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Design and Synthesis of C3-Substituted β-Carboline-Based Histone Deacetylase Inhibitors with Potent Antitumor Activities

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A series of hydroxamic acid histone deacetylase (HDAC) inhibitors in which the β-carboline motif has been incorporated were designed and synthesized. The effect of substitution at the C3 amide on HDAC inhibition and antiproliferative activities was investigated. Most of these compounds were found to display significant HDAC inhibitory effects and good antiproliferative activity, with IC_{50} values in the low-micromolar range. In particular, the HDAC inhibition IC_{50} value of *N*-(2-(dimethylamino)ethyl)-*N*-(4-(hydroxylcarbamoyl)benzyl)-1-(4-methoxyphenyl)-9*H*-pyrido[3,4-*b*]indole-3-carboxamide (**9h**) is five-fold lower than that of suberoylanilide hydroxamic acid (SAHA, vorinostat). Furthermore, **9h** was found to increase the acetylation of histone H3 and α-tubulin, and to induce DNA damage as evidenced by hypochromism and enhanced phosphorylation of histone H2AX. Compound **9h** inhibits Stat3, Akt, and ERK signaling, important cell-growth-promoting pathways that are aberrantly activated in most cancers. Finally, **9h** showed reasonable solubility and permeability in Caco-2 cells. Our findings suggest that these novel β-carboline-based HDAC inhibitors may hold great promise as therapeutic agents for the treatment of human cancers.

The design and development of single drug molecules that simultaneously interact with multiple drug targets have recently gained considerable interest in drug discovery.[1–4] This approach aims to alleviate issues commonly associated with single-target drugs, such as limited efficacies and development of resistance.[4,5] Given that different types of tumors may originate from different cell types and are frequently driven by various combinations of genetic alterations, multitarget antitumor drugs may also be effective against a wider range of cancers. Moreover, multitarget drugs as single chemical entities may achieve effective combinatorial therapies without the common disadvantages of combining multiple agents together, such as drug–drug interactions, difficulty in achieving sufficient potencies against tumor cells, and potentiation of adverse effects.[6,7]

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes that control the reversible acetylation and deacetylation of histones. DNA methylation or post-translational modification of histone proteins often leads to chromatin remodeling, which partly controls the epigenetic regulation of gene expression.[8,9] Several HDACs are overexpressed in various tumor types, indicating their importance in tumor growth.[10,11] HDACs have been shown to play an important role in carcinogenesis, including the transcription and regulation of genes involved in cell proliferation, cell-cycle regulation, and apoptosis.[10,11] HDAC inhibitors (HDACis) can induce tumor cell apoptosis, growth arrest, senescence, differentiation, and immunogenicity, and can inhibit angiogenesis.[12] Moreover, HDACis have also been reported to synergistically enhance the inhibitory effects of a number of other antitumor agents, including those targeting EGFR, DNA, or topoisomerase, in suppressing proliferation and inducing apoptosis in tumor cells.[13–16]

Several HDACis are currently on the market, the majority of which primarily target hematological malignancies, and about 20 others are in clinical trials. Based on the structural characteristics of the Zn^{2+} binding domain, HDACis can be classified into four main categories: hydroxamic acids, cyclic peptides, short-chain fatty acids, and benzamides. The most widely explored class of HDACis that have entered pre-clinical or clinical studies as anticancer agents are the hydroxamic acid based compounds, including suberoylanilide hydroxamic acid (SAHA, vorinostat; Figure 1), belinostat (PXD101), and panobinostat (LBH589), three FDA-approved drugs for the treatment of cutaneous T-cell lymphoma (CTCL) or multiple myeloma.[17–19]

Hydroxamic acid based HDACis such as SAHA commonly have an aromatic amide (CAP) group and a hydroxamic acid (ZBG) group connected through a linker. While structure–activity relationship (SAR) studies have shown that the hydroxamic acid group is required for HDAC activity, the aromatic group, such as the phenyl group in SAHA, can be replaced with various aromatic groups. We recently demonstrated that merging the key structural elements of β-carboline and SAHA resulted in hybrid compounds with increased antitumor potency and low acute toxicity.[20] Naturally occurring β-carboline alkaloids

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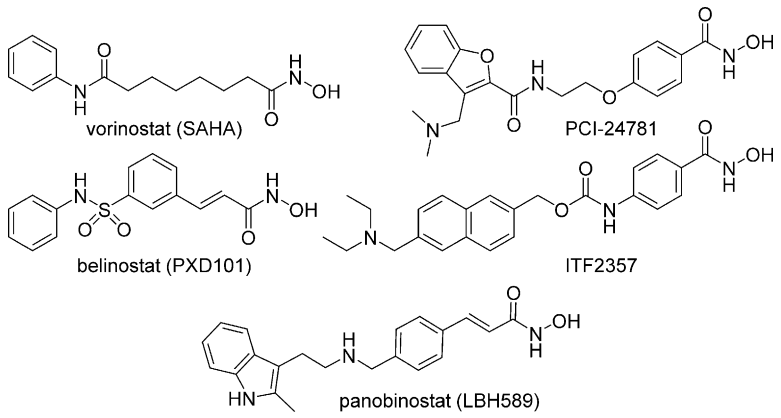


Figure 1. Structures of representative hydroxamic acid based HDAC inhibitors on the market or in clinical trials.

and synthetic analogues containing β-carboline subunits are known to have antitumor activities,[21,22] likely by DNA intercalation and inhibition of topoisomerase, cyclin-dependent kinase (CDK), or the IκB kinase complex (IKK).[20,23–26] This observed increase in antitumor potency of the SAHA–β-carboline hybrids is most likely the result of simultaneous inhibition of HDAC and induction of DNA damage. In these hybrid compounds, the β-carboline and hydroxamic acid motifs were connected via alkyl linkers of various length, as seen in SAHA. Considering that other HDACis such as PCI-24781 and ITF2357 contain aromatic rings in the linker region (Figure 1), we reasoned that connecting substituted β-carbolines and hydroxamic acids with aromatic-ring-containing linkers may lead to similar increased anticancer efficacy. Herein we report our efforts in the design, synthesis, and biological evaluation of a series of novel C3-substituted β-carboline-based hydroxamic acids (**9a–l**), and investigations into the antitumor mechanism of these compounds in multiple cancer cell lines. We also investigated the effects of substitution at the amide nitrogen atom on HDAC inhibition and antitumor activities (Figure 2).

The general route for the synthesis of the target compounds **9a–l** is depicted in Scheme 1. Reductive amination between methyl 4-formylbenzoate (**1**) and various primary amines **2a–l** in the presence of acetic acid yielded imines **3a–l**, which were subsequently reduced by sodium borohydride to afford amines **4a–l**. 1-(4-Methoxylphenyl)-3-carboxyl-β-carboline **7** was prepared in a two-step sequence. First, treatment of commercially available L-tryptophan **5** with a solution of 4-methoxyphenaldehyde in acetic acid produced the intermediate 1-(4-methoxyphenyl)-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylic acid (**6**) via Pictet–Spengler reaction, which was then oxidized by potassium permanganate in *N*,*N*-dimethylformamide to furnish compound **7**. Reaction of **7** with amines **4a–l** in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) afforded amide derivatives **8a–l**. Finally, treatment of

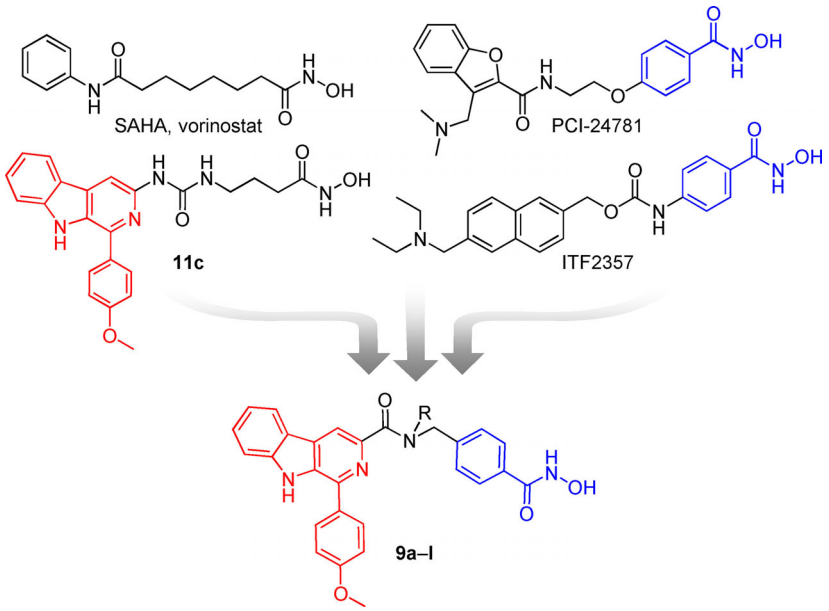
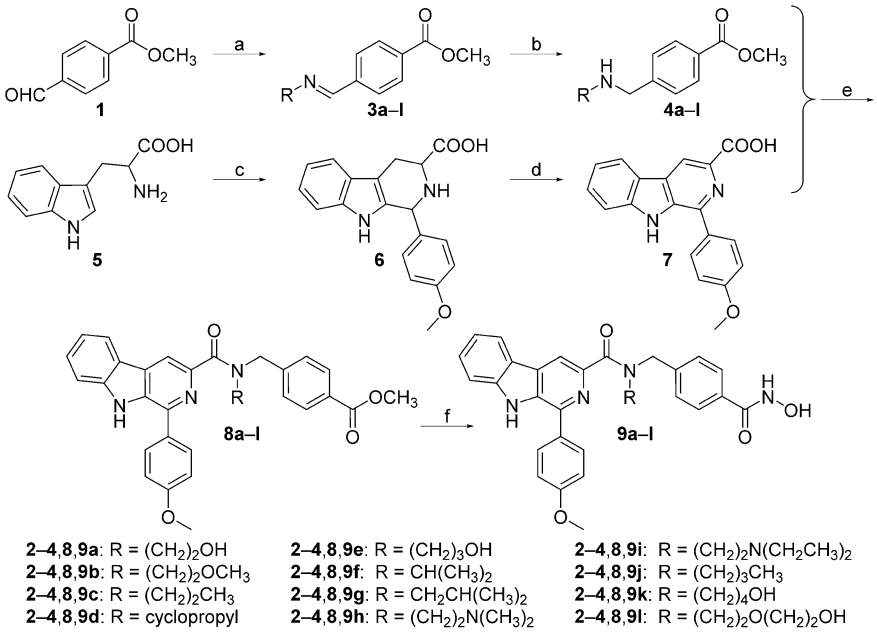


Figure 2. Design of β-carboline-based hydroxamic acids **9a–l**.



Scheme 1. Synthesis of target compounds **9a–l**. *Reagents and conditions*: a) primary amines **2a–l**, HOAc, THF, RT, 1–2 h; b) $NaBH_4$, THF, RT, 2 h, 56–73%; c) 4-methoxybenzaldehyde, HOAc, reflux, 2–4 h, 86%; d) $KMnO_4$, DMF, RT, 6 h, 70%; e) EDCI, DMAP, CH_2Cl_2, RT, 5–8 h; f) NH_2OK, MeOH, RT, 10–15 h, 52–67%.

intermediates **8a–l** with NH_2OK gave the target compounds **9a–l**. All target compounds **9a–l** were purified by column chromatography, and their structures were confirmed by IR, ^1H NMR, MS, and HRMS. All compounds were of >95% purity, as determined by high-performance liquid chromatography (HPLC).

All target compounds were first tested against HeLa cell nuclear extracts, a rich source of HDAC activity, for their HDAC inhibitory potencies (Table 1).[27] SAHA was used as a positive control. As expected, harmine, a β-carboline analogue with antitumor activity, showed no HDAC activity at concentrations up to 10 μM. All target compounds show moderate to good potency in the inhibition of HDAC, similar to that of SAHA. These results suggest that connecting β-carboline and hydroxamic acid via aromatic-ring-containing linkers permits the maintenance of HDAC activities. Compounds **9a**, **9c**, and **9h–j** all displayed IC_{50} values (0.092–0.34 μM) lower than that of SAHA (IC_{50} = 0.48 μM) in HeLa nuclear extracts. In particular, the IC_{50} value of **9h** (0.092 μM), the most potent compound of the series, was five-fold lower than that of SAHA (IC_{50} = 0.48 μM).

The inhibitory activities of these compounds against human hepatocellular carcinoma cells (HepG2 and SMMC-7721), human colon cancer cells (HCT116), and human lung cancer cells (H1299) were evaluated in vitro in MTT assays. SAHA and harmine were used as positive controls. The IC_{50} values of **9a–l** against the four human cancer cell lines are listed in Table 2. Most of the target compounds displayed similar or better anti-

Table 1. HDAC inhibition by compounds **9a–l**.

Compound	R	IC_{50} [μM][a]
SAHA	–	0.48 ± 0.06
harmine	–	> 10
9a	$(CH_2)_2OH$	0.41 ± 0.05
9b	$(CH_2)_2OCH_3$	0.65 ± 0.09
9c	$(CH_2)_2CH_3$	0.26 ± 0.03
9d	cyclopropyl	1.23 ± 0.15
9e	$(CH_2)_3OH$	0.64 ± 0.08
9f	$CH(CH_3)_2$	0.97 ± 0.12
9g	$CH_2CH(CH_3)_2$	1.35 ± 0.18
9h	$(CH_2)_2N(CH_3)_2$	0.092 ± 0.01
9i	$(CH_2)_2N(CH_2CH_3)_2$	0.12 ± 0.02
9j	$(CH_2)_3CH_3$	0.34 ± 0.05
9k	$(CH_2)_4OH$	0.93 ± 0.11
9l	$(CH_2)_2O(CH_2)_2OH$	1.56 ± 0.19

[a] Determined in HeLa cell nuclear extracts; data are the mean ± SD of three independent experiments.

Table 2. In vitro antiproliferative activity of compounds **9a–l** against four human cancer cell lines.

Compound	IC_{50} [μM][a]			
	HCT116	HepG2	SMMC-7721	H1299
SAHA	5.53 ± 0.68	6.26 ± 0.51	5.61 ± 0.57	7.78 ± 0.63
harmine	46.7 ± 3.92	51.2 ± 4.76	55.3 ± 5.01	ND[b]
9a	2.34 ± 0.32	4.10 ± 0.37	5.78 ± 0.65	7.36 ± 0.82
9b	5.60 ± 0.64	6.73 ± 0.71	8.94 ± 1.02	11.1 ± 1.05
9c	2.24 ± 0.21	2.15 ± 0.36	3.42 ± 0.31	5.56 ± 0.68
9d	> 12.5	> 12.5	> 12.5	> 12.5
9e	4.89 ± 0.13	6.06 ± 0.48	7.89 ± 0.85	> 12.5
9f	> 12.5	> 12.5	> 12.5	> 12.5
9g	> 12.5	> 12.5	> 12.5	> 12.5
9h	1.79 ± 0.15	1.60 ± 0.18	2.46 ± 0.31	4.25 ± 0.58
9i	1.81 ± 0.26	3.27 ± 0.29	4.05 ± 0.38	6.63 ± 0.70
9j	3.02 ± 0.26	3.37 ± 0.52	5.25 ± 0.60	5.37 ± 0.61
9k	> 12.5	> 12.5	> 12.5	> 12.5
9l	> 12.5	> 12.5	> 12.5	> 12.5

[a] Determined by MTT assay; data are the mean ± SD of three independent experiments. [b] Not detected.

proliferative activity against the tested cells than that of SAHA, and significantly more potent than harmine. The IC_{50} values of **9a**, **9c**, and **9h–j**, compounds with low-micromolar potencies in HDAC inhibition, were also in the low-micromolar range (1.60–7.36 μM) in antiproliferation. These values were significantly lower than both SAHA and harmine. Consistent with the HDAC inhibitory activities, **9h** [$R=(CH_2)_2NMe_2$] was also the most potent compound among the group in antiproliferative activities, with IC_{50} values of 1.60–4.25 μM, are were lower than those of SAHA (IC_{50}: 5.53–7.78 μM) in all four cell lines.

Considering the significant growth inhibitory activity of **9h** in vitro, the general toxicity of **9h** was further evaluated in normal human LO2 cells. The treatment of non-tumor LO2 cells with **9h** showed no apparent growth inhibitory activity at doses up to 25 μM, the same dose that induced the majority of HepG2 cell death, suggesting **9h** possess no significant general toxicity to normal human cells.

The fact that all target compounds are active in HDAC inhibition and antitumor assays suggests that linkers with aromatic rings, like the alkyl linkers previously studied, are well tolerated for activity. In general, the potencies of these compounds are similar to that of SAHA, but significantly more potent than harmine, confirming the importance of the hydroxamic acid for activity. The SARs of this compound series show remarkable resemblance in their inhibition of HDAC and cancer cell proliferation. The size and composition of the substituents on the amide nitrogen atom have a clear impact on the activities of these compounds. Compounds with straight-chain alkyl groups (**9c**, **9j**) are favored. However, if a terminal hydroxy group is introduced, a decrease in potency is observed (**9c** vs. **9e**, **9j** vs. **9k**). Introduction of an oxygen atom within the chain slightly decreases potency (**9b** vs. **9c** vs. **9j**). Bulkier substituents such as those of **9d**, **9f**, and **9g** also resulted in decreased potency in both HDAC and antiproliferation assays. Interestingly, if a nitrogen atom was introduced, the potency significantly increased (**9h**, **9i**), particularly in the inhibition of HDAC activity. This is likely the result of hydrogen bonding interactions between the hydrogen and the hydrogen bonding donating residue(s) of the target.

Given that the inhibition of HDACs by these compounds enhances the antiproliferative activity against tumor cells, the HDAC inhibitory effects of the most active compound **9h** on the levels of acetylation of histone H3 and α-tubulin were determined by immunoblotting assays using β-actin as a negative control (Figure 3). HepG2 cells were incubated with the vehicle alone, SAHA (5.0 μM), or **9h** (0.8, 1.6, and 3.2 μM). Compared with the control group, compound **9h** increased the expression of acetyl-histone H3 and acetyl-α-tubulin in a dose-dependent manner. Levels of acetyl-histone H3 in the groups treated with **9h** at 1.6 and 3.2 μM were higher than the values from the group treated with 5.0 μM SAHA, consistent with the results from the HDAC fluorimetric activity assay.

It is known that the planar structure of β-carbolines can bind to DNA and induce DNA damage.[23,24] To investigate whether the anticancer activities of these β-carboline derivatives resulted partly from DNA binding, UV/Vis spectroscopic titration studies were performed. In general, hypochromism, or

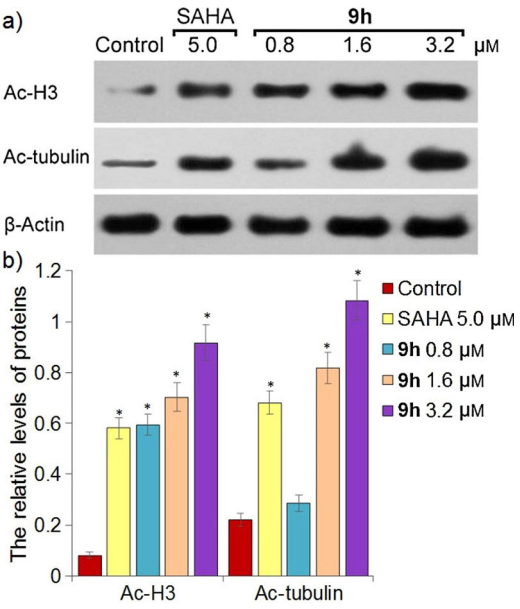


Figure 3. Immunoblot analysis of the acetylation of histone H3 and α-tubulin in vitro. a) HepG2 cells were treated with **9h** and SAHA for 48 h at the indicated concentrations. Cell lysates were prepared and subjected to SDS-PAGE and immunoblot analysis using anti-acetyl-histone H3, anti-acetyl-α-tubulin, and anti-β-actin antibodies. β-Actin was used as a loading control. b) Quantitative analysis. The levels of Ac-H3 and Ac-α-tubulin relative to β-actin control were determined by densitometric scanning. Data are the means ± SD of three separate experiments; $*p<0.01$ vs. control.

decrease in absorption, is observed when a small molecule binds to DNA by intercalation, resulting from a strong π–π stacking interaction between an aromatic chromophore from the molecule and the base pairs of DNA.[24] In our study, the **9h**–CT-DNA system showed absorption bands at λ 250, 300, and 360 nm, with the maximum absorption at 300 nm (Figure 4). Upon addition of equal increments of CT-DNA to a solution of **9h**, the intensity of all three absorption bands gradually decreased, with the strongest hypochromicity observed at 300 nm. These results clearly indicate electrostatic binding of compound **9h** to CT-DNA via intercalation.

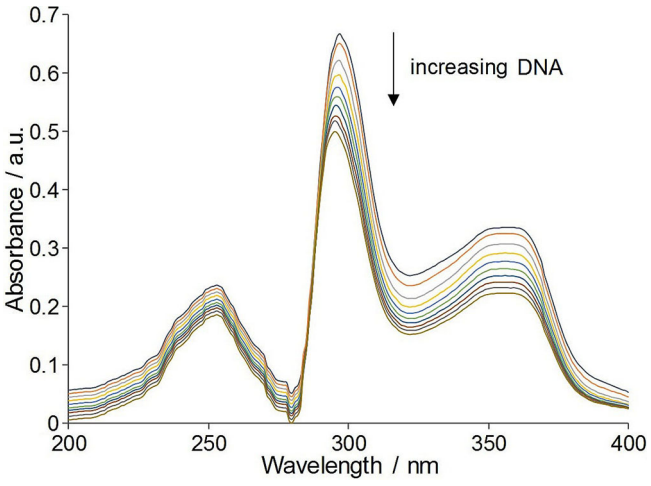


Figure 4. UV/Vis absorption spectra of **9h** (20 μM) in the presence of increasing amounts of calf thymus (CT)-DNA. Arrows indicate the changes in absorbance with increasing DNA concentrations.

To further investigate whether DNA damage contributes to the anticancer activities of these β-carboline derivatives, we examined the extent of DNA damage caused by **9h** in HepG2 cells. We used histone H2AX phosphorylation as the DNA damage marker. Harmine and SAHA were used as positive controls. HepG2 cells were treated with vehicle or **9h** for 48 h. H2AX (S139ph) levels were then detected by western blot analysis. As shown in Figure 5, treatment with **9h** dose-dependently increased the levels of H2AX phosphorylation in HepG2 cells, significantly higher than that of the SAHA group. Because DNA damage induced by the β-carboline complex is associated with H2AX (S139ph) activation, these results clearly indicate that these new β-carboline-based hydroxamic acid derivatives are involved in DNA damage.

It is known that the Stat3, Akt, and ERK signaling pathways are important cell-growth-promoting pathways which are aberrantly activated in most cancers.[28,29] To gain greater insight into the molecular mechanisms that underlie the activity of **9h**, we examined the regulatory effects of **9h** on Stat3, Akt, and ERK signaling in HepG2 cells using SAHA as a control. The cells were treated with various concentrations of **9h** or SAHA. The expression and activation of Stat3, AKT, and ERK1/2 were determined by western blotting. As shown in Figure 6, treatment with **9h** significantly inhibited Stat3, Akt, and ERK1/2 phosphorylation in a dose-dependent manner, although it did not alter the levels of Stat3, Akt, and ERK1/2 expression in cancer cells. Similarly, treatment with SAHA also significantly decreased the levels of Stat3, Akt, and ERK1/2 phosphorylation, although to a lesser extent than **9h**. Previous studies have shown that aberrant activation of Stat3 and Akt are associated with the development of human cancer, and SAHA can inhibit the proliferation of cancer cells by downregulating Stat3 and Akt activation in cancer cells.[30–32] Compound **9h** may share the same mechanisms with SAHA, but further investigations are needed.

To assess the drug-likeness of this class, we conducted preliminary in vitro pharmacokinetics studies on compound **9h**. Solubility is an important parameter to predict drug absorption, and it is commonly accepted that insufficient drug solubility can lead to poor oral absorption.[33] The most potent compound of the series, **9h**, demonstrated good aqueous solubility (32.9 mg mL^{-1}). SAHA was previously reported to have an approximate solubility of 0.5 mg mL^{-1} in a 1:1 solution of DMSO/PBS (pH 7.2).[34] The transport of compound **9** was measured to evaluate the intestinal permeation of these compounds. The human colon adenocarcinoma Caco-2 cell line is the most widely used in vitro model that mimics the absorptive properties of the intestinal epithelium.[35] Experiments were conducted at 37 °C, and measurements were performed over a 90 min incubation period. Compound **9h** showed moderate permeability, with the rate of transport (P_{app} A→B) of

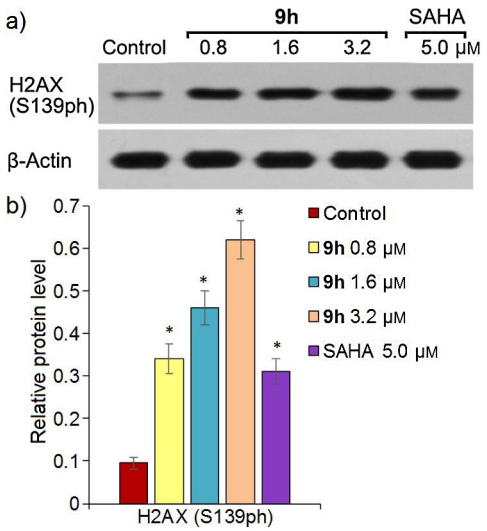


Figure 5. Immunoblot analysis of DNA damage in vitro. a) HepG2 cells were treated with vehicle (control), various doses of **9h**, or SAHA. Cells were homogenized, and their lysates were subjected to immunoblot analysis using anti-H2AX (S139ph) and anti-β-actin antibodies. β-Actin was used a loading control. b) Quantitative analysis. The levels of each signaling event relative to β-actin control were determined by densitometric scanning. Data are the means ± SD of three separate experiments; *$p < 0.01$ vs. control.

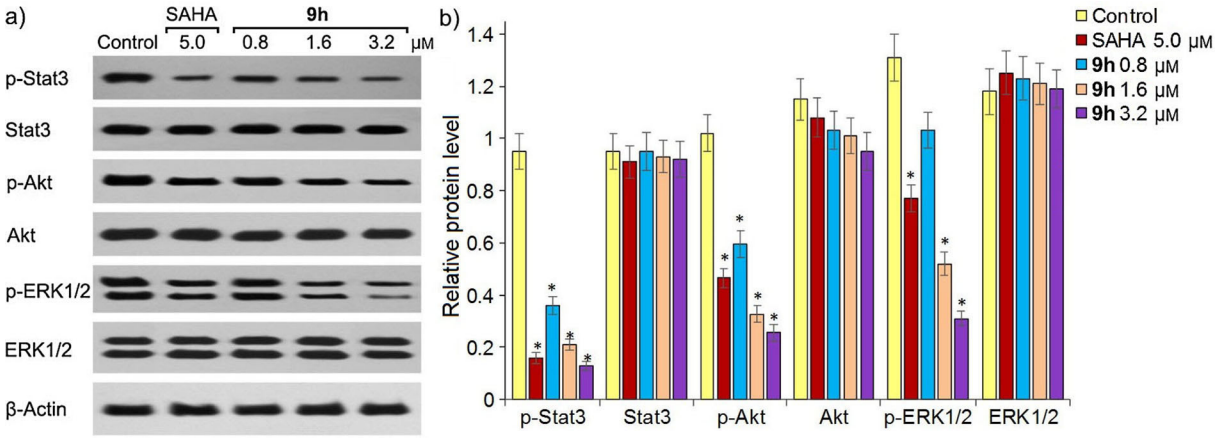


Figure 6. Effects of **9h** and SAHA on Stat3, Akt, and ERK signaling in HepG2 cells. Cells were treated with vehicle or the indicated test compound for 48 h, and the relative levels of Stat3, Akt, and ERK1/2 expression and phosphorylation were determined by western blot assays using β-actin as a control. a) Western blot analysis of the relative levels of Stat3, Akt, and ERK expression and phosphorylation. b) Quantitative analysis. Data are the means ± SD of each group of cells from three separate experiments; *$p < 0.01$ vs. vehicle-treated control.

0.58×10^{-6} cm s^{-1} at 10 μM, which is three-fold lower than the reported value for SAHA (1.70×10^{-6} cm s^{-1}).[36]

In conclusion, we recently demonstrated that the incorporation of the β-carboline structural motif into hydroxamic acid based HDACis such as SAHA resulted in agents with increased HDAC inhibitory and antiproliferative activity, where the two moieties are connected by alkyl linkers.[20] Herein we describe a series of β-carboline-based hydroxamides in which the two units are linked via aromatic-ring-containing linkers. The biological activities of these compounds were evaluated, and most of them displayed significant HDAC inhibitory effects and good antiproliferative activity, with IC_{50} values in low-micromolar range, confirming the compatibility of these linkers. SAR studies show that the size and composition of the substituents at the C3-amide position clearly affect the activities of these compounds. Introduction of a basic nitrogen atom increases the potencies of these compounds, including **9h**, which is the most potent of the series. Compound **9h** dose-dependently increased the acetylation of histone H3 and α-tubulin, as expected with HDACis. The increased antiproliferative activity of **9h** most likely results from induced DNA damage by the β-carboline structural moiety, as evidenced by hypochromism and enhanced histone H2AX phosphorylation. Compound **9h** was also found to inhibit Stat3, Akt, and ERK signaling, pathways that are aberrantly activated in most cancers. Finally, compound **9** showed reasonable solubility and moderate Caco-2 cell permeability. Together, given their potent antitumor activities, these 3-substituted β-carboline-based HDACis warrant further investigation as candidates for the potential treatment of human cancer.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antitumor agents · β-carboline · DNA damage · histone deacetylase · inhibitors

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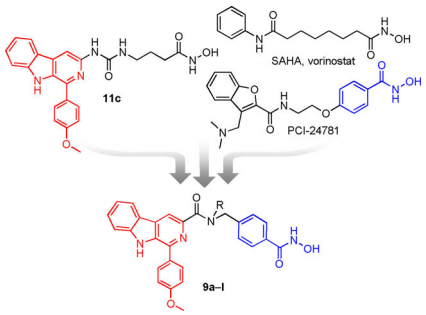
COMMUNICATIONS

It takes two: A series of hydroxamic acid histone deacetylase (HDAC) inhibitors containing β-carboline motifs were designed and synthesized. The most potent compound was found to increase the acetylation of histone H3 and α-tubulin, to induce DNA damage, and to inhibit important cell growth signaling pathways such as Stat3, Akt, and ERK. Our findings suggest that these new β-carboline-based HDAC inhibitors have great potential as therapeutic agents for the treatment of human cancers.



Y. Ling, J. Feng, L. Luo, J. Guo, Y. Peng, T. Wang, X. Ge, Q. Xu, X. Wang, H. Dai, Y. Zhang**

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Design and Synthesis of C3-Substituted β-Carboline-Based Histone Deacetylase Inhibitors with Potent Antitumor Activities

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