Influence of the adamantyl moiety on the activity of biphenylacrylohydroxamic acid-based HDAC inhibitors

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Abstract

To investigate the influence of the adamantyl group on the biological properties of known HDAC inhibitors with a 4-phenylcinnamic skeleton, a series of compounds having the adamantyl moiety in the cap structure were synthesized and compared to the corresponding hydroxamic acids lacking this group. An unexpected finding was the substantial reduction of inhibitory activity toward the tested enzymes, in particular HDAC6, following the introduction of the adamantyl group. In spite of the reduced ability to function as HDAC inhibitors, the compounds containing the adamantane still retained a good efficacy as antiproliferative and proapoptotic agents. A selected compound (**2c**; ST3056) of this series exhibited an appreciable antitumor activity against the colon carcinoma xenograft HCT116.

1. Introduction

In a recent study [1] we have reported novel structurally simple histone deacetylase (HDAC) inhibitors with a hydroxamic acid as a zinc chelating head group, a cinnamic linker domain and an aromatic ring as a cap structure (**1**, Chart 1). The compounds were designed on the basis of a model of the HDAC2 binding site, based on the homology model derived and validated by Wang et al.[2]. Our modelling studies indicated that the proximal phenyl ring of prototype compound **1a** (R = H) exhibited a π–π stacking interaction with the benzyl side chain of Phe151, Phe206 and Tyr304 of HDAC2. The distal phenyl ring of **1** (cap structure) appeared to accommodate in a large cavity,

without any significant steric clash. In vitro assay with the isoenzyme HDAC2 validated the model, confirming that a variety of substitutions in the distal ring were well tolerated [1].

Chart 1

Therefore, in order to produce highly potent and/or receptor-selective HDAC inhibitors, we mainly concentrated our efforts at modifying the cap portion of the HDAC pharmacophore, i.e. the distal phenyl ring of **1**.

Among various substituents a growing interest in adamantyl derivatives is gaining prominence, since the incorporation of this moiety into various molecules resulted in compounds with modified and/or improved biological availability [3].

The advantages of introducing this bulky group mainly reside in an enhancement of lipophilicity and a protection from metabolic cleavage of functional groups in its proximity (e.g. a phenolic OH), thus enhancing the duration of action. On the other hand, the adamantyl group being "biocompatible", since metabolism can take place in the liver, toxic effects by accumulation upon chronic treatment are not expected [4]. Most adamantyl-based drugs are, in fact, excreted largely unmodified, presenting therefore the added benefit that potential side effects arising from bioactive metabolites are intrinsically improbable [5].

Seven adamantyl -derived drugs are available in the market: Amantadine for use both as antiviral and antiparkinsonian drug [6]; Memantine, a NMDA-type glutamate receptors inhibitor [7], active in Alzheimer's disease; Rimantadine and Tromantadine as antiviral drugs [8]; Vildagliptin and Saxagliptin, as oral anti-diabetic drugs [9]; and Adapalene, a retinoid for topic use against acne [10] (Chart 2).

Chart 2

After the discovery of amantadine as an antiviral drug [11], the exploitation of the properties of the bulky, apolar adamantyl group has been pioneered in a series of papers from Gerzon et al. [12], followed by a large number of studies on compounds for diverse medicinal applications [3][4].

Interestingly, in a recent paper from Gopalan et al. [13], adamantyl-based HDAC inhibitors were found to exhibit cytotoxicity in the nanomolar range.

On the basis of these considerations, we selected some of the most active compounds among our previously synthesized HDAC inhibitors [1] (compounds **1a-d**), and prepared the corresponding analogues **2a-d**, having the adamantyl moiety in the cap structure (Scheme 1).

Scheme 1

2. Results and discussion

2.1. Chemistry

Compounds **1a-d** were synthesized according to the procedures described previously [1]. Compounds **2a-d** were prepared by reacting the corresponding carboxylic acids [14] with hydroxylamine using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (WSC) and 1-hydroxybenzotriazole hydrate (HOBt) as coupling agents (**2a-c**) or from the corresponding esters via reaction with *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine ($THPONH_2$), followed by hydrolysis with *p*-toluene sulfonic acid monohydrate (PTSA) (**2d**) (Scheme 2).
Successively, a series of derivatives with different substituents on the phenolic oxygen (**2e-h**) and with a naphthalene instead of a cinnamic spacer (**2i-l**) (Scheme 3) were synthesized following standard coupling procedures.

Scheme 2
Scheme 3

2.2. Biological assays

To understand the role of the adamantyl group as a determinant of the antitumor activity, we performed a comparative study of compounds containing or lacking this group (i.e. **1a-d** vs **2a-d**).
The compounds were tested for their inhibitory activity towards HDAC2 isoform and for antiproliferative activities against a panel of tumor cell lines from different tissue origin: H460 (human lung carcinoma cell line), HCT116 (human colon cancer cell line), IGROV-1 and its subline resistant to cis-platinum IGROV-1/Pt1 (human ovarian carcinoma cell lines)
Derivatives **2a-d** showed inhibitory activities in the HDAC2 assay lower than the corresponding compounds lacking the adamantyl group. However, compounds **2a-d** exhibited significant antiproliferative profile against H460 cell line, **2b-d** showing higher activity than the corresponding compounds **1b-d**. A comparable effect between the two series was found against HCT116 and IGROV-1 cell lines. The growth-inhibitory activities were apparently p53-independent, because the cellular effects were comparable in cells with functional (IGROV-1) or p53 defective (IGROV-1/Pt1). SAHA, used as reference compound, exhibited similar antiproliferative effects under the same treatment conditions (72 h exposure) (Table 1).

Table 1

A series of derivatives with different substituents on the phenolic oxygen (**2e-h**) and with a naphthalene instead of a cinnamic spacer (**2i-l**) (Scheme 3) were also synthesized and tested for antiproliferative activity. (Table 2)

Table 2

Interestingly, these compounds also exhibited a significant antiproliferative profile on all the cell lines, in spite of a low inhibitory activity in the HDAC2 assay ($IC_{50} > 5\mu M$).

In order to unveil whether compounds **2** were active on other HDAC isoforms, a comparative study on the different HDAC isoforms was also carried out (Table 3).

Compounds lacking the adamantyl group, e.g. **1b** and **1c**, exhibited preferential inhibition of HDAC6 (IC_{50} ca. 40 nM), an effect consistent with tubulin acetylation. The introduction of the adamantylmoiety reduced the inhibitory activity against all the isoforms, including HDAC6 and HDAC8.

These findings confirmed the detrimental effect of the adamantly group on the HDAC inhibitory activity for this class of compounds.

Table 3

In light of the promising results obtained from the antiproliferative activity evaluation, further studies on selected compounds were carried out.

The ability of compounds **1c**,**d** vs **2c**,**d** to induce apoptosis was investigated in IGROV-1 cells following 72 h exposure to equitoxic concentrations, i.e. drug concentrations (deduced from dose-response curve) which produced 80% cell growth inhibition (IC_{80}). As shown in figure 1, all compounds were able to induce high levels of apoptosis (i.e. superior to that obtained with SAHA). Compounds lacking the adamantyl group (i.e. **1c** and **1d**) were more effective as apoptosis inducers than the analogues containing the adamantly moiety. The former compounds induced apoptotic cell death in 90% of treated cells.

Figure 1

As shown in figure 2, compound **1c** was found to be a potent inducer of acetylation of tubulin, an effect consistent with a preferential inhibition of HDAC6 isoform [15], even if to a lesser extent with expect of SAHA. A specific feature of these compounds was their ability to induce an early acetylation of p53. Conversely, compounds **2c** and **2d** exhibited a complete loss of ability to induce acetylation of tubulin and histone H4 at equitoxic doses, IC_{80} (Figure 2). This observation is consistent with the reduced potency of compounds **2** as HDAC inhibitors.

Figure 2

Since compound **2c** is structurally related to the atypical retinoid containing the adamantly group **7** [16], which is known to induce DNA damage as detected by the cellular response (i.e. activation of p53 and induction of phosphorylation of H2AX histone), we examined the markers of DNA damage response in IGROV-1 cells (Figure 3).

In contrast to the atypical retinoid, which induces an early phosphorylation of γ-H2Ax, RPA-2 cleavage and p53 phosphorylation (at Ser15) [16], **2c** did not exhibit the same profile, phosphorylation of p53 being detected only.

Figure 3

In spite of the different pattern on protein acetylation, all compounds exhibited a comparable cell-cycle perturbation with evidence of a marked sub-G1 peak and cell accumulation in G1 (Figure 4).

Figure 4

On the basis of the above results, the therapeutic potential of a representative derivative as an anticancer agent was analysed.

In a previous study we have reported the good antitumor activity of **1c** following oral administration in a panel of human tumor xenografts [1]. Therefore, we evaluated the efficacy of **2c** against the human colon carcinoma model HCT116 under similar treatment conditions. A daily oral administration (5 day/week) for five weeks produced a significant tumor growth inhibition (60-70%) at the end of treatment without manifestation of toxicity. The antitumor activity of **2c** was comparable to that of the analogue lacking the adamantly group (**1c**) (Figure 5). The good tolerability profile allowed prolonged treatment in order to achieve optimal antitumor response.

Figure 5

3. Conclusions

The present study was designed to investigate the influence of the adamantyl group on the biological properties of known HDAC inhibitors with a 4-phenylcinnamic skeleton [1].

Indeed, hydrophobic bulky groups in the cap region of HDAC inhibitors have been reported to confer a lipophilic nature and some isoform selectivity to the parent compounds. An unexpected finding of our study was the substantial reduction of inhibitory activity toward the tested enzymes, in particular HDAC6, following the introduction of the adamantyl group. This was consistent with a marginal (if any) ability of the modified compounds to induce acetylation of tubulin (Fig. 2). In spite of the reduced ability to function as HDAC inhibitors, the compounds containing the adamantly moiety still retain a good efficacy as antiproliferative and proapoptotic agents.

Indeed, a selected compound of this series (**2c**; ST3056) exhibited an appreciable antitumor activity against colon carcinoma xenograft HCT116. Compound **2c** was found to induce a dose-dependent phosphorylation of histone H2AX but this effect, observed after 24h exposure, could reflect the onset of apoptosis. It should be emphasized that zinc-dependent proteins other than HDACs may be targeted by hydroxamic-acid-based compounds, but the biological relevance of off-target effects remains to be explored.

Notwithstanding the fact that the molecular mechanism of action of the reported compounds remains to be defined, it has been confirmed that the presence of the adamantly moiety sustains the antiproliferative effect found for the unsubstituted series of hydroxamic acids. While a better understanding of the mechanism of action will provide a basis for rational design of antitumor agents, structure-activity information inferred from our study could be the starting point for their optimization.

4. Experimental section

4.1 Chemistry

All reagents and solvents were of 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. 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. Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and diethyl ether (Et_2O) were

obtained by distillation from sodium-benzophenone ketyl; dry methylene chloride was obtained by distillation from phosphorus pentoxide. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and all glassware were oven dried and/or flame dried. Isolation and purification of the compounds were performed by flash column chromatography on silica gel 60 (230-400 mesh) or RP-18 silica gel. Analytical thin-layer chromatography (TLC) was conducted on Fluka TLC plates (silica gel 60 F_{254}, aluminum foil).

Compounds **4** [16], **5**[14], **7**[16], **10a** [16], **10b** [14], **11a-b** [17], and **15** [18] were obtained following the procedures reported in the literature.

*4.2. General procedure for the synthesis of compounds **2a-c***

To a suspension of the appropriate acid (0.53 mmol) in dry DMF (8.3 mL) HOBt (86 mg, 0.64 mmol) and WSC (132 mg, 0.69 mmol) were added. The mixture was stirred at room temperature under nitrogen for 1.5-24 h, then $NH_2OH \cdot HCl$ (185 mg, 2.66 mmol) and TEA (368 µL, 2.66 mmol) were added. The reaction was stirred at room temperature for 1-4 h. After addition of water, a precipitate formed, which was filtered and dried to obtain the desired hydroxamic acid.

*4.2.1. 3-(3'-Adamantan-1-yl-biphenyl-4-yl)-N-hydroxyacrylamide (**2a**).*

Stirred for 1.5h, then 1h after the addition of hydroxylamine. Yield: 33%. M.p. 132-134 °C. ^{1}H NMR (DMSO-d_6) δ: 9.10 (1H, brs); 7.75-7.35 (9H, m); 6.51 (1H, d, J = 16.1 Hz); 2.10 (3H, s); 1.95 (6H, s); 1.74 (6H, s). Anal. Calcd for $C_{25}H_{27}NO_2$: C, 80.40; H, 7.29; N, 3.75. Found: C, 80.62; H, 7.32; N, 3.80.

*4.2.2. 3-(3'-Adamantan-1-yl-4'-hydroxybiphenyl-4-yl)-N-hydroxyacrylamide (**2b**).*

Stirred for 3h, then 4h after the addition of hydroxylamine. The crude precipitate was purified by flash chromatography with CH_3OH/H_2O 75:25 v/v on RP-18 silica gel. Yield: 54%. M.p. 184 °C. ^{1}H NMR (DMSO-d_6) δ: 10.70 (1H, brs); 9.50 (1H, s); 9.01 (1H, brs); 7.65-7.50 (4H, m); 7.45 (1H, d, J = 16.2 Hz); 7.35-7.25 (2H, m); 6.82 (1H, d, J = 8.8 Hz); 6.45 (1H, d, J = 16.2 Hz); 2.12 (6H, s); 2.04 (3H, s); 1.72 (6H, s). ^{13}C NMR (DMSO-d_6) δ: 162.9, 156.4, 141.9, 138.1, 136.0, 132.7, 129.9, 128.1, 126.4, 124.9, 124.7, 118.3, 117.0, 38.7, 36.7, 36.3, 28.4. Anal. Calcd for $C_{25}H_{27}NO_3$: C, 77.09; H, 6.99; N, 3.60. Found: C, 77.20; H, 6.93; N, 3.62.

*4.2.3. 3-(3'-Adamantan-1-yl-4'-methoxybiphenyl-4-yl)-N-hydroxyacrylamide (**2c**).*

Stirred for 24 h, then 4h after the addition of hydroxylamine. The solid obtained was filtered and washed with acetone. Yield: 75%. ^{1}H NMR (DMSO-d_6) δ: 10.72 (1h, brs); 9.03 (1H, brs); 7.70-7.39 (7H, m); 7.07 (1H, d, J = 8.6 Hz); 6.47 (1H, d, J = 16.0 Hz); 3.84 (3H, s); 2.10 (6H, s); 2.05 (3H, s); 1.74 (6H, s). ^{13}C NMR (DMSO-d_6) δ: 162.8, 158.5, 141.5, 137.9, 133.1, 131.4, 128.1, 126.6, 125.2, 124.6, 118.6, 112.7, 55.4, 36.6, 28.4. Anal. Calcd for $C_{26}H_{29}NO_3$: C, 77.39; H, 7.24; N, 3.47. Found: C, 77.51; H, 7.27; N, 3.41.

*4.3. 5-(3'-Adamantan-1-yl-4'-methoxybiphenyl-4-yl)penta-2,4-dienoic acid (tetrahydropyran-2-yloxy)amide (**9**)*

To a solution of **6** (68 mg, 0.21 mmol) in dioxane (1.5 mL) bis(pinacolato)diboron (59 mg, 0.23 mmol), KOAc (62 mg, 0.63 mmol) and $PdCl_2$(dppf) (5 mg, 0.006 mmol) were added. The mixture was refluxed for 1.45 h under nitrogen. The solution was cooled at room temperature, then compound **8** (144 mg, 0.41 mmol), 2M Na_2CO_3 (270 µL, 0.53 mmol) and $PdCl_2$(dppf) (5 mg, 0.006 mmol) were added. The resulting mixture was refluxed for 4 h. 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 which was purified by flash chromatography (hexane /ethyl acetate 60:40) to obtain 91 mg of **9**. Yield: 86%. M. p. 190-192 °C.

^{1}H NMR (DMSO-d_6) δ: 11.20 (1H, brs); 7.69-7.60 (4H, m); 7.55 (1H, m); 7.43 (1H, d, J = 1.9 Hz); 7.30 (1H, m); 7.12-7.00 (3H, m); 6.05 (1H, d, J = 16.1 Hz); 4.90 (1H, m); 3.92 (3H, s); 3.55 (2H, m); 2.12 (6H, s); 2.05 (3H, s); 1.75 (6H, s); 1.72 (2H, m); 1.58 (4H, m). Anal. Calcd for $C_{33}H_{39}NO_4$: C, 77.16; H, 7.65; N, 2.73. Found: C, 77.01; H, 7.69; N, 2.78.

*4.4. 5-(3'-Adamantan-1-yl-4'-methoxybiphenyl-4-yl)penta-2,4-dienoic acid hydroxyamide (**2d**).*

To a solution of compound **9** (50 mg, 0.1 mmol) in methanol (1.5 mL) p-toluenesulfonic acid monohydrate (PTSA) (6 mg, 0.03 mmol) was added and the mixture was stirred at room temperature for 4 h. The solid formed was filtered and dried to give 19 mg of compound **2d**. Yield: 44%. M.p. 242-244 °C (dec). ^{1}H NMR (DMSO-d_6) δ: 10.71 (1H, s); 8.95 (1H, s); 7.65-7.59 (4H, m); 7.55 (1H, dd, J = 8.8, 1.9 Hz); 7.42 (1H, d, J = 1.9 Hz); 7.25 (1H, m); 7.15-6.90 (3H, m); 6.02 (1H, d, J = 16 Hz),; 3.85 (3H, s); 2.12 (6H, s); 2.05 (3H, s); 1.75 (6H, s). ^{13}C NMR (DMSO-d_6) δ: 162.9, 158.4, 140.5, 139.0, 137.9, 137.7, 134.6, 131.5, 127.6, 126.7, 126.5, 125.1, 124.5, 122.1, 112.7, 55.3, 36.6, 28.4. Anal. Calcd for $C_{28}H_{31}NO_3$: C, 78.29; H, 7.27; N, 3.26. Found: C, 78.11; H, 7.23; N, 3.22.

*4.5. 3-[4-(8-Adamantan-1-yl-2,3-dihydrobenzo[1,4]dioxin-6-yl)-phenyl]-N-hydroxyacrylamide (**2e**)*

A solution of HATU (55 mg, 0.14 mmol), acid **10a** (60 mg, 0.14 mmol) and DIPEA (0.05 mL, 0.23 mmol) was stirred for 2 min under nitrogen. $NH_2OH{\cdot}HCl$ (40 mg, 0.58 mmol) was added and the resulting solution was stirred at room temperature overnight. The solvent was evaporated, water was added and the suspension was stirred 1 h at room temperature. The white solid formed was filtered. Yield: 65% M.p. 211-213 °C. ^{1}H NMR (DMSO-d_6) δ: 10.75 (1H, s); 9.04 (1H, s); 7.68-7.52 (4H, m); 7.47 (1H, d, *J* = 16.0 Hz); 7.03 (1H, d, *J* = 2.1 Hz); 7.00 (1H, d, *J* = 2.1 Hz); 6.47 (1H, d, *J* = 16.0 Hz); 4.27 (4H, s); 2.10 (6H, s); 2.05 (3H, s); 1.74 (6H, s). ^{13}C NMR (DMSO-d_6) *δ*: 162.8, 144.0, 142.7, 141.2, 138.7, 138.0, 133.3, 131.6, 128.0, 126.7, 118.6, 116.6, 113.2, 63.7, 63.5, 36.7, 36.5, 28.4. Anal. Calcd for $C_{27}H_{29}NO_4$: C, 75.15; H, 6.77; N, 3.25. Found: C, 75.25; H, 6.73; N, 3.21.

*4.6. 3-(3'-Adamantan-1-yl-2-chloro-4'-hydroxybiphenyl-4-yl)-N-hydroxyacrylamide (**2f**)*

Prepared from **10b** following the procedure described for **2e**. Yield 26%. M.p. 160 °C. ^{1}H NMR (DMSO-d_6) δ: 10.77 (1H, s); 9.58 (1H, s); 9.10 (1H, s); 7.71 (1H, d, *J* = 1.8 Hz); 7.56 (1H, d, *J* = 8.2 Hz); 7.51-7.36 (2H, m); 7.20-7.13 (2H, m); 6.84 (1H, d, *J* = 8.2 Hz); 6.51 (1H, d, *J* = 16.0 Hz); 2.09 (6H, s); 2.02 (3H, s), 1.72 (6H, s). ^{13}C NMR (DMSO-d_6) *δ*: 167.7, 156.2, 141.8, 141.5, 135.1, 134.5, 131.8, 129.5, 128.4, 127.5, 126.8, 120.8, 116.1, 36.6, 36.3, 28.4. Anal. Calcd for $C_{25}H_{26}ClNO_3$: C, 70.83; H, 6.18; N, 3.30. Found: C, 70.66; H, 6.13; N, 3.33.

*4.7. 4-{2-[3-Adamantan-1-yl-4'-(2-hydroxycarbamoylvinyl)-biphenyl-4-yloxy]ethyl}-morpholin-4-ium toluene-4-sulfonate (**2g**)*

A solution of **11a** (153 mg, 0.3 mmol), THP-ONH_2 (36 mg, 0.3 mmol), 1.06 M LiHMDS (0.6 mL) in dry THF (4 mL) was stirred at -78 °C for 2h. After warming to room temperature, water was added and the aqueous phase was extracted with ethyl acetate. (3 x 5mL). The collected organic layers were dried and evaporated to give 3-[3'-adamantan-1-yl-4'-(2-morpholin-4-yl-ethoxy)biphenyl-4-yl]-N-(tetrahydropyran-2-yloxy)acrylamide as a white solid. Yeld 98%. M.p. 139-141 °C.

A suspension of the above compound (176 mg, 0.3 mmol) and PTSA (56 mg, 0.3 mmol) in MeOH (25 mL) was stirred at room temperature for 24 h. The solvent was evaporated and the residue was washed with ethyl acetate and water to give the title compound as a white solid. Yield 63%. ^{1}H NMR (DMSO-d_6) δ: 10.77 (1H, s); 10.16 (1H, s); 9.05 (1H, s); 7.74-7.40 (9H, m); 7.21-7.06 (3H, m); 6.48 (1H, d, *J* = 16.0 Hz); 4.52-4.32 (2H, m); 4.17-3.89 (2H, m); 3.86-3.49 (4H, m), 2.28 (3H, s); 2.08 (9H, s), 1.76 (6H, s). ^{13}C NMR (DMSO-d_6) *δ*: 162.8, 157.0, 145.6, 141.2, 138.2, 138.0,

137.7, 133.2, 132.2, 128.1, 126.7, 125.5, 125.2, 124.9, 118.7, 114.0, 63.9, 55.4, 52.2, 36.6, 36.5, 28.4, 20.8.

*4.8. 3-(3'-Adamantan-1-yl-4'-cyanomethoxybiphenyl-4-yl)-N-hydroxyacrylamide (**2h**)*

A solution of compound **11b** (240 mg, 0.56 mmol) and $LiOH \cdot H_2O$ (117 mg, 2.8 mmol) in THF:H_2O 1:1 v/v (24 mL) was stirred overnight at room temperature. THF was evaporated and the aqueous phase was acidified with 1N HCl to pH 1. The precipitate was filtered to give 3-(3'-adamantan-1-yl-4'-cyanomethoxybiphenyl-4-yl)acrylic acid as a light brown solid. Yield 93%. M.p. 333-334 °C. The above compound was coupled with hydroxylamine hydrochloride following the general procedure described for the synthesis of compounds **2a-c** to give compound **2h** as a white solid. Yield 33%. M.p. 172-173 °C. ^{1}H NMR (DMSO-d_6) δ: 9.35 (1H, s); 7.70-7.40 (6H, m); 7.32 (1H, d, *J* = 1.9 Hz); 6.98 (1H, d, *J* = 8.8 Hz); 6.49 (1H, d, *J* = 16.1 Hz); 4.52 (2H, s); 2.10 (6H, s), 2.05 (3H, s); 1.74 (6H, s). ^{13}C NMR (DMSO-d_6) *δ*: 169.7, 162.8, 157.1, 141.3, 138.3, 137.9, 133.2, 132.1, 128.1, 126.7, 125.2, 124.8, 118.6, 113.8, 67.4, 36.6, 36.5, 28.4. Anal. Calcd for $C_{27}H_{28}N_2O_3$: C, 75.68; H, 6.59; N, 6.54. Found: C, 75.75; H, 6.54; N, 6.57.

*4.9. 6-(3-Adamantan-1-yl-4-hydroxyphenyl)-naphthalene-2-carboxylic acid benzyloxyamide (**13**)*

To a solution of 6-bromo-naphthalene-2-carboxylic acid (154 mg, 0.61 mmol) in anhydrous THF (3 mL) at 0°C ethyl chloroformate (0.88 mL, 0.92 mmol) and triethylamine (0.138 mL, 0.99 mmol) were added. The resulting mixture was stirred for 10 min. then a solution of *O*-benzyl-hydroxylamine in CH_3OH (obtained from *O*-benzyl-hydroxylamine hydrochloride (147 mg, 0.92 mmol), KOH (52 mg, 0.92 mmol) in 0.8 mL of CH_3OH) was added. The reaction was stirred for 1 hour, the solvent was evaporated and the residue was washed with ethyl acetate and water. The crude was washed with diethyl ether to obtain 150 mg of 6-bromonaphthalene-2-carboxylic acid benzyloxyamide (**13**). Yield: 69%. M. p. 171 °C. ^{1}H NMR (DMSO-d_6) δ: 11.80 (1H, bs); 8.32 (1H, d, *J* = 1.9 Hz); 8.25 (1H, d, *J* = 1.9 Hz); 8.02-7.87 (2H, m); 7.38 (1H, d, *J* = 8.2 Hz); 7.67 (1H, m); 7.52-7.25 (5H, m); 4.92 (2H, s).

To a solution of **12** (123 mg, 0.40 mmol) in dioxane (2.4 mL) were added bis(pinacolato)diboron (112 mg, 0.44 mmol), $PdCl_2$(dppf) (8.8 mg, 0.012 mmol), KOAc (118 mg, 1.2 mmol) and the resulting solution was refluxed for 2h. After addition of **13** (285 mg, 0.8 mmol) and 2M Na_2CO_3 (0.5 mL), the solution was refluxed for further 2 h. Ethyl acetate (2 mL) was added and the organic phase was washed with water, 1M HCl (4 mL) and brine, then dried and evaporated. Purification by flash column chromatography (hexane: ethyl acetate 2:1) afforded compound **14** as a white solid. Yield 28%. M.p. 135-138°C. ^{1}H NMR (DMSO-d_6) δ: 11.90 (1H, bs); 9.57 (1H, bs); 8.33 (1H, d, *J* =

1.9 Hz); 8.14 (1H, d, *J* = 1.9 Hz); 8.12-7.97 (2H, m); 7.80-7.72 (2H, m); 7.63-7.28 (7H, m); 6.93 (1H, d, *J* = 8.19 Hz); 4.97 (2H, s); 2.46-1.98 (9H, m); 1.90-1.59 (6H, m). Anal. Calcd for $C_{34}H_{33}NO_3$: C, 81.08; H, 6.60; N, 2.78. Found: C, 81.29; H, 6.64; N, 2.76.

*4.10. 6-(3-Adamantan-1-yl-4-hydroxyphenyl)-naphthalene-2-carboxylic acid hydroxyamide (**2i**)*

Compound **14** (24 mg, 0.05 mmol) was hydrogenated for 36 h at room temperature in MeOH (5 mL) using 20% Pd/C as a catalyst (5 mg). The catalyst was filtered and the solvent evaporated. The crude was purified by flash chromatography (hexane:ethyl acetate 1:2 v/v, then MeOH) to give the title compound as a white solid . Yield 70%. M.p. 204 °C. ^{1}H NMR (DMSO-d_6) δ: 9.65 (1H, brs); 8.35 (1H, d, *J* = 1.9 Hz); 8.12 (1H, d, *J* = 1.9 Hz); 8.10-7.95 (2H, m); 7.88-7.76 (2H, m); 7.52-7.45 (2H, m); 6.95 (1H, d, *J* = 8.6 Hz); 2.19 (6H, s); 2.07 (3H, s); 1.75 (6H, s). ^{13}C NMR (DMSO-d_6) *δ*: 164.3, 156.4, 139.8, 136.1, 134.7, 130.8, 130.1, 129.5, 129.3, 128.1, 126.9, 125.8, 125.4, 125.2, 124.0, 123.6, 117.0, 36.7, 36.4, 28.4. Anal. Calcd for $C_{27}H_{27}NO_3$: C, 78.42; H, 6.58; N, 3.39. Found: C, 78.56; H, 6.53; N, 3.34.

*4.11. 6-(3-Adamantan-1-yl-4-methoxyphenyl)naphthalene-2-carboxylic acid hydroxyamide (**2l**)*

The title compound was obtained from **15** following the same procedure described for the synthesis of **2e**. The crude was purified by RP-18 silica gel chromatography (MeOH :H_2O 85:15 v/v). Yield 30%. M.p. 222 °C. ^{1}H NMR (DMSO-d_6) *δ*: 9.10 (1H, s); 8.35 (1H, d, *J* = 1.9 Hz); 8.19 (1H, d, *J* = 1.9 Hz); 8.10-8.00 (2H, m); 7.88 (1H, dd, *J* = 8.6, 1.9 Hz); 7.83 (1H, dd, *J* = 8.6, 1.9 Hz); 7.65 (1H, dd, *J* = 8.9, 1.9 Hz); 7.57 (1H, d, *J* = 1.9 Hz); 7.12 (1H, d, *J* = 8.6 Hz); 3.86 (3H, s); 2.14 (6H, s); 2.07 (3H, s); 1.76 (6H, s). ^{13}C NMR (DMSO-d_6) *δ*: 164.2, 158.5, 139.5, 138.0, 134.6, 131.6, 130.9, 129.7, 129.4, 128.2, 126.9, 125.9, 125.7, 125.0, 124.1, 123.6, 112.7, 55.4, 36.63, 36.59, 28.4. Anal. Calcd for $C_{28}H_{29}NO_3$: C, 78.66; H, 6.84; N, 3.28. Found: C, 78.81; H, 6.81; N, 3.25.

4.12. 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 x g) for 10 min at 4 ºC 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 4ºC. Protein A-Sepharose (10 µl of settled beads) were added and the components mixed on a rotor overnight at 4 ºC. 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 ºC for 40 min. The reaction was quenched with the addition of 50 µl HDAC-FDL Developer (KI-105, Biomol) 20X 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.

4.13. Histone Deacetylase Profiling (HDAC1, HDAC3-11)

HDAC profiling was performed by Reaction Biology Corp. (Malvern, PA) against 10 isolated isoforms of human HDAC (HDAC1, HDAC3-11) in the presence of the fluorogenic tetrapeptide RHKKAc (p53 residues 379-382) as the substrate (50 µM). Isolated human HDACs were obtained by standard purification, with the exception of HDAC3, which was isolated in complex with NCOR2 and used as such. TSA and SAHA were used as reference compounds. Each compound was dissolved in DMSO (1:3 diluition; 10 doses), and sequentially diluted solutions were used for testing. IC50 values were calculated from the resulting sigmoidal dose-response inhibition slopes.

4.14. 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.

4.15. Cell cycle analysis

The cell cycle distribution was analyzed in propidium iodide-stained cells by FACScan flow cytometry, as described [19].

4.16. 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).

4.17 Western blotting

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

4.18 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 2x2x6 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

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Appendix A. Supplementary data

Supplementary data related to this article can be found at…. These data include the experimental procedures for the synthesis of compounds **4**, **5**, **7**, **10a-b**, **11a-b**, **15**.

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Figure captions

Figure 1. Apoptosis induced in ovarian carcinoma cells IGROV-1 by selected compounds at equitoxic concentrations (concentrations causing comparable cell growth inhibition, IC_{80}) after 72 h exposure. SAHA is shown as a reference HDAC inhibitor. Apoptosis was determined after 72 h exposure by TUNEL assay and flow cytometry analysis.

Figure 2. Effects of selected compounds on acetylation of tubulin, histone H4 and p53 in IGROV-1 cells, where each compound was treated at IC_{80} dosage, for the indicated exposure times.

Figure 3. Effect of compound **2c** on the modulation of proteins implicated in cellular response to DNA damage. Compound **7** is an atypical retinoid containing the adamantly group.

Figure 4. Time course of cell cycle perturbation in ovarian carcinoma cells, IGROV-1, following exposure to equitoxic concentrations (IC_{80}) of each compound. The cell cycle was analyzed by FACScan^{TM} analysis of PI-stained cells.

Figure 5. Antitumor effect of compound **2c** against the human colon carcinoma HCT 116. Animals were treated by oral route with 50 mg/Kg (Once-daily administration for 4-5 days a week for 5 weeks, for a total of 24 doses).

Tables

Table 1 Inhibition of HDAC2 (IC_{50}, μM) and antiproliferative activity (IC_{50}, μM) of compounds **1a-d** and **2a-d** and of reference HDAC inhibitor (SAHA) against a panel of tumor cell lines.

Cpd.	HDAC-2 assay	H460	HCT-116	IGROV-1	IGROV-1/Pt1
SAHA	0.1±0.02	3.4±0.8	0.31±0.02	2.2±0.3	2.2±0.2
1a	0.82±0.14	5.4±0.1	1.58±0.3	3.5±0.6	4.3±0.7
2a	>10	>10	10.3±1.2	1.23±0.3	5.7±0.9
1b	0.59±0.2	6.0±0.9	0.33±0.05	7.6±3.5	6.5±2.0
2b	2.64±0.5	0.83±0.28	0.58±0.01	1.1±0.6	1.3±0.6
1c	1.16±0.27	3.7±0.1	0.22±0.01	0.75±0.4	2.3±0.3
2c	2.3±0.6	1.42±0.23	1.3±0.1	0.96±0.35	1.8±0.07
1d	>5	>20	2.4±0.1	0.57±0.09	2.01±0.91
2d	>10	1.4±0.02	1.8±0.4	1.3±0.1	2.03±0.4

Table 2. Antiproliferative activity (IC_{50}, μM) of compounds **2e-l** and of reference HDAC inhibitor (SAHA) against a panel of various tumor cell lines**.**

Cpd.	H460	HCT-116	IGROV-1	IGROV-1/Pt1
SAHA	3.4±0.8	0.31±0.02	2.2±0.3	2.2±0.2
2e	1.0±0.04	2.55±0.03	1.2±0.04	0.8±0.2
2f	5.4±0.3	5.4±0.9	1.34±0.04	1.6±0.16
2g	2.4±0.2	2.5±0.3	0.38±0.03	1.36±0.16
2h	2.7±0.02	2.5±0.01	0.9± 0.2	1.6±0.1
2i	1.0±0.06	0.7	0.65±0.11	1.1±0.3
2l	1.3±0.1	1.4±0.1	1.5±0.5	4.16

Table 3. Inhibition of HDAC isoforms by selected compounds.[a]

	HDAC-1	HDAC-3	HDAC-4	HDAC-5	HDAC-6	HDAC-7	HDAC-8	HDAC-9	HDAC-10	HDAC-11
					IC_{50} (M)					
A[b]	7.12×10^{-9}	1.03×10^{-8}	1.21×10^{-8}	1.65×10^{-8}	4.19×10^{-10}	2.25×10^{-8}	8.95×10^{-8}	3.81×10^{-8}	2.01×10^{-8}	1.52×10^{-8}
B[c]	2.58×10^{-7}	3.50×10^{-7}	4.93×10^{-7}	3.78×10^{-7}	2.86×10^{-8}	3.44×10^{-8}	2.43×10^{-7}	3.16×10^{-7}	4.56×10^{-7}	3.62×10^{-7}
1b	5.51×10^{-6}	4.57×10^{-6}	1.71×10^{-5}	1.12×10^{-5}	4.18×10^{-8}	1.56×10^{-5}	1.62×10^{-7}	8.92×10^{-6}	1.10×10^{-5}	5.01×10^{-6}
2b	$> 10^{-4}$	5.56×10^{-5}	6.14×10^{-5}	7.84×10^{-5}	2.80×10^{-6}	7.51×10^{-5}	6.96×10^{-6}	4.14×10^{-5}	$> 10^{-4}$	5.72×10^{-5}
1c	8.55×10^{-6}	8.45×10^{-5}	9.94×10^{-5}	2.96×10^{-5}	3.68×10^{-8}	2.13×10^{-4}	6.98×10^{-8}	3.93×10^{-5}	9.10×10^{-5}	7.30×10^{-5}
2c[d]	-	-	-	-	-	-	-	-	-	7.70×10^{-5}
1d	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	3.26×10^{-6}	$> 10^{-4}$	1.56×10^{-6}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$
2d	6.01×10^{-5}	$> 10^{-4}$	$> 10^{-4}$	$> 10^{-4}$	1.67×10^{-5}	7.72×10^{-5}	3.06×10^{-5}	$> 10^{-4}$	$> 10^{-4}$	3.81×10^{-5}
2h	4.85×10^{-5}	3.40×10^{-4}	4.01×10^{-5}	1.24×10^{-4}	4.08×10^{-7}	4.92×10^{-5}	2.91×10^{-6}	4.23×10^{-5}	6.39×10^{-5}	6.34×10^{-5}

[a]Assay condition for compounds is to start with 50 μM, 1:3 dilution, 10 doses.

[b]Trichostatin A (TSA): Assay condition is to start with 5 μM, 1:3 dilution, 10 doses.

[c]SAHA

[d]Compound **2c** has a fluorescent background at 50 μM.

Figures:

Figure 1

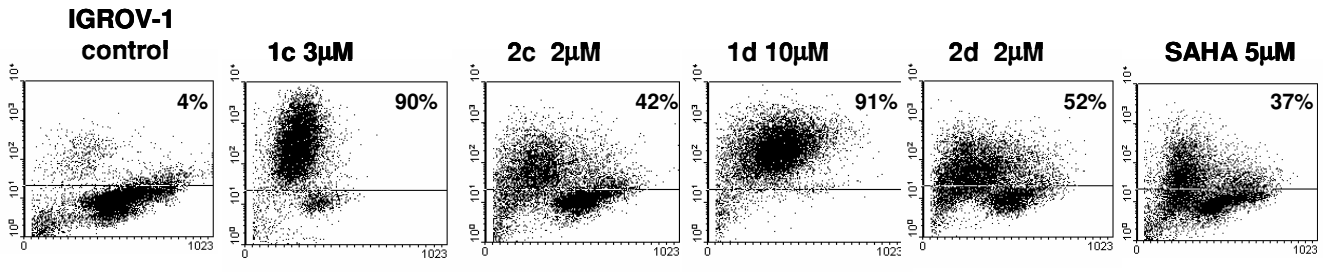


Figure 2

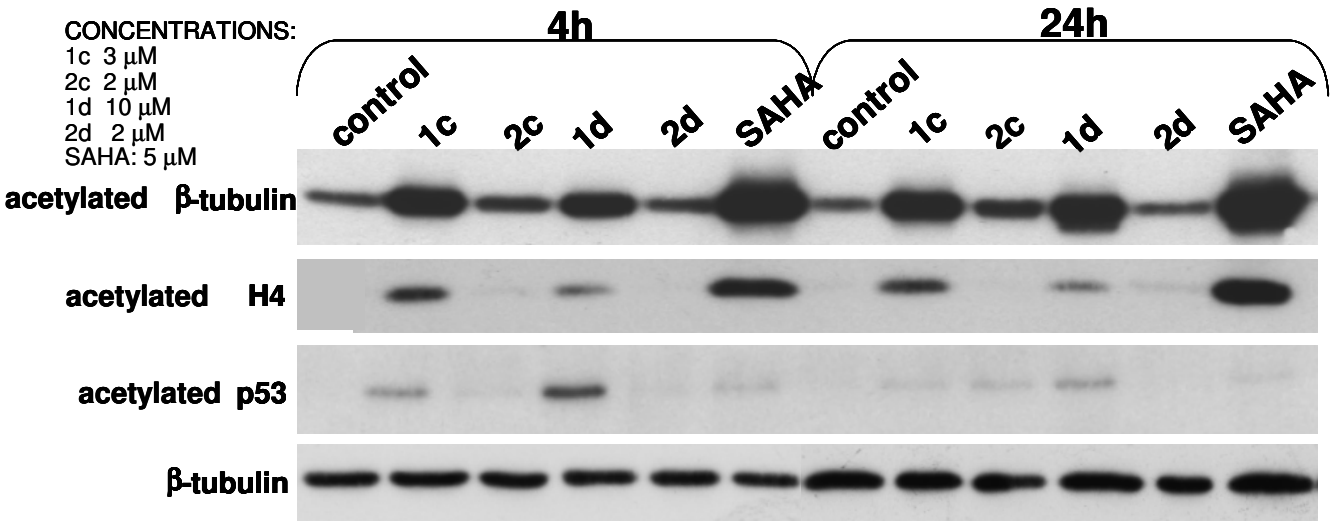


Figure 3

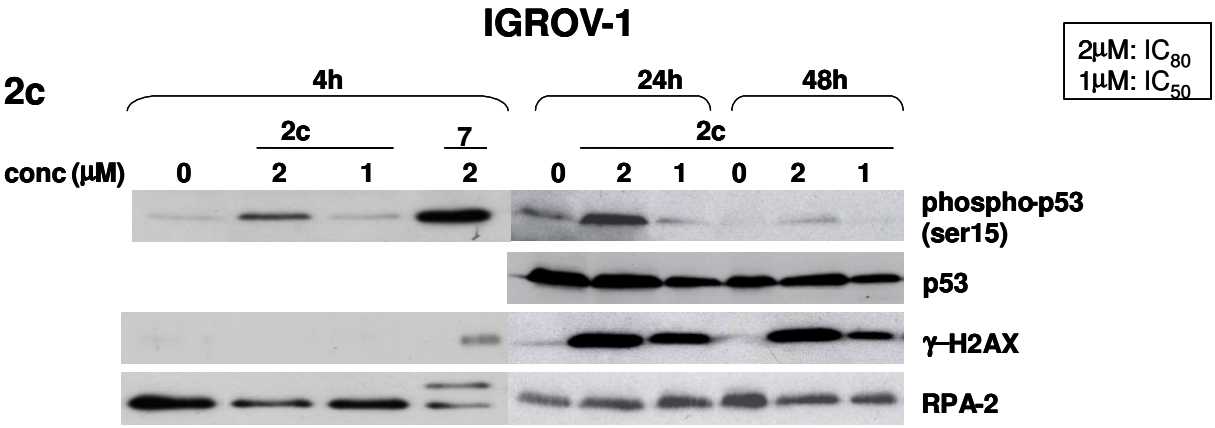


Figure 4

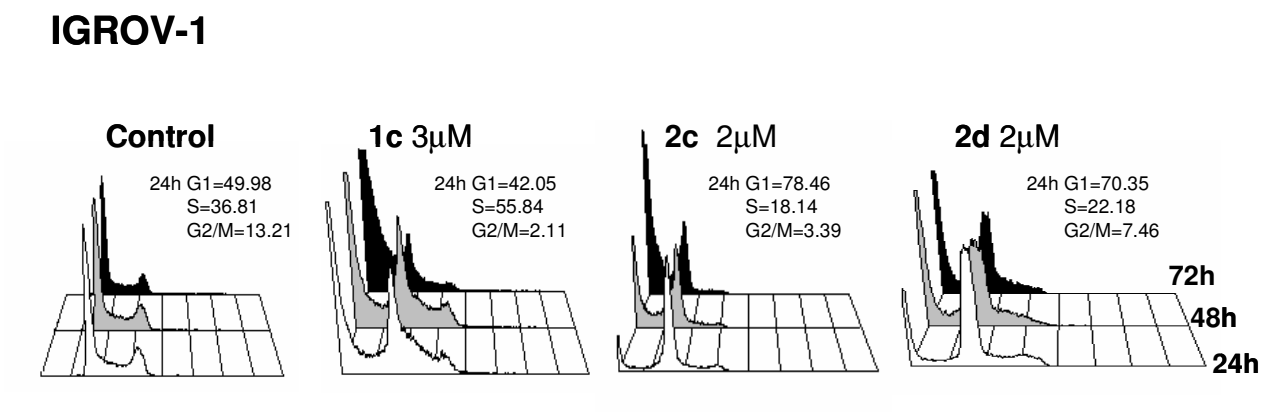
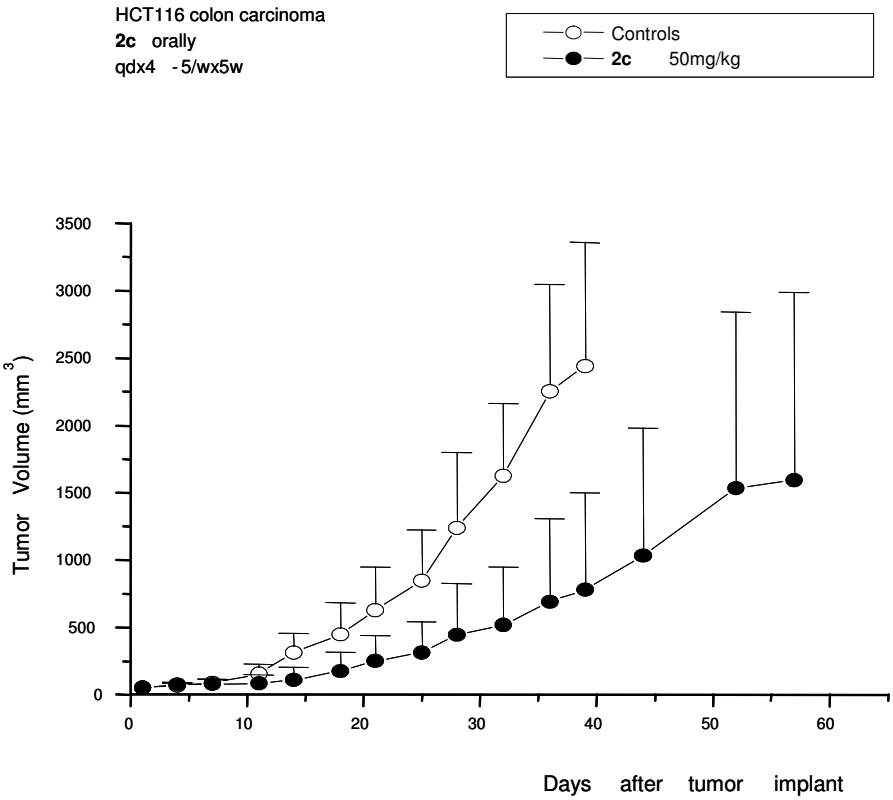
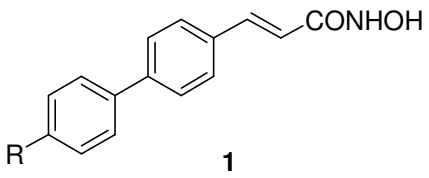


Figure 5


HCT116 colon carcinoma
2c orally
qdx4 - 5/wx5w
Controls
2c 50mg/kg
Tumor Volume (mm 3)
3500
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
50
60
Days after tumor implant

Charts and schemes:



1

Chart 1. Structure of biphenyl-4-yl-acrylohydroxamic acids.

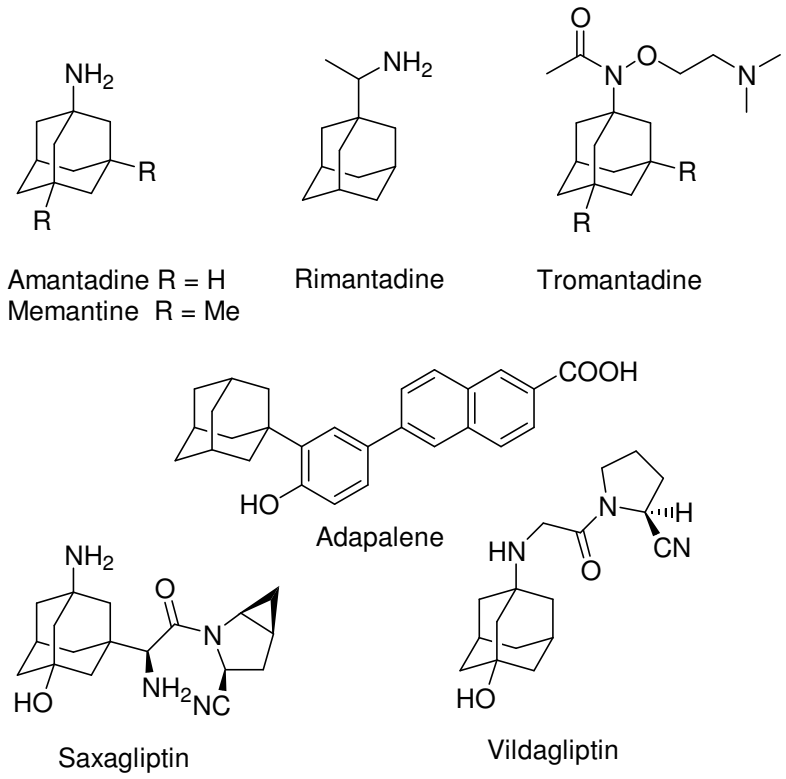
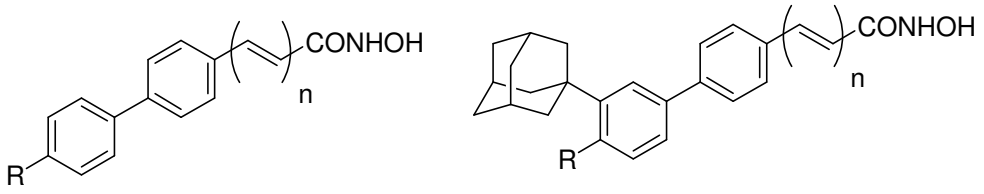


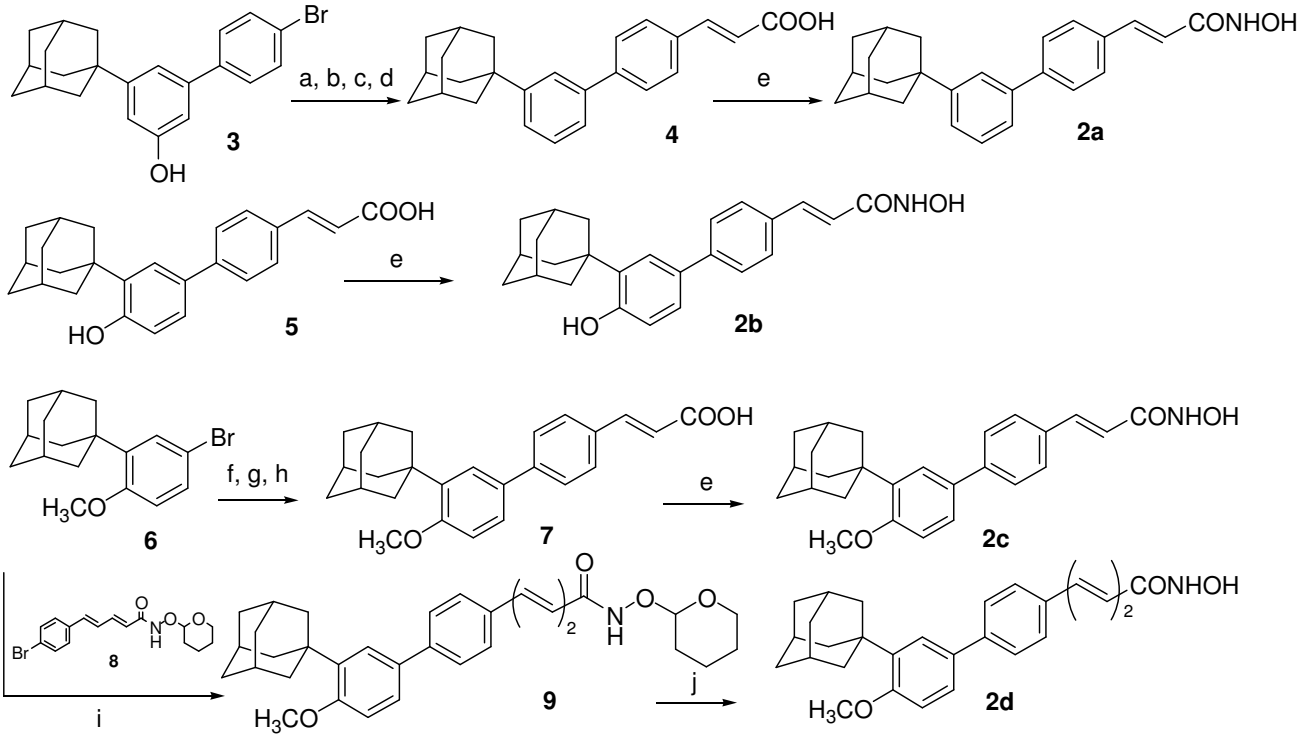
Chart 2. Adamantyl-derived drugs in clinical practice.



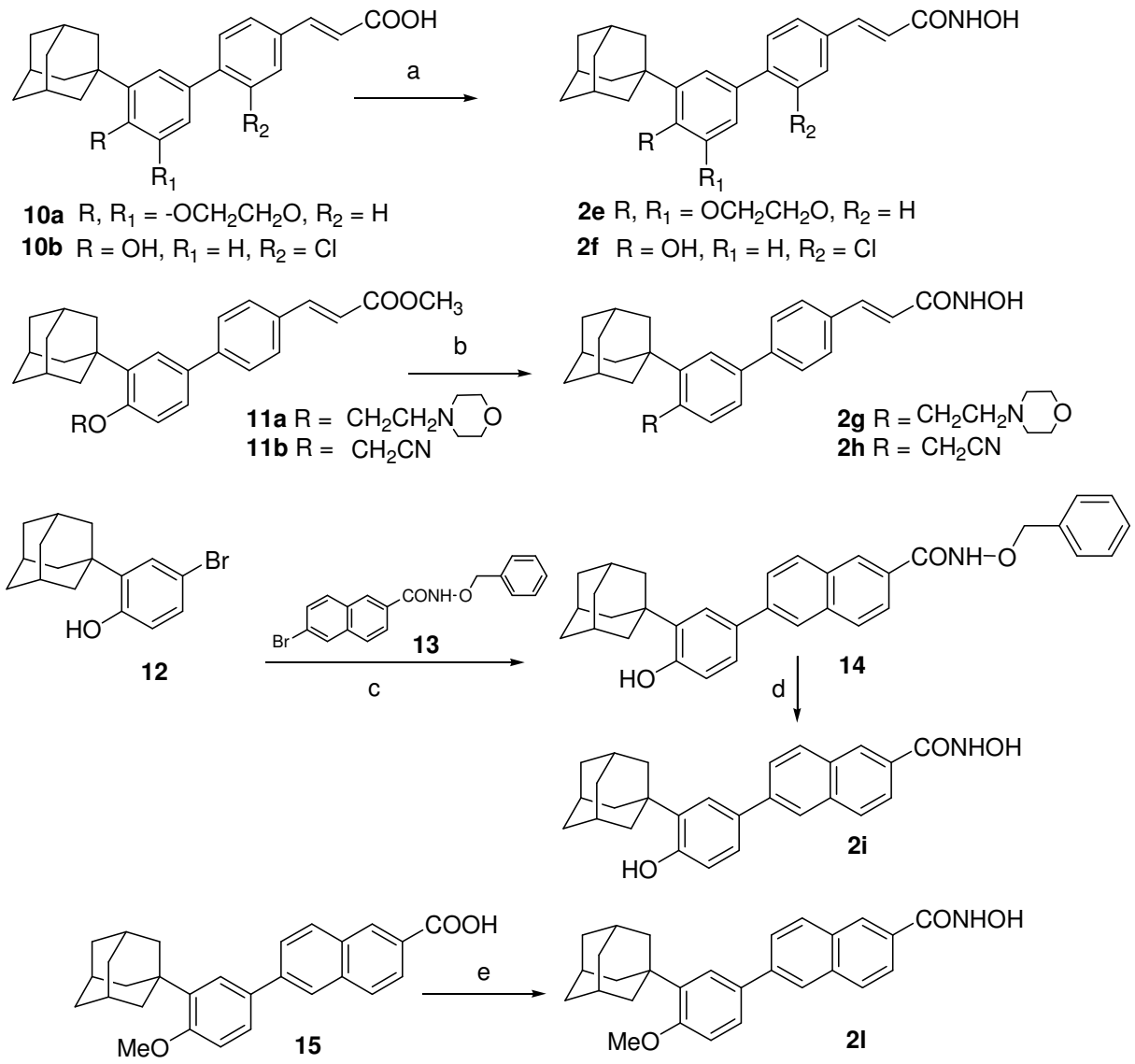
1a n = 1 R = H
1b n = 1 R = OH
1c n = 1 R = OMe
1d n = 2 R = OMe

2a n = 1 R = H
2b n = 1 R = OH
2c n = 1 R = OMe
2d n = 2 R = OMe

Scheme 1. Structures of biphenylacrylohydroxamic acids lacking (**1**) or containing (**2**) the adamantyl moiety in the cap structure.



Scheme 2. Synthesis of compounds **2a-d**. Reagents and conditions: a) TfO_2, Py, 3h, rt, 87%; b) Et_3SiH, dppp, $Pd(OAc)_2$, DMF, 24h, 60°C, 42%; c) tri (*o*-tolyl)phosphine, $Pd(OAc)_2$, TEA, methyl acrylate, 3h, reflux, 67%; d) $LiOH.H_2O$, THF/H_2O, rt, overnight, 63%; e) WSC, HOBt, DME, rt, 1.5-24h, then $NH_2OH.HCl$, 1-4h, Et_3N, 40-75%; f) Pd tetrakis, 4-formylbenzeneboronic acid, 2M Na_2CO_3, toluene, 2h, reflux, 72%; g) $Ph_3PCH{=}COOMe$, chloroform, 3h, reflux, 86%; h) NaOH, methanol, 7h, reflux, 70%; i) Bis(pinacolato)diboron, $PdCl_2(dppf)$, KOAc, dioxane, 100°C, 13h, then **8**, 2M Na_2CO_3, $PdCl_2(dppf)$, reflux, 4h, 86%; j) PTSA, MeOH, rt, 4h, 44%.



Scheme 3. Synthesis of compounds **2e-l**. Reagents and conditions: a) 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium-3-oxid hexafluorophosphate (HATU), DIPEA, $NH_2OH{\cdot}HCl$, DMF, rt, 4h, 65% for **2e**; 1.5h, 26% for **2f**; b) for **2g**: $THPONH_2$, LiHMDS, THF, -78°C, 2h, 100%, then PTSA, MeOH, rt, 24h, 63%; for **2h**: $LiOH{\cdot}H_2O$, THF/H_2O, rt, overnight, 93%, then WSC, HOBt, DMF, $NH_2OH{\cdot}HCl$, TEA, rt, overnight, 33%; c) Bis(pinacolato)diboron, $PdCl_2$(dppf), KOAc, reflux, 2h, then 6-bromonaphthalene-2-carboxylic acid benzyloxyamide (**13**), 2M Na_2CO_3, 2h, 28%; d) H_2/Pd/C, MeOH, 36h, rt, 70%; e) HATU, DIPEA, $NH_2OH{\cdot}HCl$, DMF, rt, overnight, 30%.