenation, followed by basic hydrolysis and coupling with hydroxylamine using HBTU and DIPEA.

Synthesis of compound **10a** (Scheme 2) was accomplished by Wadsworth–Horner–Emmons condensation of 4-phenylbenzaldehyde with ethyl 4-(diethoxyphosphoryl)-but-2-enoate, followed by hydrolysis and coupling with HBTU and DIPEA. Compounds **10b** and **10c** were synthesized by hydrolysis of the common intermediate 5-(4-bromophenyl)-penta-2,4-dienoic acid (tetrahydropyran-2-yloxy)-amide **9**, on its turn prepared from 4-bromobenzaldehyde, following the sequence described in Scheme 2. The saturated derivative **11** was obtained by catalytic hydrogenation of the dienoic ester and usual hydrolysis and coupling.

Insertion of an oxygen atom between the double bond and the biphenyl system led to compound **7** whose synthesis started from addition of 4-phenylphenol to ethyl propiolate, followed by the aforementioned conventional steps. The synthesis of the analog (**13**) of **4a** with the phenyl substituent shifted to the meta-position is shown in Scheme 3. It was obtained from the Suzuki coupling of methyl 3-bromocinnamate with 4-hydroxyphenylboronic acid, followed by the usual steps.

A synthetic route towards the derivative with a saturated proximal ring **17** is outlined in Scheme 4. Acetylation of 4-(4-hydroxyphenyl)cyclohexanone followed by Wittig reaction with (methoxymethyl)triphenylphosphonium chloride gave, after hydrolysis, aldehyde **14**. Reaction with methyl(triphenylphosphoranylidene)acetate, hydrolysis and coupling with HBTU and DIPEA furnished **17**. Catalytic hydrogenation of the acid **16** gave, after coupling, compound **18**.

4. Results and discussion

The synthesized compounds were tested for their inhibitory activity towards HDAC2 isoform (Table 1), and for antiproliferative activities against a panel of tumor cell lines of different tissue origin: NB4 (human acute Promyelocytic leukemia), H460 (human lung carcinoma cell line), HCT116 (human colon cancer cell line), IGROV-1 and its subline resistant to cisplatinum IGROV-1/Pt1 (human ovarian carcinoma cell lines) (Table 2). The growth-inhibitory activities were apparently p53-independent, because the cellular effects were comparable in cells with functional (IGROV-1) or defective p53 (IGROV-1/Pt).

Substitution at the distal phenyl ring of compound **1** was first assessed (compounds **3a**–**m**). Most of these compounds (except **3c**,**e**,**i**) showed an inhibitory activity in the HDAC2 assay in the same order of magnitude, or slightly lower than SAHA (Table 1). Compounds **3a**,**b**,**m**, among the most potent in this group, were predicted by the docking to have a strong interaction energy, similarly to SAHA.

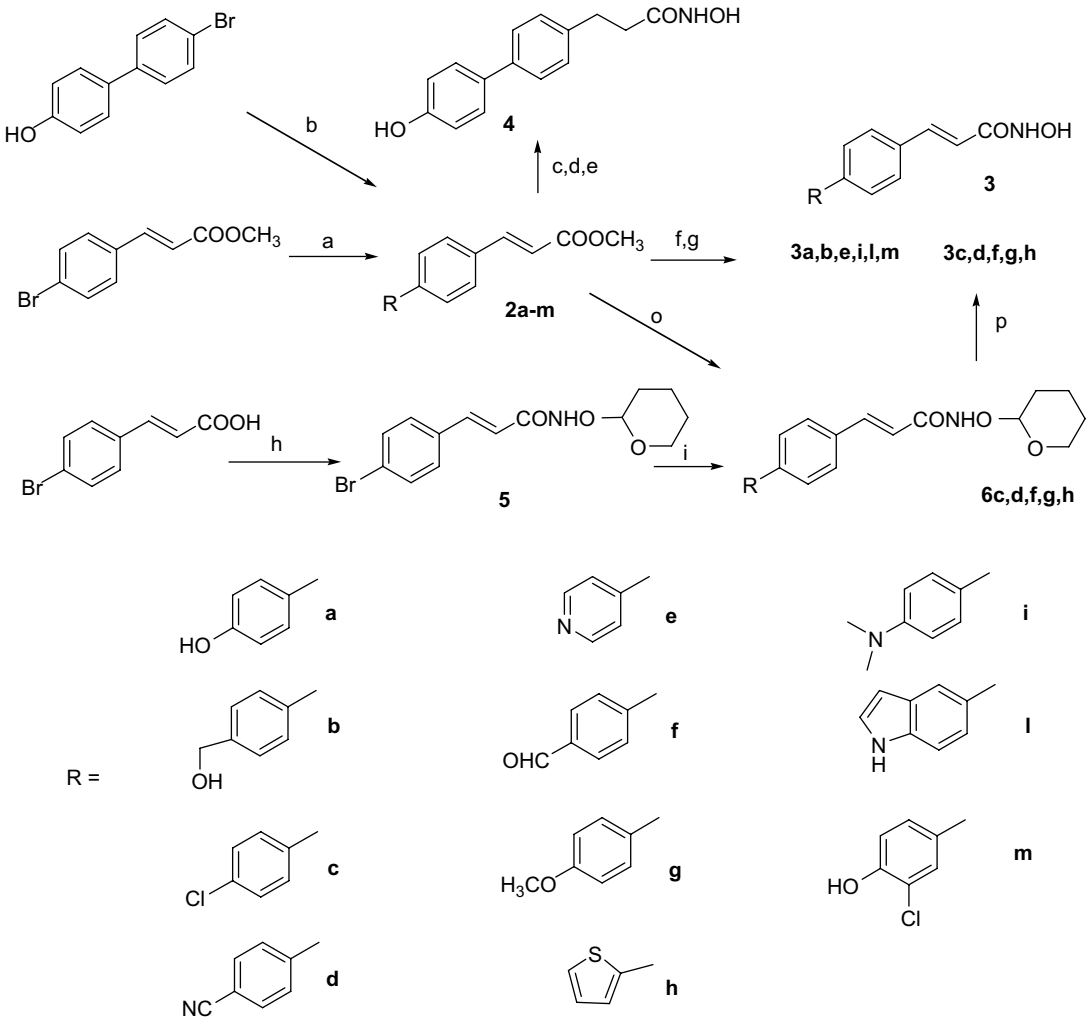
We then investigated the linker domain focusing on altering saturation and chain length. First, we studied the effect of inserting an ethyl bridge instead of the cinnamyl double bond, thus allowing further flexibility of the side chain (compound **4**). Also in this case docking studies and HDAC2 assays are in agreement, indicating that the unsaturation in the side chain makes the ligand more active (compare compounds **3a** and **4**). We then investigated the effect of the elongation of the linker by two carbon units (**10a**), finding that it is detrimental for the activity (as evidenced both by docking studies and HDAC2 assays). The shift of the position of the side chain to the adjacent position on the proximal ring (compound **13**) seemed not to affect the interaction with the isoenzyme. In general, within the compounds with a cinnamic spacer, a pattern emerged of reasonable correlation of the docking energy with the inhibitory activity against HDAC2.

The results of the antiproliferative activity tests are reported in Table 2. Generally, it was found that a wide array of functionalities can be tolerated, particularly as concerns substituents of the distal phenyl ring, most compounds showing an antiproliferative activity comparable with that of SAHA.

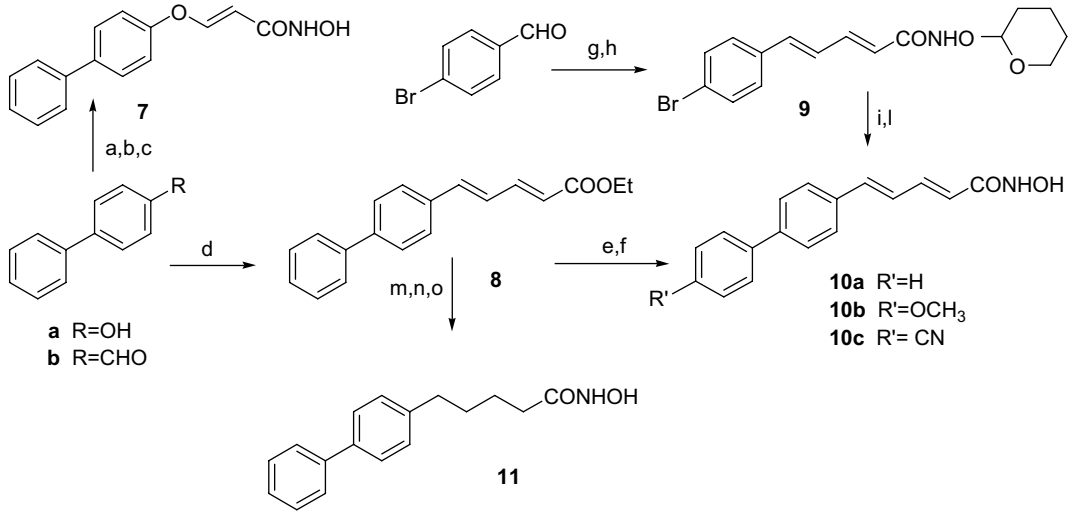
Because we hypothesized that the placement of an appropriate spacer between the *cap* group and the zinc binder would modulate the HDAC inhibitory activity, we varied the chain length of the spacer. Compounds **10a**–**c**, which contain a dienic system [17], showed a cytotoxicity of the same order as that of **1**, depending on the cell line. This result indicates that a longer chain is not only tolerated but in some cases, e.g. **10a**, increases the cytotoxicity. Also insertion of an oxygen atom between the double bond and the biphenyl system (7) was tolerated, as well as changing the proximal planar aromatic ring into a cyclohexyl ring (**17**). On the contrary, reduction of the double bond in the chain, which in principle should confer additional flexibility to the spacer, was not, in fact, beneficial (compounds **3a** vs. **4**, **10a** vs. **11**, **17** vs. **18**). These results seem to emphasize the importance of the double bond adjacent to the hydroxamic group. It is of interest to note that the position of the chain was important for the antiproliferative activity. In fact compound **13** showed reduced potency when compared to **3a**.

These data show that no precise correlations could be found between the inhibitory potency against HDAC2 and the antiproliferative potency in the tested cell lines. This observation is not surprising, because a) the antiproliferative potency could reflect several factors, in particular cellular pharmacokinetics, which could critically influence cellular response; b) a number of indirect evidences support the similarity of the profile of HDAC inhibition by compounds of this series with SAHA known to be a pan-HDAC inhibitor [18]; c) the cellular effects are expected to be dependent on the pattern of inhibition among the various HDAC isoenzymes and on the profile of HDAC expression in the various cell systems.

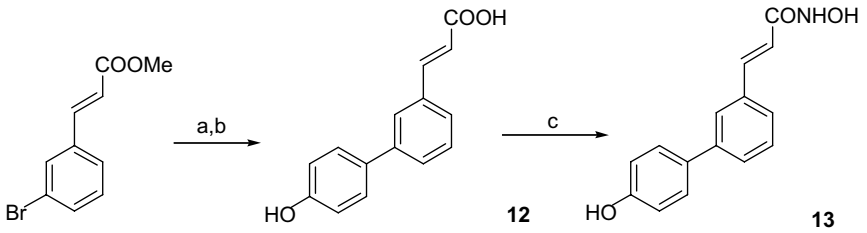
The most potent compounds, as antiproliferative agents, were also studied for their ability to induce apoptosis in the ovarian carcinoma cell line, IGROV-1, carrying a functional wild-type p53



Scheme 1. Reagents and conditions: a) for **2b**–**h**: RPhB(OH)$_2$, Pdtetrakis(PPh)$_3$, 2 N Na_2CO_3, toluene, 2–8 h, reflux; for **2i**,**l**,**m**: RPhBr, bis(pinacolato)diboron, KOAc, $PdCl_2$(dppf), 2 N Na_2CO_3, dioxane, reflux, 17–48 h; b) methyl acrylate, Pd(OAc)$_2$, TEA, tri-*o*-tolylphosphine, 100 °C, 14 h, 85%; c) H_2, PtO_2, AcOEt, rt, 1 h, 77%; d) LiOH, THF:H_2O 1:1, rt, 24 h, dark, 100%; e) HBTU, DIPEA, DMF, $NH_2OH \cdot HCl$, rt, 4 h, 92%; f) LiOH, THF:H_2O 1:1, rt, 24 h, dark; g) for **3a**: HOBt, WSC, DMF, rt, 3 h, then $NH_2OH \cdot HCl$, TEA, DMF, rt, overnight, 13%; for **4e**,**i**,**l**: HBTU, DIPEA, $NH_2OH \cdot HCl$, DMF, rt, 5–12 h, 36–68%; for **3b**,**f**,**m**: HATU, DIPEA, $NH_2OH \cdot HCl$, DMF, rt, 3–8 h, 25–65%; h) HOBT, WSC, DMF, rt, 3 h, the $THPONH_2$, 50 °C, 1 h, 84%; i) for **6d**: RPhBr, bis(pinacolato)diboron, KOAc, $PdCl_2$(dppf), dioxane, 100 °C, 1 h, then **8**, $PdCl_2$(dppf), 2 N Na_2CO_3, 100 °C, 4 h, 82%; for **6f**,**g**,**h**: RPhB(OH)$_2$, Pdtetrakis(PPh)$_3$, 2 N Na_2CO_3, toluene, reflux, 3 h, 88–100%; o) for **6c**,**d**,**g**,**h**: $THPONH_2$, Li hexamethyldisilazane, THF, −78 °C, 10 min–1 h, 53–100%; p) for **3c**,**d**,**h**: MeOH, pTSA, rt, 12–48 h, 46–62%.



Scheme 2. Reagents and conditions: a) ethyl propiolate, 4-methylmorpholine, MeCN, rt, 20 min, 91%; b) LiOH, THF:H_2O 1:1, rt, 24 h, dark, 53%; c) HBTU, DIPEA, DMF, $NH_2OH \cdot HCl$, rt, 48 h, 19%; d) triethyl 4-phosphonocrotonate, NaH, THF dry, 0 °C, 30 min, then rt 4 h, 61%; e) LiOH, THF:H_2O 1:1, rt, 24 h, dark, 95%; f) for **10a**: HBTU, DIPEA, DMF, $NH_2OH \cdot HCl$, rt, 12 h, 31%; g) triethyl 4-phosphonocrotonate, NaH, THF dry, 0 °C, 30 min, then rt, 2 h, 58%; h) $THPONH_2$, Li hexamethyldisilazane, THF, −78 °C, 40 min, 96%; i) RPhB(OH)$_2$, Pdtetrakis(PPh)$_3$, 2 N Na_2CO_3, toluene, 2 h, reflux, 34–42%; l) MeOH, pTSA, rt, 12 h, 56–58%; m) H_2/Pd/C, MeOH/AcOH 1:1, rt, 12 h, 100%; n) LiOH, THF:H_2O 1:1, rt, 24 h, dark, 95%; o) HBTU, DIPEA, DMF, $NH_2OH \cdot HCl$, rt, 12 h, 62%.



Scheme 3. Reagents and conditions: a) 4-HO–PhB(OH)$_2$, Pdtetrakis(PPh)$_3$, 2 N Na_2CO_3, toluene, 6, reflux, 25%; b) LiOH, THF:H_2O 1:1, rt, 24 h, dark, 64%; c) HBTU, DIPEA, DMF, $NH_2OH \cdot HCl$, rt, 4 h, 23%.

and characterized by an efficient susceptibility to apoptosis under stress conditions [19,20]. Our analysis also included compounds exhibiting lower growth-inhibitory activity ($IC_{50} \leq 8$ μM) in an attempt to establish possible correlation between apoptosis and antiproliferative effect. Most of the tested compounds induced a substantially higher level of apoptotic cell death than SAHA at concentrations causing equivalent antiproliferative effects (IC_{80} after 72 h exposure) (Table 3). Indeed, the percentage of drug-induced apoptotic cells ranged between 40% and 90%. The most potent inducers of apoptosis were **3c**, **3i**, **10a** which was markedly effective (80–90% apoptosis) at 2–3 μM. Although these compounds were among the most potent in inhibiting cell growth, only a rough correlation was found between antiproliferative and proapoptotic effects. The proapoptotic activity was also supported by the presence of sub-G1 peak detected in the cell cycle analysis, already evident at 24 h following drug exposure (Fig. 2).

This analysis also shows that two representative compounds of our series (**3d** and **10a**) induced G1 arrest in IGROV-1 cells with functional wild-type p53. This cellular response was likely mediated by p21 induction, possibly as a consequence of p53 activation (not shown). This interpretation was consistent with a different cell cycle perturbation observed in IGROV-1/Pt1 with mutant p53, exhibiting a partial accumulation of cells also in S-phase and in G2/M.

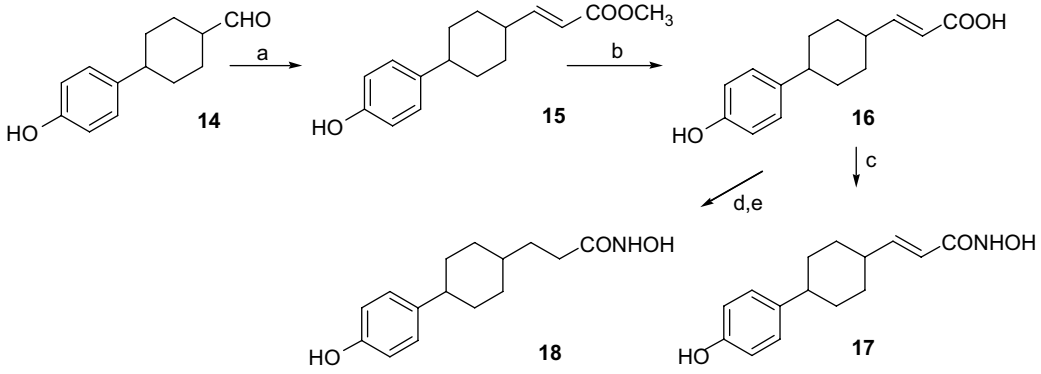
In an attempt to understand the cellular response to treatment we evaluated the acetylation state of histone H4 and α-tubulin after short-term exposure (4 h) (Fig. 3). The selected compounds, at IC_{80} and IC_{50} concentrations respectively, caused a comparable increase in the acetylation state of α-tubulin and of histone H4. As HDAC6 is known to deacetylate lysine 40 of α-tubulin [20] this finding supports that the selected compounds were also effective HDAC6 inhibitors.

The modulation of the acetylation of α-tubulin was more evident after a prolonged exposure (up to 48 h) (Fig. 4). Under these conditions all examined compounds were more effective than SAHA in inducing a persistent tubulin acetylation. The same observation applies to the p53 acetylation analyzed at equitoxic or equimolar concentrations. Since compounds very effective as apoptosis inducers (e.g., **3h**, **3i** and **1b**) caused a marginal acetylation of p53, it is conceivable that modulation of p53 function is not a primary event in activation of apoptotic pathways.

On the basis of the above results, we thought it worthwhile to examine the therapeutic potential of some representative derivatives as anticancer agents. Therefore compounds, **3a**, **3d** and **3g** were selected for *in vivo* studies (Table 4).

The antitumor efficacy was evaluated in 3 human tumor xenografts (s.c. implanted) derived from tumor cell lines used in the antiproliferative assay. Using a daily oral administration (5 day/week for a total of 10 doses), the methoxyderivative **3g** was more effective than SAHA against the H460 model. The i.p. treatment was less effective in this preliminary evaluation. All tested compounds were well tolerated and did not produce toxic deaths. Using a prolonged treatment schedule (4 weeks) a transient animal body weight loss was observed with **3a**. Again compound **3g** was effective in the treatment of the colon carcinoma HCT116 and even more effective against the ovarian carcinoma IGROV-1, producing 71% growth inhibition at the end of the treatment (Fig. 5). Significant antitumor activity was observed in a wide range of well tolerated doses delivered by oral route (20–100 mg/kg) with a prolonged treatment schedule, thus suggesting a good therapeutic index. The improved activity of tested compounds over SAHA could reflect a better bioavailability in oral administration.

In conclusion, we have designed and synthesized a new class of hydroxamic acid HDAC inhibitors. *In vitro* assay with the isoenzyme HDAC2 and molecular docking with a model of HDAC2 have been performed. The good correlation between the two validates the use of the model as a useful tool in the design of further analogues. The synthesized compound were tested for antiproliferative activities against a panel of tumor cell lines, from which general features of structure–activity relationships can be discerned. The 4-phenylcinnamic scaffold appears to be required for a good cytotoxic activity on different cell lines. Modification of the position of the side chain, or replacement of the proximal ring with a cyclohexyl,



Scheme 4. Reagents and conditions: a) methyl(triphenylphosphoranylidene)acetate, THF, rt then reflux, 4 h, 94%; b) LiOH, THF:H_2O 1:1, rt, 24 h, dark, 81%; c) HBTU, DIPEA, DMF, $NH_2OH \cdot HCl$, rt, 48 h, 13%; d) H_2/Pd/C, MeOH, rt, 4 h, 80%; e) HBTU, DIPEA, DMF, $NH_2OH \cdot HCl$, rt, 12 h, 35%.

Table 1
Selected ligands – HDAC2 model intermolecular interaction energies (kcal/mol) and HDAC2 inhibitory activity.

Entry	HDAC2 assay (IC_{50}, μM)	Interaction energy (kcal/mol)
SAHA	0.1 ± 0.02	−18.90
1	0.82 ± 0.14	−25.33
3a	0.59 ± 0.2	−25.65
3b	0.22 ± 0.06	−27.75
3m	0.46 ± 0.14	−23.15
4	1.7 ± 0.064	−17.86
10a	>5	−14.34
13	0.32 ± 0.024	−23.09

led to substantial decrease of the activity. The length and type of the chain tolerated some changes. A variety of substitutions in the distal ring were also well tolerated. Selected compounds of this series exhibited a promising antitumor activity.

5. Experimental

5.1. General chemical methods

All reagents and solvents were reagent grade or were purified by standard methods before use. Melting points were determined in open capillaries on a Büchi melting point apparatus and are uncorrected. Column chromatography was carried out on flash silica gel (Merck 230–400 mesh). TLC analysis was conducted on silica gel plates (Merck $60F_{254}$). NMR spectra were recorded at 300 MHz with a Bruker instrument. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and Hz, respectively. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values.

5.2. General procedure for one-pot Suzuki condensation (method A)

A small amount of the appropriate aryl bromide (0.6 mmol), 166 mg (0.65 mmol) of bis(pinacolato)diboron, 174 mg (1.78 mmol) of KOAc and 13 mg (0.018 mmol) of $PdCl_2$(dppf) were dissolved in 36 mL of dioxane and the mixture was stirred at 100 °C for 1–2 h under nitrogen. The solution was cooled at room temperature, and methyl 4-bromocinnamate (287 mg, 1.19 mmol), $PdCl_2$(dppf) (13 mg, 0.018 mmol) and 2 N Na_2CO_3 (1.48 mmol, 0.74 mL) were added and the mixture was heated at 80–100 °C for 1–4 h. The solution was cooled at room temperature, diluted with water, acidified with 2 N HCl (2.4 mL) and extracted with ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, filtered and evaporated. The crude product was purified, when necessary, by flash chromatography.

Table 2
Inhibition of HDAC2[a] (IC_{50}, μM) and antiproliferative activity[b] (IC_{50}, μM) of selected compounds and of reference HDAC inhibitor (SAHA) against a panel of various tumor cell lines.

Cpd.	HDAC2 assay	NB4	H460	HCT-116	IGROV-1	IGROV-1/Pt1
SAHA	0.1 ± 0.02	0.7 ± 0.03	3.4 ± 0.8	0.31 ± 0.02	2.2 ± 0.3	2.2 ± 0.2
1	0.82 ± 0.14	0.90 ± 0.1	5.4 ± 0.1	1.58 ± 0.3	3.5 ± 0.6	4.0 ± 0.3
3a	0.59 ± 0.2	2.3 ± 0.02	6.0 ± 0.9	0.33 ± 0.05	7.6 ± 3.5	6.5 ± 2.0
3b	0.22 ± 0.06	1.81 ± 0.1	3.8 ± 0.2	0.8 ± 0.04	8.0 ± 0.4	5.5 ± 0.4
3c	1.86 ± 0.17	0.76 ± 0.03	nd	0.51 ± 0.1	0.61 ± 0.14	1.36 ± 0.04
3d	0.33 ± 0.001	nd	5.7 ± 0.4	nd	2.7 ± 0.8	3.2 ± 1.6
3e	>5	3.29 ± 0.2	nd	nd	nd	nd
3f	nd	nd	nd	nd	12.1 ± 0.7	20.3 ± 1.5
3g	1.16 ± 0.27	nd	3.7 ± 0.1	0.22 ± 0.01	0.75 ± 0.4	2.3 ± 0.3
3h	0.45 ± 0.04	nd	2.8 ± 0.1	nd	2.8 ± 1.8	3.2 ± 1.6
3i	>5	0.86 ± 0.05	nd	0.7 ± 0.4	1.1 ± 0.5	3.5 ± 0.3
3l	1.2 ± 0.46	6.5 ± 0.5	14 ± 2.0	nd	22.1 ± 0.9	29.3 ± 2.5
3m	0.46 ± 0.14	0.58 ± 0.09	3.1 ± 0.1	1.46 ± 0.25	4.1 ± 0.5	3.9 ± 0.4
4	1.7 ± 0.064	3.9 ± 0.5	15.2 ± 2.0	0.26 ± 0.14	0.8 ± 0.2	0.95 ± 0.1
7	nd	nd	nd	3.9 ± 0.8	5.4 ± 0.7	5.9 ± 1.3
10a	>5	0.66 ± 0.003	1.9 ± 0.05	0.98 ± 0.03	1.4 ± 0.3	2.4 ± 1.2
1b	>5	nd	>20	nd	0.57 ± 0.09	2.01 ± 0.91
10c	1.2 ± 0.02	nd	3.3 ± 0.2	nd	1.05 ± 0.5	3.1 ± 0.6
11	0.72 ± 0.12	0.86 ± 0.03	4.7 ± 0.6	2.7 ± 0.4	8.0 ± 1.5	9.1 ± 2.9
13	0.32 ± 0.024	2.85 ± 0.05	5.6 ± 0.3	1.64 ± 0.2	>10	>10
17	0.31 ± 0.003	0.9 ± 0.1	2.2 ± 0.3	0.37 ± 0.17	2.5 ± 0.5	2.6 ± 0.4
18	nd	6.9 ± 0.1	9.5 ± 0.7	4.27 ± 0.3	31.9	23.9

nd = Not determined.
[a] HDAC activity determined using HDAC2 from HeLa cells as indicated in Section 5.
[b] Cells were exposed for 72 h. Antiproliferative activity was measured by cell counting and expressed as concentration required for 50% inhibition of cell growth (IC_{50}). IC_{50} values were determined by dose–response curves.

Table 3
Apoptosis induction in IGROV-1 cell by selected HDAC inhibitors.[a]

Cpd.	IC_{80} (μM)	Apoptosis
Untreated		2 ± 2
SAHA	5	15 ± 5
SAHA	10	45 ± 5
1b	10	90 ± 8
3a	20	40 ± 8
3b	15	90 ± 2
3c	2	77 ± 6
3d	10	90 ± 5
3g	3	52 ± 3
3h	7	90 ± 4
3i	3	89 ± 7
4	10	45 ± 6
10a	3	89 ± 3
11	12	65 ± 6

[a] Apoptosis was determined at 72 h following exposure to IC_{80} by TUNEL assay and analysis by flow cytometry. SAHA was used as a control at two concentrations, 5 μM (IC_{80}) and 10 μM (IC_{90}). The results are expressed as percentage of apoptotic cells vs. total cell number. Values are the mean ± standard deviation of three independent experiments.

5.3. General procedure for Suzuki coupling (method B)

To a solution of methyl 4-bromocinnamate (883 mg, 3.66 mmol) in 8 mL of toluene, 4.7 mL of a 2 M solution of Na_2CO_3, 141 mg (0.122 mmol) of Pd(tetrakis)triphenylphosphine and 4.1 mmol of the appropriate boronic acid (previously dissolved in 2 mL of EtOH) were added. The solution was refluxed for 2 h to 2 days under nitrogen. After addition of ethyl acetate, the organic phase was washed with water, brine, dried over Na_2SO_4 and filtered. The solvent was removed under reduced pressure to give a crude ester which was purified, when necessary, by flash chromatography.

5.4. General procedure for ester hydrolysis (method C)

The appropriate ester (1 mmol) was suspended in a solution of 219 mg (5.2 mmol) of $LiOH \cdot H_2O$ in 4 mL of THF/H_2O 1:1 and stirred at room temperature in the dark overnight. After evaporation of THF the remaining aqueous phase was washed with hexane, diethyl ether and then acidified with HCl 2 N (0.4 mL). The precipitated white solid was filtered and dried. No further purification was necessary.

5.5. General procedure for HATU or HBTU condensation (method D)

The appropriate acid (0.89 mmol) was dissolved under nitrogen in 9 mL of DMF, then 0.89 mmol of HATU or HBTU and 308 μL (1.78 mmol) of DIPEA were added and the solution was stirred at room temperature for 2 min. After addition of hydroxylamine hydrochloride (68 mg, 0.98 mmol), the mixture was stirred at room temperature for 3–72 h. DMF was removed under reduced pressure and the residue was washed with water to obtain, after filtration, a crude product which was purified, when necessary, by flash chromatography.

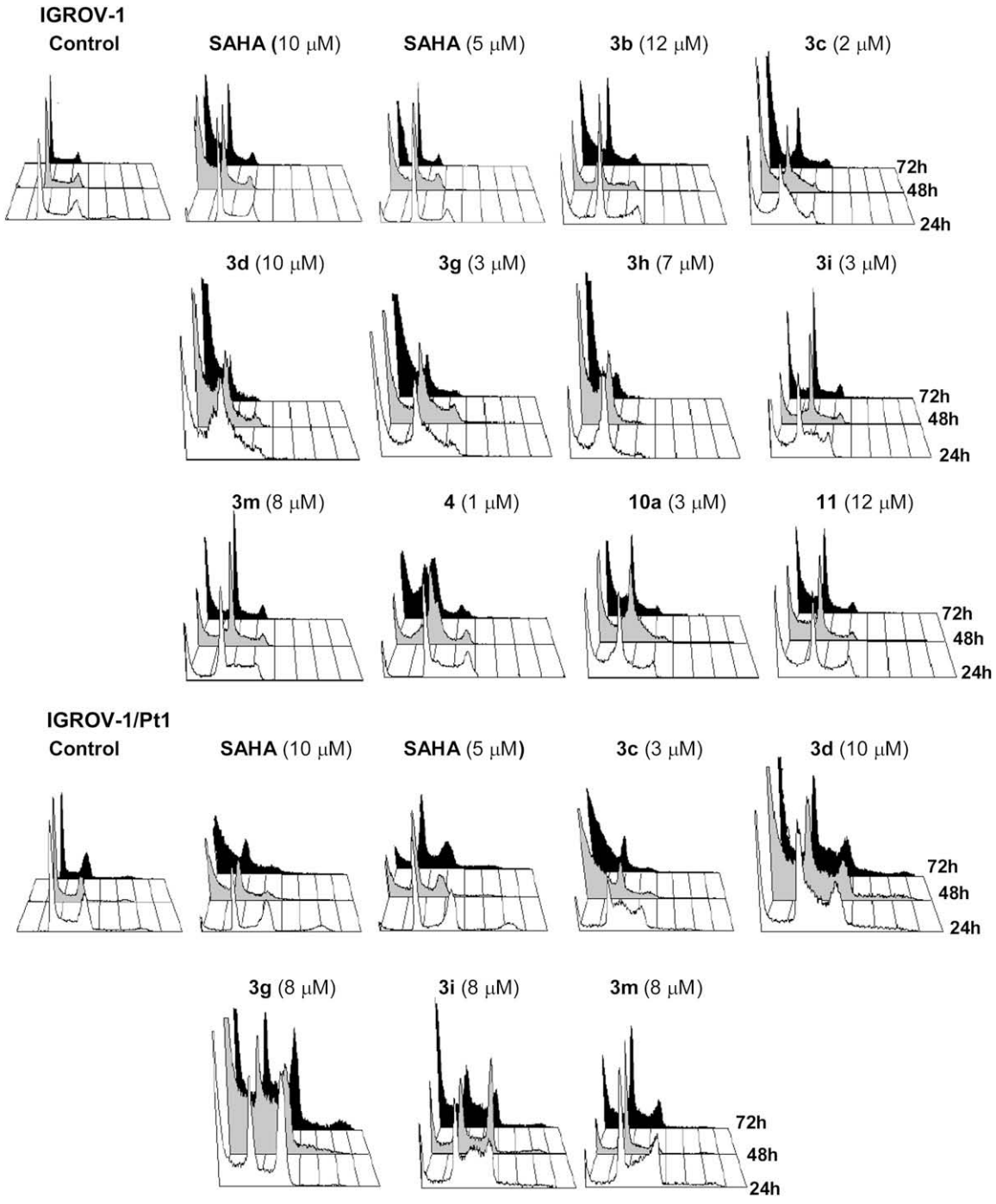


Fig. 2. Time course of cell cycle perturbations in ovarian carcinoma cells, IGROV-1, and in a cisplatin-resistant subline, IGROV-1/Pt1, following treatment with selected compounds. The comparison was performed in cells treated with IC_{80} for each drug and the cell cycle was analyzed by FACScan analysis of PI-stained cells at 24, 48 and 72 h of treatment. In the case of SAHA a higher concentration (10 μM), corresponding to IC_{90}, was also shown. One experiment representative of three is reported.

5.6. General procedure for the synthesis of hydroxamic acids from esters via N-*(tetrahydropyran-2-yloxy) amides (method E)*

The appropriate ester (1.02 mmol) was dissolved in 14 mL of anhydrous THF, then *O*-tetrahydropyranylhydroxylamine (120 mg, 1.02 mmol) was added. The solution was cooled to −78 °C and treated with 1.07 mL (2.14 mmol) of lithium hexamethyldisilazane. The mixture was stirred for 1 h under nitrogen, then was quenched with NH_4Cl solution. Once at room temperature, the mixture was extracted with ethyl acetate and the organic phase was dried over Na_2SO_4. The solvent was evaporated under reduced pressure to give the crude *N*-(tetrahydropyran-2-yloxy) amide, which was purified, when necessary, by flash chromatography. This product (0.603 mmol) was suspended in 9 mL of MeOH, then 34 mg (0.181 mmol) of *p*-toluenesulfonic acid monohydrate was added and the mixture was stirred overnight at room temperature. The solid formed was filtered, washed with Et_2O and dried to give the hydroxamic acid.

5.6.1. N-*Hydroxy*-E-*3-(biphenyl-4-yl)-acrylamide (**1**)*

E-4-Phenylcinnamic acid (200 mg, 0.89 mmol) was condensed with hydroxylamine hydrochloride according to procedure D, using

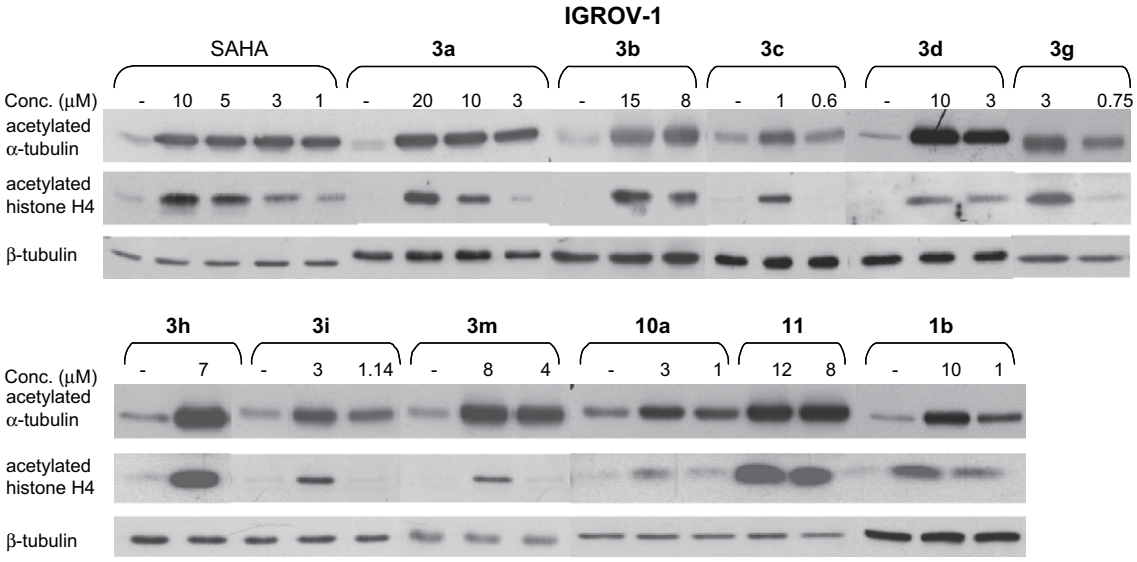


Fig. 3. Effects of selected compounds on acetylation of α-tubulin and histone H4 in IGROV-1 cells after 4 h exposure to IC_{80} and IC_{50} for each compound. β-tubulin is shown as a control of protein loading.

HATU, to obtain 220 mg of the title compound as a white solid. Yield 100%, mp 168–170 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.75 (s, 1H), 9.03 (br s, 1H), 7.79–7.56 (m, 6H), 7.53–7.29 (m, 4H), 6.47 (d, 1H, $J = 16$ Hz). Anal. $C_{15}H_{13}NO_2$ (C, H, N).

5.6.2. N-*Hydroxy*-E-*3-(4′-hydroxybiphenyl-4-yl)-acrylamide (**3a**)*

E-4-(4′-Hydroxyphenyl)cinnamic acid [21] (250 mg, 1.04 mmol) was dissolved under nitrogen in 10 mL of DMF, then 169 mg (1.25 mmol) of HOBt and 259 mg (1.35 mmol) of WSC were added and the solution stirred at room temperature for 3 h. After addition of hydroxylamine hydrochloride (361 mg, 5.2 mmol), followed by 0.72 mL (5.2 mmol) of TEA, the mixture was stirred at room temperature overnight. DMF was removed under reduced pressure and the residue was washed with water to obtain 263 mg of a crude product. Purification by flash chromatography on RP-18 (40–63 μm) reverse phase (CH_3OH/H_2O 50:50) afforded 34 mg of the title compound as a white solid. Yield 13%, mp > 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.73 (s, 1H), 9.62 (s, 1H), 9.00 (s, 1H), 7.69–7.48 (m, 6H), 7.43 (d, 1H, $J = 16$ Hz), 6.82 (d, 2H, $J = 8.19$ Hz), 6.44 (d, 1H, $J = 16$ Hz). MS (EI) *m*/*z*: 255 (M^+, 40), 91 (100), 73 (40), 57 (40). Anal. $C_{15}H_{13}NO_3$ (C, H, N).

5.6.3. N-*Hydroxy*-E-*3-[4′-hydroxymethylbiphenyl-4-yl]-acrylamide (**3b**)*

Methyl 4-bromocinnamate was reacted with 4-hydroxymethylbenzeneboronic acid (method A) to give, after flash chromatography (hexane/ethyl acetate 80:20) methyl *E*-3-(4′-hydroxymethylbiphenyl-4-yl)-acrylate as a white solid. Yield 51%,

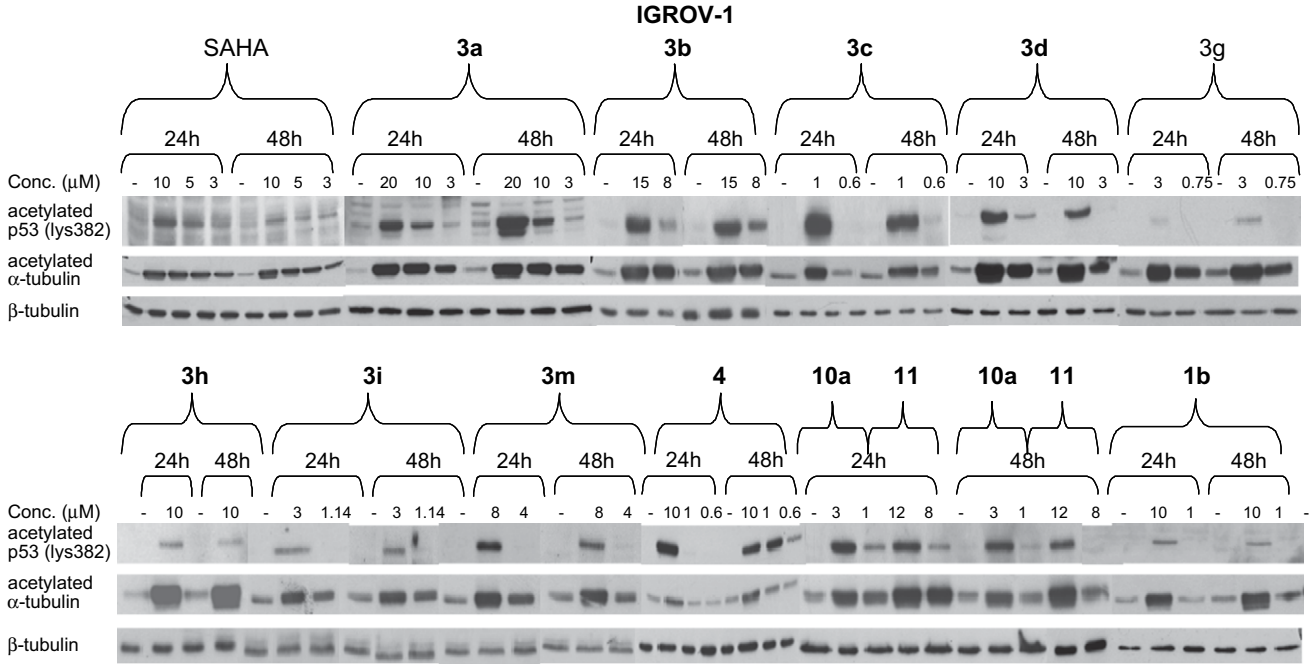


Fig. 4. Effects of selected compounds on acetylation of α-tubulin and p53 in IGROV-1 cells after 24 and 48 h exposure to IC_{80} and IC_{50} for each compound. β-tubulin is shown as a control of protein loading.

Table 4
Antitumor activity of selected HDAC inhibitors.

Tumor model	Compound	Schedule[a]	Dose (mg/kg)	Route	TVI%[b]	BWL%[c]
H460 (nonSCLC)	SAHA	qd × 5 d/week	100	p.o.	34	2
	3g	×2 weeks	100	p.o.	57	1
	3g		100	i.p.	39	4
HCT116 (colon carcinoma)	SAHA	qd × 5 d/week	100	p.o.	48	4
	3a	×4 weeks	40	p.o.	65	8
	3g		20	p.o.	35	1
	3g		20	i.p.	50	2
IGROV-1 (ovarian carcinoma)	**3a**	qd × 5 d/week	20	p.o.	44	2
	3a	×4 weeks	40	p.o.	49	6
	3g		20	p.o.	71	0
	3d		20	p.o.	37	0

[a] qd × 5 d/week, daily treatment for 5 consecutive days in each week. The treatment was repeated for the indicated weeks.
[b] Tumor volume inhibition (TVI%) was determined within the first week after treatment.
[c] Body weight loss.

mp 180–182 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.72 (d, 1H, $J = 16.00$ Hz), 7.66–7.53 (m, 6H), 7.45 (d, 2H, $J = 7.82$ Hz), 6.45 (d, 1H, $J = 16.00$ Hz), 4.75 (s, 2H), 3.81 (s, 3H). The above ester was hydrolyzed (method C) to the corresponding acid. Yield 85%, mp 265–266 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.77–7.58 (m, 7H), 7.37 (d, 2H, $J = 7.82$ Hz), 6.52 (d, 1H, $J = 16.00$ Hz), 5.20 (t, 1H, $J = 5.58$ Hz), 4.50 (d, 2H, $J = 5.58$ Hz). This acid was condensed with hydroxylamine hydrochloride (method D, HATU) to give the title compound as a white solid. Yield 65%, mp 220–223 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.75 (s, 1H), 9.03 (s, 1H), 7.77–7.54 (m, 6H), 7.47 (d, 1H, $J = 16.00$ Hz), 7.38 (d, 2H, $J = 7.82$ Hz), 6.47 (d, 1H, $J = 16.00$ Hz), 5.20 (t, 1H, $J = 5.58$ Hz), 4.51 (2H, d, $J = 5.58$ Hz). Anal. $C_{16}H_{15}NO_3$ (C, H, N).

5.6.4. N-*Hydroxy*-E-*3-(4′-chlorobiphenyl-4-yl)-acrylamide* (***3c***)

Methyl 4-bromocinnamate was reacted with 4-chlorobenzeneboronic acid (method B) to give, after flash chromatography (hexane/ethyl acetate 92:8) methyl *E*-3-(4′-chlorobiphenyl-4-yl)-acrylate. Yield 46%, mp 155–156 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.71 (d, 1H, $J = 16.00$ Hz), 7.63–7.47 (m, 6H), 7.41 (d, 2H, $J = 8.34$ Hz), 6.47 (d, 1H, $J = 16.00$ Hz), 3.81 (s, 3H). This ester was reacted with *O*-tetrahydropyranylhydroxylamine (method E) to give *E*-3-(4′-chlorobiphenyl-4-yl)-*N*-(tetrahydropyran-2-yloxy)-acrylamide. Yield 100%, mp 120–122 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.73 (d, 1H, $J = 15.63$ Hz), 7.62–7.46 (m, 6H), 7.37 (d, 2H, $J = 8.34$ Hz), 6.43 (br s, 1H), 5.12 (br s, 1H), 4.02–3.87 (m, 1H), 3.71–3.57 (m, 1H), 1.92–1.73 (m, 4H), 1.70–1.31 (m, 2H). This compound was treated according to method E to give the title compound as a white solid. Yield 46%, mp 200–202 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.50 (s, 1H), 9.10 (s, 1H), 7.75–7.50 (m, 8H), 7.49 (s, 1H, $J = 16.00$ Hz), 6.50 (s, 1H, $J = 16.00$ Hz). Anal. $C_{15}H_{12}ClNO_2$ (C, H, N).

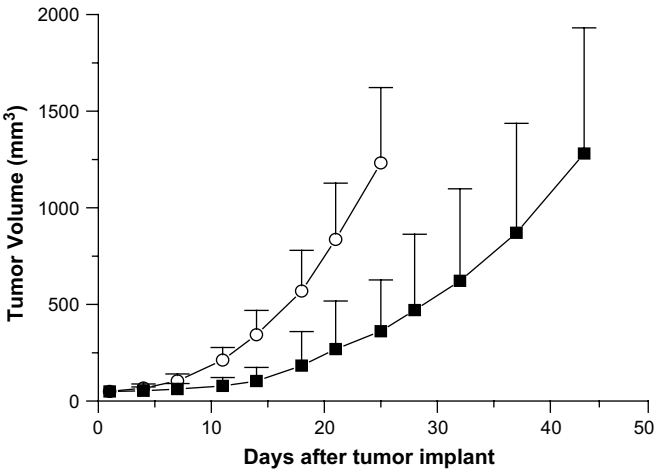


Fig. 5. Antitumor activity of compound **3g** against the ovarian carcinoma model IGROV-1. Animals were treated by oral route with 20 mg/kg daily (5 daily administration for week for a total of 20 doses). (○) control (solvent-treated animals); (■) drug-treated animals. Drug treatment started when tumors were just measurable (~ 100 mm^3).

5.6.5. N-*Hydroxy*-E-*3-(4′-cyanobiphenyl-4-yl)-acrylamide* (***3d***)

Methyl 4-bromocinnamate was reacted with 4-cyanobenzeneboronic acid (method B) to give, after flash chromatography (hexane/ethyl acetate 90:10) methyl *E*-3-(4′-cyanobiphenyl-4-yl)-acrylate as a white solid. Yield 35%, mp 150–152 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.81–7.57 (m, 9H), 6.50 (d, 1H, $J = 16.00$ Hz), 3.82 (s, 3H). The above ester was reacted with *O*-tetrahydropyranylhydroxylamine (method E) to give, after flash chromatography (hexane/ethyl acetate 60:40) *E*-3-(4′-cyanobiphenyl-4-yl)-*N*-(tetrahydropyran-2-yloxy)-acrylamide. Yield 53%, mp 211–213 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.83–7.62 (m, 5H), 7.61–7.50 (m, 4H), 6.49 (br s, 1H), 5.12–4.93 (m, 1H), 4.05–3.90 (m, 1H), 3.73–3.58 (m, 1H), 1.97–1.74 (m, 3H), 1.72–1.52 (m, 3H). Alternatively, this intermediate could be obtained by reacting 4-bromobenzonitrile with *E*-3-(4′-bromophenyl)-*N*-(tetrahydropyran-2-yloxy)-acrylamide (method A). Yield 82%. The product was treated according to method E to give the title compound. Yield 62%, mp 212–214 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.80 (s, 1H), 9.05 (s, 1H), 8.00–7.80 (m, 4H), 7.82 (d, 2H, $J = 8.20$ Hz), 7.69 (d, 2H, $J = 8.20$ Hz), 7.51 (d, 1H, $J = 15.30$ Hz), 6.54 (d, 1H, $J = 15.30$ Hz). Anal. $C_{16}H_{12}N_2O_2$ (C, H, N).

5.6.6. N-*Hydroxy*-E-*3-(4-pyridin-4-yl-phenyl)-acrylamide* (***3e***)

Methyl 4-bromocinnamate was reacted with pyridine-4-boronic acid (method B) to give, after flash chromatography (hexane/ethyl acetate 50:50) methyl *E*-3-(4-pyridin-4-yl-phenyl)-acrylate. Yield 63%, mp133–134 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.70 (d, 2H, $J = 4.84$ Hz), 7.80–7.58 (m, 7H), 6.51 (d, 1H, $J = 16.00$ Hz), 3.82 (s, 3H). This ester was hydrolysed (method C) to give the corresponding acid as a white solid. Yield 85%, mp 270 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 8.87 (d, 2H, $J = 4.84$ Hz), 8.21 (d, 2H, $J = 4.84$ Hz), 8.02 (d, 2H, $J = 8.19$ Hz), 7.90 (d, 2H, $J = 8.19$ Hz), 7.66 (d, 1H, $J = 16.00$ Hz), 6.71 (d, 1H, $J = 16.00$ Hz). This acid was condensed with hydroxylamine hydrochloride (method D, HBTU) to obtain, after flash chromatography on reverse phase (CH_2Cl_2/MeOH 95:5), the title compound. Yield 36%, mp 180 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.82 (s, 1H), 9.11 (br s, 1H), 8.64 (d, 2H, $J = 4.84$ Hz), 7.86 (d, 2H, $J = 8.19$ Hz), 7.79–7.66 (m, 4H), 7.51 (d, 1H, $J = 16.00$ Hz), 6.54 (d, 1H, $J = 16.00$ Hz). MS (EI) m/z: 241 (M^+, 100). Anal. $C_{14}H_{12}N_2O_2$ (C, H, N).

5.6.7. N-*Hydroxy*-E-*3-(4′-formylbiphenyl-4-yl)-acrylamide* (***3f***)

Methyl 4-bromocinnamate was reacted with 4-formylbenzeneboronic acid (method B) to give, after flash chromatography (hexane/ethyl acetate 90:10), methyl *E*-3-(4′-formylbiphenyl-4-yl)-acrylate as a yellow solid. Yield 32%, mp 160–162 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 10.06 (s, 1H), 7.96 (d, 2H, $J = 8.19$ Hz), 7.76 (d, 2H, $J = 8.19$ Hz), 7.73 (d, 1H, $J = 16.00$ Hz), 7.67–7.62 (m, 4H), 6.50 (d, 1H, $J = 16.00$ Hz), 3.82 (s, 3H). This ester was hydrolyzed (method C) to the corresponding acid. Yield 22%, mp 209–212 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.06 (s, 1H), 7.94–7.74 (m, 9H). This acid was condensed (method D, HATU) with hydroxylamine hydrochloride to afford 8 mg of the title compound. Yield 25%, mp

188–190 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 11.30 (br s, 1H), 10.78 (s, 1H), 9.08 (br s, 1H), 8.10–7.37 (m, 9H), 6.52 (d, 1H, J = 15.26 Hz). Anal. $C_{16}H_{13}NO_3$ (C, H, N).

5.6.8. N-*Hydroxy*-E-*3-(4′-methoxybiphenyl-4-yl)-acrylamide (**3g**)*

Methyl 4-bromocinnamate was reacted with 4-methoxybenzeneboronic acid (method B) to give, after flash chromatography (hexane/ethyl acetate 95:5 then hexane/ethyl acetate 80:20), methyl *E*-3-(4′-methoxybiphenyl-4-yl)-acrylate as a pale yellow solid. Yield 51%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.71 (d, 1H, J = 16.00 Hz), 7.65–7.50 (m, 6H), 6.97 (d, 2H, J = 8.39 Hz), 6.46 (d, 1H, J = 16.00 Hz), 3.85 (s, 3H), 3.81 (s, 3H). This ester was reacted with *O*-tetrahydropyranylhydroxylamine (method E) to give *E*-3-(4′-methoxybiphenyl-4-yl)-*N*-(tetrahydropyran-2-yloxy)-acrylamide as a yellow solid. Yield 100%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.74 (d, 1H, J = 15.64 Hz), 7.64–7.47 (m, 6H), 6.97 (d, 2H, J = 8.39 Hz), 6.44 (br s, 1H), 5.13–4.93 (m, 1H), 4.06–3.90 (m, 1H), 3.84 (s, 3H), 3.74–3.61 (m, 1H), 1.99–1.75 (m, 3H), 1.74–1.45 (m, 3H). Alternatively, this compound could be prepared by reacting *E*-3-(4′-bromophenyl)-*N*-(tetrahydropyran-2-yloxy)-acrylamide with 4-methoxybenzeneboronic acid (method B). Yield 100%. The product was treated according to method E to give the title compound as a white solid. Yield 88%. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.76 (s, 1H), 9.05 (s, 1H), 7.73–7.56 (m, 6H), 7.48 (d, 1H, J = 15.60 Hz), 7.03 (d, 2H, J = 8.90 Hz), 6.47 (d, 1H, J = 15.60 Hz), 3.80 (s, 3H). Anal. $C_{16}H_{15}NO_3$ (C, H, N).

5.6.9. N-*Hydroxy*-E-*3-(4′-thiophen-2-yl-phenyl)-acrylamide (**3h**)*

Methyl 4-bromocinnamate was reacted with thiophene-2-boronic acid (method B) to give methyl-*E*-3-(4′-thiophen-2-yl-phenyl)-acrylate as a pale yellow solid. Yield 43%, mp 158–162 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.68 (d, 1H, J = 16.00 Hz), 7.62 (d, 2H, J = 8.19 Hz), 7.53 (d, 2H, J = 8.19 Hz), 7.42–7.28 (m, 2H), 7.15–7.05 (m, 1H), 6.43 (d, 1H, J = 16.00 Hz), 3.83 (s, 3H). This ester was reacted with *O*-tetrahydropyranylhydroxylamine (method E) to give *E*-3-(4′-thiophen-2-yl)phenyl)-*N*-(tetrahydropyran-2-yloxy)-acrylamide. Yield 100%, mp 120–122 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.77–7.57 (m, 2H), 7.55–7.44 (m, 3H), 7.41–7.27 (m, 2H), 7.13–7.04 (m, 1H), 6.39 (br s, 1H), 5.08–4.94 (m, 1H), 4.03–3.89 (m, 1H), 3.72–3.60 (m, 1H), 1.94–1.77 (m, 3H), 1.72–1.52 (m, 3H). Alternatively, this intermediate could be prepared by reacting *E*-3-(4′-bromophenyl)-*N*-(tetrahydropyran-2-yloxy)-acrylamide with thiophene-2-boronic acid (method B). Yield 88%. The product was treated according to method E to give the title compound. Yield 60%, mp 182–184 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.77 (s, 1H), 9.07 (br s, 1H), 7.70 (d, 2H, J = 8.19 Hz), 7.65–7.55 (m, 4H), 7.46 (d, 1H, J = 16.00 Hz), 7.15 (t, 1H, J = 4.47 Hz), 6.48 (d, 1H, J = 16.00 Hz). Anal. $C_{13}H_{11}NO_2S$ (C, H, N).

5.6.10. N-*Hydroxy*-E-*3-[4′-dimethylaminobiphenyl-4-yl] acrylamide (**3i**)*

4-Bromo-*N*,*N*-dimethylaniline was reacted with methyl 4-bromocinnamate according to method A to give methyl *E*-3-(4′-dimethylaminobiphenyl-4-yl)-acrylate. Yield 8%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.73 (d, 1H, J = 16.00 Hz), 7.70–7.53 (m, 8H), 6.48 (d, 1H, J = 16.00 Hz), 3.83 (s, 3H), 3.16 (s, 3H), 2.97 (s, 3H). The above ester was hydrolyzed (method C) to give the corresponding acid. Yield 77%, mp 314–315 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.75–7.55 (m, 7H), 7.10–6.90 (m, 2H), 6.54 (d, 1H, J = 16 Hz), 3.00 (s, 6H). This acid was condensed with hydroxylamine hydrochloride (method D, HBTU) to give the title compound. Yield 68%, mp 260–263 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.73 (s, 1H), 9.03 (s, 1H), 7.70–7.52 (m, 6H), 7.46 (d, 1H, J = 16.38 Hz), 6.80 (d, 2H, J = 8.93 Hz), 6.44 (d, 1H, J = 16.38 Hz), 2.95 (s, 6H). Anal. $C_{17}H_{18}N_2O_2$ (C, H, N).

5.6.11. N-*Hydroxy*-E-*3-[4-(1H-indol-5-yl)-phenyl]-acrylamide (**3l**)*

4-Bromoindole was reacted with methyl 4-bromocinnamate according to procedure A to give methyl *E*-3-[4-(1*H*-indol-5-yl)-phenyl]-acrylate. Yield 26%, mp 162–164 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.21 (br s, 1H), 7.88 (s, 1H), 7.73 (d, 1H, J = 16 Hz), 7.70–7.64 (m, 2H), 7.63–7.54 (m, 3H), 7.46 (br s, 2H), 6.64–6.57 (m, 1H), 6.46 (d, 1H, J = 16 Hz), 3.81 (s, 3H). The above ester was hydrolysed (method C) to give the corresponding acid. Yield 70%, mp 230 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 11.18 (s, 1H), 7.88 (s, 1H), 7.78–7.70 (m, 5H), 7.62 (d, 1H, J = 16.00 Hz), 7.49–7.43 (m, 2H), 7.39 (d, 1H, J = 2.61 Hz), 6.58–6.47 (m, 2H). This product was condensed with hydroxylamine hydrochloride (method D, HBTU) to give the title compound as a brown solid. Yield 55%, mp 215 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 11.18 (s, 1H), 10.75 (br s, 1H), 9.04 (br s, 1H), 7.87 (s, 1H), 7.72 (d, 2H, J = 7.82 Hz), 7.61 (d, 2H, J = 7.82 Hz), 7.48–7.34 (m, 4H), 6.55–6.42 (m, 2H). Anal. $C_{17}H_{14}N_2O_2$ (C, H, N).

5.6.12. N-*Hydroxy*-E-*3-[3′-chloro-4′-hydroxybiphenyl-4-yl]-acrylamide (**3m**)*

Condensation of methyl 4-bromocinnamate (463 mg, 1.92 mmol) with 2-chloro-4-bromophenol (200 mg, 0.96 mmol) was accomplished according to procedure A to give ester **2m** as a white solid (40%) after flash chromatography (hexane/ethyl acetate 8:2), mp 153–154 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.71 (d, 1H, J = 16 Hz), 7.50–7.65 (m, 5H), 7.43 (dd, 1H, J = 2.23, 8.56 Hz), 7.09 (d, 1H, J = 8.56 Hz), 6.46 (d, 1H, J = 16 Hz), 3.81 (3H, s). The ester was hydrolyzed (procedure C) to obtain the corresponding acid in 94% yield, mp 233–234 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.45 (br s, 1H), 10.45 (br s, 1H), 7.60–7.80 (m, 5H), 7.69 (d, 1H, J = 16 Hz), 7.53 (dd, 1H, J = 1.86, 8.56 Hz), 7.05 (d, 1H, J = 8.56 Hz), 6.55 (d, 1H, J = 16 Hz). The acid was condensed with hydroxylamine hydrochloride according to procedure D (HATU). Purification by flash chromatography RP-18 (40–63 μm) reverse phase (CH_3OH/H_2O 50:50) and crystallization from diethyl ether afforded the title compound as a white solid. Yield 24%, mp 172–175 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.77 (s, 1H), 10.42 (s, 1H), 9.06 (br s, 1H), 7.77–7.40 (m, 7H), 7.05 (d, 1H, J = 8.56 Hz), 6.47 (d, 1H, J = 15.63 Hz). Anal. $C_{15}H_{12}ClNO_3$ (C, H, N).

5.6.13. N-*Hydroxy-3-(4′-hydroxybiphenyl-4-yl)-propionamide (**4**)*

3-(4′-Hydroxybiphenyl-4-yl)-acrylic acid methyl ester [21] (500 mg, 1.97 mmol) was dissolved in 250 mL of ethyl acetate, then PtO_2 (291 mg, 1.3 mmol) was added and the mixture was hydrogenated for 1 h. After filtration of the catalyst, the solvent was evaporated to obtain 390 mg of 3-(4′-hydroxybiphenyl-4-yl)-propionic acid methyl ester. Yield 77%, mp 127–128 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.56–7.37 (m, 4H), 7.32–7.17 (m, 2H), 6.87 (d, 2H, J = 8.56 Hz), 3.67 (s, 3H), 2.97 (t, 2H, J = 7.44 Hz), 2.68 (t, 2H, J = 7.44 Hz). The above ester (390 mg, 1.52 mmol) was hydrolyzed according to method C. The solid formed was filtered and dried to give 368 mg of 3-(4′-hydroxybiphenyl-4-yl)-propionic acid. Yield 100%, mp 207–208 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 12.13 (s, 1H), 9.62 (s, 1H), 7.50–7.40 (m, 4H), 7.23 (d, 2H, J = 7.82 Hz), 6.81 (d, 2H, J = 8.56 Hz), 2.80 (t, 2H, J = 7.44 Hz), 2.52 (t, 2H, J = 7.44 Hz). The above acid was condensed according to method D (HBTU), to give the title compound as a white solid. Yield 92%, mp 180–182 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.38 (s, 1H), 9.49 (s, 1H), 8.71 (s, 1H), 7.49–7.39 (m, 4H), 7.21 (d, 2H, J = 7.82 Hz), 6.82 (d, 2H, J = 8.56 Hz), 2.81 (t, 2H, J = 7.44 Hz), 2.26 (t, 2H, J = 7.44 Hz). Anal. $C_{15}H_{15}NO_3$ (C, H, N).

5.6.14. E-*3-(4-Bromophenyl)*-N-*(tetrahydropyran-2-yloxy)-acrylamide (**5**)*

E-4-Bromocinnamic acid (1.28 g, 5.68 mmol) was dissolved under nitrogen in 100 mL of anhydrous DMF, then 1.15 g (8.53 mmol) of HOBt and 1.30 g (6.82 mmol) of WSC were added and the solution was stirred at room temperature for 3 h. After addition of *O*-tetrahydropyranylhydroxylamine (1,00 g, 8.53 mmol), the mixture was heated at 50 °C for 1 h. DMF was evaporated under reduced pressure and the residue was taken up with CH_2Cl_2. The

organic phase was washed with satd. aq. $NaHCO_3$, dried over Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure to give a crude product which was purified by flash chromatography (CH_2Cl_2/CH_3OH 99:1) to afford 1.56 g of the title compound as a white solid. Yield 84%, mp 119–120 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.31 (br s, 1H), 7.66 (d, 1H, J = 16.00 Hz), 7.50 (d, 2H, J = 7.82 Hz), 7.36 (d, 2H, J = 7.82 Hz), 6.37 (br s, 1H), 4.99 (br s, 1H), 4.01–3.88 (m, 1H), 3.71–3.59 (m, 1H), 1.92–1.77 (m, 3H), 1.70–1.57 (m, 3H).

5.6.15. E-*3-(Biphenyl-4-yloxy)*-N-*hydroxy-acrylamide (**7**)*

4-Phenylphenol (160 mg, 0.94 mmol) was dissolved in 2 mL of CH_3CN and treated with 6 μL (0.05 mmol) of 4-methylmorpholine. After stirring for 20 min at room temperature, an amount of 115 μL (1.13 mmol) of ethyl propiolate was dropped. Dilution with water, extraction with ethyl acetate, washing with brine, drying over Na_2SO_4, filtering, evaporating, and flash chromatography (hexane/ethyl acetate 80:20) gave 230 mg of ethyl *E*-3-(biphenyl-4-yloxy)-acrylate. Yield 91%, mp 74–76 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.82 (d, 1H, J = 12.28 Hz), 7.63–7.50 (m, 5H), 7.48–7.38 (m, 2H), 7.13 (d, 2H, J = 8.56 Hz), 5.58 (d, 1H, J = 12.28 Hz), 4.19 (q, 2H, J = 7.07 Hz), 1.28 (t, 3H, J = 7.07 Hz). The above ester was hydrolysed (method C) to give the corresponding acid. Yield 53%, mp 161–162 °C. ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.87 (d, 1H, J = 12.29 Hz), 7.73 (d, 2H, J = 9.00 Hz), 7.68–7.61 (m, 2H), 7.50–7.41 (m, 2H), 7.39–7.34 (m, 1H), 7.28 (d, 2H, J = 9.00 Hz), 5.54 (d, 1H, J = 12.29 Hz). This acid was condensed with hydroxylamine hydrochloride (method D, HBTU) to afford the title compound. Yield 19%, mp 123–125 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.44 (br s, 1H), 8.87 (br s, 1H), 7.85–7.57 (m, 5H), 7.55–7.19 (m, 5H), 5.56 (d, 1H, J = 12.28 Hz).

*5.6.16. 5-(4-Bromophenyl)-penta-2,4-dienoic acid (tetrahydropyran-2-yloxy)-amide (**9**)*

A solution of 700 mg (2.5 mmol) of 5-(4-bromophenyl)-penta-2,4-dienoic acid ethyl ester [22] and 293 mg (2.5 mmol) of *O*-tetrahydropyranylhydroxylamine in 33 mL of anhydrous THF was cooled to −78 °C, then treated with 5.12 mL (5.25 mmol) of lithium hexamethyldisilazane. The mixture was stirred for 40 min under nitrogen, then was quenched with NH_4Cl saturated solution. Once at room temperature, the product was extracted with ethyl acetate (3 × 30 mL) and the organic phase was dried over Na_2SO_4. The solvent was evaporated under reduced pressure to afford the title compound as a yellow solid. Yield 96%. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.23 (s, 1H), 7.60–7.47 (m, 4H), 7.33–7.04 (m, 2H), 6.96 (d, 1H, J = 15.26 Hz), 6.06 (d, 1H, J = 15.26 Hz), 4.87 (br s, 1H), 4.01–3.86 (m, 1H), 3.58–3.47 (m, 1H), 1.75–1.62 (m, 3H), 1.60–1.46 (m, 3H).

5.6.17. E,E-*5-Biphenyl-4-yl-penta-2,4-dienoic acid* N-*hydroxyamide (**10a**)*

5-Biphenyl-4-yl-penta-2,4-dienoic acid [23] was condensed with hydroxylamine hydrochloride (method D, HBTU) to afford the title compound. Yield 31%, mp 178–180 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.75 (s, 1H), 9.00 (s, 1H), 7.75–7.60 (m, 6H), 7.50–7.45 (m, 2H), 7.40 (m, 1H), 7.40–6.90 (m, 3H), 6.02 (s, 1H, J = 14.89 Hz). Anal. $C_{17}H_{15}NO_2$ (C, H, N).

5.6.18. E,E-*5-(4′-Methoxybiphenyl-4-yl)-penta-2,4-dienoic acid* N-*hydroxyamide (**10b**)*

5-(4-Bromophenyl)-penta-2,4-dienoic acid (tetrahydropyran-2-yloxy)-amide **9** was reacted with 4-methoxybenzeneboronic acid (method B) to give, after flash chromatography (ethyl acetate/hexane 70:30) 5-(4′-methoxybiphenyl-4-yl)-penta-2,4-dienoic acid (tetrahydropyran-2-yloxy)-amide as a yellow solid. Yield 34%, mp 137–139 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.41 (br s, 1H), 7.59–7.41 (m, 6H), 7.29 (d, 1H, J = 8.56 Hz), 6.96–6.79 (m, 4H), 6.04 (d, 1H, J = 15.26 Hz), 5.08–4.87 (m, 1H), 4.05–3.90 (m, 1H), 3.84 (s, 3H), 3.71–3.56 (m, 1H), 1.94–1.75 (m, 3H), 1.71–1.50 (m, 3H). This compound was treated according to method E to afford the title compound. Yield 58%, mp 200–202 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.73 (s, 1H), 8.96 (s, 1H), 7.73–7.53 (m, 6H), 7.25 (dd, 1H, J = 14.89, 10.42 Hz), 7.02–6.97 (m, 4H), 6.01 (d, 1H, J = 14.89 Hz), 3.80 (s, 3H). Anal. $C_{18}H_{17}NO_3$ (C, H, N).

5.6.19. E,E-*5-(4′-Cyano-biphenyl-4-yl)-penta-2,4-dienoic acid* N-*hydroxyamide (**10c**)*

5-(4-Bromophenyl)-penta-2,4-dienoic acid (tetrahydropyran-2-yloxy)-amide **9** was reacted with 4-cyanobenzeneboronic acid (method B) to give, after flash chromatography (ethyl acetate/hexane 60:40), 5-(4′-cyanobiphenyl-4-yl)-penta-2,4-dienoic acid (tetrahydropyran-2-yloxy)-amide as a yellow solid. Yield 42%, mp 193–195 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.22 (s, 1H), 7.79–7.64 (m, 4H), 7.63–7.46 (m, 5H), 6.97–6.88 (m, 2H), 6.05 (br s, 1H), 5.04–4.90 (m, 1H), 4.02–4.89 (m, 1H), 3.71–3.60 (m, 1H), 1.93–1.77 (m, 3H), 1.71–1.57 (m, 3H). This compound was treated according to method E to give the title compound. Yield 56%, mp 202–205 °C dec. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.75 (s, 1H), 8.99 (s, 1H), 8.05–7.86 (m, 4H), 7.79 (d, 2H, J = 8.19 Hz), 7.69 (d, 2H, J = 8.19 Hz), 7.35–7.10 (m, 2H), 7.01 (d, 1H, J = 14.51 Hz), 6.05 (d, 1H, J = 14.14 Hz). Anal. $C_{18}H_{14}N_2O_2$ (C, H, N).

5.6.20. 5-(Biphenyl-4-yl)-pentanoic acid N-*hydroxyamide (**11**)*

5-Biphenyl-4-yl-pentanoic acid [24] was condensed with of hydroxylamine hydrochloride (method D, HBTU) to afford the title compound. Yield 62%, mp 148–151 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.37 (s, 1H), 8.70 (s, 1H), 7.65 (d, 2H, J = 8.34 Hz), 7.57 (d, 2H, J = 7.44 Hz), 7.47 (t, 2H, J = 7.44 Hz), 7.36 (d, 1H, J = 7.44 Hz), 7.27 (d, 2H, J = 8.34 Hz), 2.65–2.55 (m, 2H), 2.02–1.90 (m, 2H), 1.66–1.45 (m, 4H). Anal. $C_{17}H_{19}NO_2$ (C, H, N).

5.6.21. N-*hydroxy*-E-*3-[4′-Hydroxybiphenyl-3-yl]-acrylamide (**13**)*

Methyl *E*-3-bromophenylcinnamate was reacted with 4-hydroxybenzeneboronic acid according to method B and the product was purified by flash chromatography (hexane/ethyl acetate 85:15) to afford methyl *E*-3-(4′-hydroxybiphenyl-3-yl)-acrylate as a pale yellow solid. Yield 25%, mp 128–129 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.73 (d, 1H, J = 15.6 Hz), 7.66 (s, 1H), 7.57–7.38 (m, 5H), 6.91 (d, 2H, J = 8.2 Hz), 6.48 (d, 1H, J = 15.6 Hz), 3.81 (s, 3H). The above ester was hydrolyzed according to method C to give *E*-3-(4′-hydroxybiphenyl-3-yl)-acrylic acid. Yield 64%, mp 229–230 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.86 (s, 1H), 7.70–7.62 (m, 5H), 7.45 (t, 1H, J = 8.56 Hz), 6.86 (d, 2H, J = 8.19 Hz), 6.63 (d, 1H, J = 15.63 Hz). The acid was condensed with hydroxylamine hydrochloride according to method D (HBTU) to give, after flash chromatography on reverse phase (CH_2Cl_2/MeOH 95:5), the title compound as a white solid. Yield 23%, mp 127–128 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.73 (s, 1H), 9.59 (s, 1H), 9.05 (s, 1H), 7.74 (s, 1H), 7.65–7.37 (m, 6H), 6.85 (d, 2H, J = 8.19 Hz), 6.53 (d, 1H, J = 15.63 Hz). Anal. $C_{15}H_{13}NO_3$ (C, H, N).

5.6.22. N-*Hydroxy*-E-*3-[4-(4-hydroxyphenyl)-cyclohexyl]-acrylamide (**17**)*

To a solution of 4-(4-hydroxyphenyl)-cyclohexylaldehyde [25] (204 mg, 1 mmol) in 10 mL of THF was added 501 mg (1.5 mmol) of methyl(triphenylphosphoranylidene)acetate, and the mixture refluxed 4 h. Adding water (1 mL), extracting with AcOEt (5 mL) and flash chromatography with hexane/AcOEt 85:15 gave 214 mg (82%) of ethyl *E*-3-[4-(4-hydroxyphenyl)-cyclohexyl]-acrylate (**15**). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.10 (d, 2H, J = 8.3 Hz), 6.85 (dd, 1H, J = 7.0, 15.6 Hz), 6.73 (d, 2H, J = 8.3 Hz), 5.77 (d, 1H, J = 15.6 Hz), 3.72 (s, 3H, OMe), 2.5–1.2 (10H), and 5% of the *Z* diastereoisomer. This ester was hydrolyzed (method C) to give the corresponding acid (**16**). Yield 81%, mp 222–225 °C. ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.04 (d, 2H, J = 8.34 Hz), 6.91 (dd, 1H, J = 7.03, 15.59 Hz), 6.74 (d, 2H,

$J = 8.34$ Hz), 5.80 (d, 1H, $J = 15.59$ Hz), 2.51–2.33 (m, 1H), 2.32–2.14 (m, 1H), 1.93–1.75 (m, 4H), 1.60–1.41 (m, 2H), 1.40–1.23 (m, 2H). The above acid was condensed with hydroxylamine hydrochloride (method D, HBTU) to give the title compound. Yield 13%, mp 182–185 °C. ^{1}H NMR (300 MHz, acetone-d_6) δ: 10.10 (br s, 1H), 8.04 (br s, 1H), 7.05 (d, 2H, $J = 7.72$ Hz), 6.84–6.69 (m, 3H), 5.84 (d, 1H, $J = 15.44$ Hz), 2.50–2.33 (m, 1H), 2.28–2.11 (m, 1H), 1.96–1.76 (m, 4H), 1.61–1.41 (m, 2H), 1.40–1.31 (m, 2H). Anal. $C_{15}H_{19}NO_3$ (C, H, N).

5.6.23. N-*Hydroxy-3-[4-(4-hydroxyphenyl)-cyclohexyl]-propionamide (**18**)*

3-[4-(4-Hydroxyphenyl)-cyclohexyl]-acrylic acid (38 mg, 0.157 mmol) was dissolved in 1.5 mL of MeOH, then a catalytic amount of Pd/C 10% was added and the mixture was hydrogenated for 40 min. After filtration of the catalyst, the solvent was evaporated to obtain 30 mg of 3-[4-(4-hydroxyphenyl)-cyclohexyl]-propionic acid. Yield 80%, mp 178–183 °C. ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.01 (d, 2H, $J = 8.54$ Hz), 6.71 (d, 2H, $J = 8.54$ Hz), 2.45–2.17 (m, 5H), 1.92–1.67 (m, 5H), 1.67–0.95 (m, 4H). The above acid was condensed with hydroxylamine hydrochloride (method D, HBTU) to give the title compound. Yield 35%, mp 149–152 °C. ^{1}H NMR (300 MHz, acetone-d_6) δ: 9.99 (br s, 1H), 8.20 (br s, 1H), 7.01 (d, 2H, $J = 8.54$ Hz), 6.71 (d, 2H, $J = 8.54$ Hz), 2.44–2.27 (m, 1H), 2.18–2.05 (m, 2H), 1.91–1.66 (m, 5H), 1.58–0.97 (m, 4H). Anal. $C_{15}H_{21}NO_3$ (C, H, N).

5.7. Molecular modelling

Three-dimensional molecular models of the designed compounds were built on a Silicon Graphics O2, using the programs Insight II and Discover (Accelrys Inc., San Diego, CA). Minimizations were performed with the AMBER all-atom forcefield [23] and the conjugate gradients algorithm. For atomic partial charges of the ligand atoms Mulliken charges calculated on the minimised structure using the MOPAC program [26] with the MNDO Hamiltonian were used. The model of the active site of HDAC2 was based on the homology model derived and validated by Wang et al. [16] using HDLP as a template. The model consists essentially of all amino acid residues within a radius of 12 Å around SAHA, inserted overlapping the histidines 142 and 143 in the catalytic pocket of the crystal structure of HDAC8 complexed with SAHA (PDB entry 1T69) [13a] with the corresponding histidines 141 and 142 in the HDAC2 model, and then deleting the structure of HDAC8. During energy minimization the backbone of the amino acids of the site model was frozen, while the rest was allowed to relax. The intermolecular interaction energy between the appropriate compound and the HDAC2 model, after energy minimization of the complex, was obtained using the docking module in Insight II, where the nonbonding energy, including Van der Waals and electrostatic energy, is calculated.

5.8. HDAC2 assay

HDAC2 was immunoprecipitated from HeLa cells. Whole cell lysates were obtained by lysing the cells in a buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 1 mM PMSF, protease inhibitors cocktail (Roche). Lysates were clarified by centrifugation (12,000 × *g*) for 10 min at 4 °C and were diluted with TBST (20 mM Tris–HCl pH 7.5, 150 mM NaCl and 0.1% Tween 20) containing 1 mM PMSF. Purified IgG from rabbit antisera to HDAC2 (Santa Cruz Biotechnology sc-7899) were then added and immune complexes allowed to form for 1 h at 4 °C. Protein A-Sepharose (10 μL of settled beads) were added and the components mixed on a rotor overnight at 4 °C. Immune complexes were collected by centrifugation and washed with cold TBST. HDAC2 activity was assayed with a pan-HDAC substrate (KI-104; Biomol Research Laboratories Inc., PA, USA) and the reaction was carried out in half-volume white 96-well plates. The assay components were incubated at 37 °C for 40 min. The reaction was quenched with the addition of 50 μL HDAC-FDL Developer (KI-105, Biomol) 20× stock diluted in KI-143 buffer with 2 μM TSA. The plates were incubated 30 min at room temperature to allow the fluorescent signal to develop. The fluorescence generated was monitored at 355/460 nm (excitation/emission) wavelength.

5.9. Cellular sensitivity to drugs

Cellular sensitivity to drugs was evaluated by growth-inhibition assay after 72-h drug exposure. Cells in the logarithmic phase of growth were seeded in duplicate into 6-well plates. Twenty-four hours after seeding, the drug was added to the medium. Cells were harvested 72 h after drug exposure and counted with a cell counter. IC_{50} is defined as the drug concentration causing a 50% reduction of cell number compared with that of untreated control.

5.10. Cell cycle analysis

The cell cycle distribution was analyzed in propidium iodide-stained cells by FACScan flow cytometry, as described [27].

5.11. TUNEL assay

Apoptosis was determined in ovarian carcinoma IGROV-1 cells by TUNEL assay following 72-h exposure to the drug and fixed in 4% paraformaldehyde for 45 min, at room temperature. The in situ cell death detection kit fluorescein (Roche, Mannheim, Germany) was used according to manufacturers instructions. Samples were analyzed by flow cytometry (Becton Dickinson).

5.12. Western blotting

Cells treated with various concentrations of HDAC inhibitors for 4 h were collected and lysed as previously described [19].

5.13. Tumor models and evaluation of antitumor activity

The experiments were performed using female athymic Swiss nude mice. Mice were maintained in laminar flow rooms keeping temperature and humidity constant. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to institutional guidelines.

Exponentially growing tumor cells (10^7 cells/mouse) were s.c. injected into the right flank of athymic nude mice. Tumor lines were achieved by serial s.c. passages of fragments (about 2 × 2 × 6 mm) from growing tumors into healthy mice. Animals were treated 3 days after tumor implantation. Compounds were dissolved in DMSO and diluted in PBS containing 5% Cremophor to a final concentration of 10% DMSO.

Acknowledgements

This work was partially supported by Associazione Italiana per la Ricerca sul Cancro, Ministero della Salute, and by Fondazione CARIPLO, Milan, Italy.

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