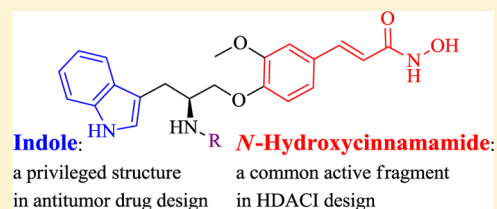


Development of *N*-Hydroxycinnamamide-Based Histone Deacetylase Inhibitors with an Indole-Containing Cap GroupYingjie Zhang,<sup>†,⊥</sup> Penghui Yang,<sup>†,⊥</sup> C. James Chou,<sup>‡</sup> Chunxi Liu,<sup>§</sup> Xuejian Wang,<sup>||</sup> and Wenfang Xu<sup>\*,†</sup><sup>†</sup>Department of Medicinal Chemistry, School of Pharmacy, Shandong University, Ji'nan, Shandong, 250012, People's Republic of China<sup>‡</sup>Department of Drug Discovery and Biomedical Sciences, South Carolina College of Pharmacy, Medical University of South Carolina, Charleston, South Carolina, 29425, United States United States<sup>§</sup>Department of Pharmaceutics, School of Pharmacy, Shandong University, Ji'nan, Shandong, 250012, People's Republic of China<sup>||</sup>Postdoctoral Workstation, Biomedical Industry Park Management Office, Weifang, Shandong, 261205, People's Republic of China

## Supporting Information

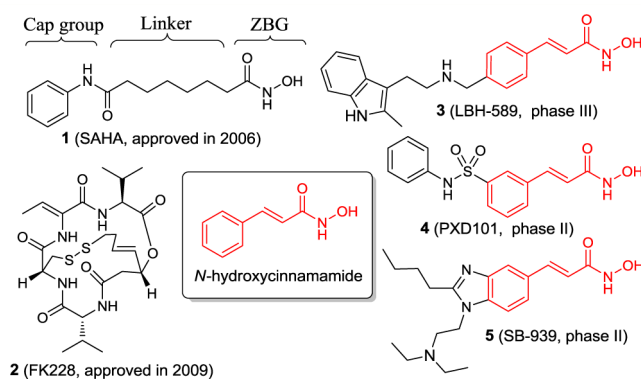
**ABSTRACT:** A novel series of histone deacetylase inhibitors combining *N*-hydroxycinnamamide bioactive fragment and indole bioactive fragment was designed and synthesized. Several compounds (17c, 17g, 17h, 17j, and 17k) exhibited comparable, even superior, total HDACs inhibitory activity and in vitro antiproliferative activities relative to the approved drug SAHA. A representative compound 17a with moderate HDACs inhibition was progressed to isoform selectivity profile, Western blot analysis, and in vivo antitumor assay. Although HDACs isoform selectivity of 17a was similar to that of SAHA, our Western blot results indicated that intracellular effects of 17a at 1  $\mu$ M were class I selective. It was noteworthy that the effect on histone H4 acetylation of SAHA decreased with time, while the effect on histone H4 acetylation of 17a was maintained and even increased. Most importantly, compound 17a exhibited promising in vivo antitumor activity in a U937 xenograft model.

**KEYWORDS:** histone deacetylases, inhibitor, *N*-hydroxycinnamamide, indole



The epigenetic regulation of histone and DNA plays an important role in chromatin structure and control of gene expression. Altered patterns of epigenetic modification are very common in many diseases, including cancer.<sup>1</sup> As with genetic information, epigenetic modifications are heritable; in contrast, however, they are reversible and catalyzed by pairs of enzymes with converse activity. Among these epigenetic enzymes, histone deacetylases (HDACs) have now emerged as a promising new class of therapeutic targets.<sup>2</sup>

Four classes were identified in the HDACs family, characterized by different cellular localization and substrate specificity. The common feature of classes I (HDAC1–3 and -8), II (HDAC4–7, -9, and -10) and IV (HDAC11) active sites are characterized by the presence of a catalytic Zn<sup>2+</sup> ion, and the class III HDACs (sirtuins 1–7) are NAD<sup>+</sup>-dependent. These HDACs isoforms perform their multiple functions by either epigenetic mechanism (deacetylation of histones) or nonepigenetic mechanism (deacetylation of nonhistone substrates). It has been revealed that Zn<sup>2+</sup>-dependent isozymes, especially class I and class II, are closely related to tumorigenesis and development.<sup>3</sup> In the past 10 years, over 490 clinical trials of more than 20 histone deacetylase inhibitor (HDACI) candidates have been initiated, culminating in the approval of two antitumor drugs, vorinostat (SAHA) and romidepsin (FK228) (Figure 1).<sup>4,5</sup>



**Figure 1.** Approved and clinical HDACIs with the *N*-hydroxycinnamamide fragment highlighted in red.

*N*-Hydroxycinnamamide (Figure 1) is a very common active fragment in HDACI design.<sup>6,7</sup> This fragment could not only form bichelation with the active site Zn<sup>2+</sup> by its hydroxamic acid group but also form a sandwichlike  $\pi$ - $\pi$  interaction by inserting its vinyl benzene group into two parallel phenylalanine residues of HDAC. Currently, there are three HDACIs

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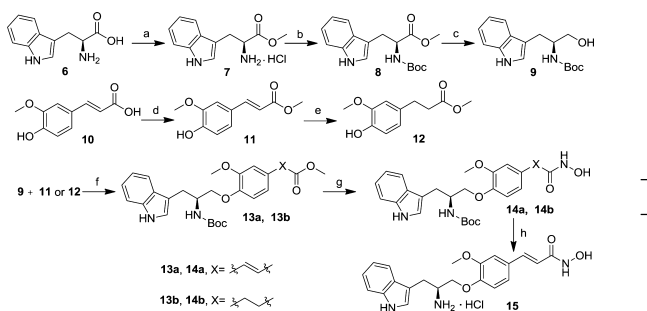
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containing *N*-hydroxycinnamamide fragment in clinical trials (Figure 1). Indole is recognized as a privileged structure in drug design,<sup>8,9</sup> and its derivatives have been found to exhibit anticancer activity by interacting with different targets.<sup>10</sup> Kinds of natural and synthetic HDACs containing an indole scaffold, especially in the cap group, possessed promising *in vitro* and *in vivo* potency.<sup>11–14</sup> Recently, comprehensive HDACs structure–activity relationship (SAR) studies revealed that *N*-hydroxycinnamamide-based compounds were more stable than their straight chain analogues, and compounds having indole groups exhibited the best *in vivo* efficacy and tolerability.<sup>15</sup> On the basis of the aforementioned information, we designed a novel series of *N*-hydroxycinnamamide-based HDACs with an indole-containing cap group.

Compounds **14a**, **14b**, and **15** were synthesized following the procedures described in Scheme 1. Methyl ester protection and

### Scheme 1. Synthesis of Compounds **14a**, **14b**, and **15**<sup>a</sup>



<sup>a</sup>Reagents and conditions: (a)  $\text{SOCl}_2$ ,  $\text{CH}_3\text{OH}$ , 98%. (b)  $(\text{Boc})_2\text{O}$ ,  $\text{Et}_3\text{N}$ ,  $\text{DCM}$ , 80%. (c)  $\text{LiAlH}_4$ , anhydrous  $\text{THF}$ ,  $0^\circ\text{C}$ , 86%. (d)  $\text{PTSA}$ ,  $\text{CH}_3\text{OH}$ ,  $80^\circ\text{C}$ , 96%. (e)  $\text{H}_2$ , 10%  $\text{Pd-C}$ ,  $\text{CH}_3\text{OH}$ . (f)  $\text{PPh}_3$ ,  $\text{DEAD}$ , anhydrous  $\text{THF}$ ,  $0^\circ\text{C}$ , 75% for **8a**, 79% for **8b**. (g)  $\text{NH}_2\text{OK}$ ,  $\text{CH}_3\text{OH}$ , 51% for **9a**, 49% for **9b**. (h)  $\text{HCl}$ , anhydrous  $\text{EtOAc}$ , 90%.

*tert*-butyloxycarbonyl (Boc) protection of *L*-tryptophan (**1**) followed by  $\text{LiAlH}_4$  reduction provided the intermediate **9**. Methyl ester protection of ferulic acid (**10**) afforded compound **11**, which was hydrogenized to give compound **12**. Both **11** and **12** could be connected with **9** under Mitsunobu reaction conditions to get **13a** and **13b**, which were converted to corresponding hydroxamic acid compounds **14a** and **14b**, respectively. Subsequent *N*-deprotection of **14a** afforded compound **15**.

The preliminary HDACs inhibitory assay was tested against HeLa cell nuclear extract, mainly containing HDAC1 and HDAC2. Results listed in Table 1 revealed that compound **14a** ( $\text{IC}_{50} = 0.65 \mu\text{M}$ ) was more potent than its analogues **14b** ( $\text{IC}_{50} = 2.09 \mu\text{M}$ ) and **15** ( $\text{IC}_{50} = 2.91 \mu\text{M}$ ), which indicated that both the vinyl benzene group and the substituent located on the amine atom (the Boc group in **14a**) were advantageous to HDACs inhibition. Therefore, using **14a** as lead, we kept its *N*-hydroxycinnamamide group unchanged and derivatized its Boc group to other functional groups. Such derivatizations were performed according to the methods in Scheme 2. *N*-Deprotection of **13a** with trifluoroacetic acid (TFA) and subsequent amide condensation or sulfonylation gave intermediates **16a–u**, which were treated with  $\text{NH}_2\text{OK}$  to get target compounds **17a–u**.

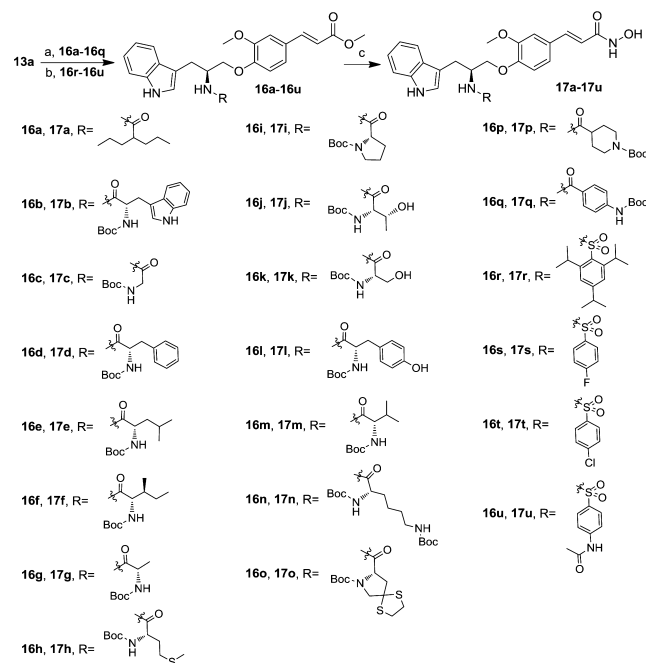
As shown in Table 1, we found that compounds **17b–n** with *N*-Boc-protected natural  $\alpha$ -amino acid residues as R group except **17n** exhibited more potent activity than their parent compound **14a**. Most importantly, the total HDACs inhibitory

Table 1. HeLa Cell Nuclear Extract Inhibitory Activity

compd	$\text{IC}_{50}$ ( $\mu\text{M}$ ) <sup>a</sup>	compd	$\text{IC}_{50}$ ( $\mu\text{M}$ ) <sup>a</sup>
<b>14a</b>	$0.65 \pm 0.12$	<b>17j</b>	$0.18 \pm 0.03$
<b>14b</b>	$2.09 \pm 0.31$	<b>17k</b>	$0.14 \pm 0.03$
<b>15</b>	$2.91 \pm 0.34$	<b>17l</b>	$0.32 \pm 0.05$
<b>17a</b>	$1.08 \pm 0.20$	<b>17m</b>	$0.43 \pm 0.05$
<b>17b</b>	$0.49 \pm 0.09$	<b>17n</b>	$1.17 \pm 0.21$
<b>17c</b>	$0.17 \pm 0.04$	<b>17o</b>	$0.80 \pm 0.17$
<b>17d</b>	$0.62 \pm 0.13$	<b>17p</b>	$1.12 \pm 0.23$
<b>17e</b>	$0.57 \pm 0.09$	<b>17q</b>	$0.73 \pm 0.16$
<b>17f</b>	$0.38 \pm 0.05$	<b>17r</b>	<i>b</i>
<b>17g</b>	$0.16 \pm 0.04$	<b>17s</b>	$1.06 \pm 0.20$
<b>17h</b>	$0.18 \pm 0.04$	<b>17t</b>	$1.47 \pm 0.36$
<b>17i</b>	$0.43 \pm 0.05$	<b>17u</b>	$0.71 \pm 0.19$
SAHA	$0.19 \pm 0.03$		

<sup>a</sup>Assays were performed in replicate ( $n \geq 2$ ). Data are shown as means  $\pm$  SDs. <sup>b</sup>Compound **17r** was undissolved under our test condition.

### Scheme 2. Synthesis of Compounds **17a–u**<sup>a</sup>



<sup>a</sup>Reagents and conditions: (a) (i) TFA,  $\text{Et}_3\text{N}$ ,  $\text{DCM}$ ; (ii)  $\text{R}'\text{COOH}$ , TBTU,  $\text{Et}_3\text{N}$ ,  $\text{THF}$ , 52–75% for two steps. (b) (i) TFA,  $\text{Et}_3\text{N}$ ,  $\text{DCM}$ ; (ii)  $\text{R}'\text{SO}_2\text{Cl}$ ,  $\text{Et}_3\text{N}$ ,  $\text{DCM}$ , 60–76% for two steps. (c)  $\text{NH}_2\text{OK}$ ,  $\text{CH}_3\text{OH}$ , 31–54%.

activities of compounds **17c**, **17g**, **17h**, **17j**, and **17k** were comparable, even more potent than that of SAHA. However, replacing the Boc group of **14a** with valproyl group (**17a**), unnatural amino acid residues (**17o–q**) and sulfur group (**17r–u**) were detrimental to activity.

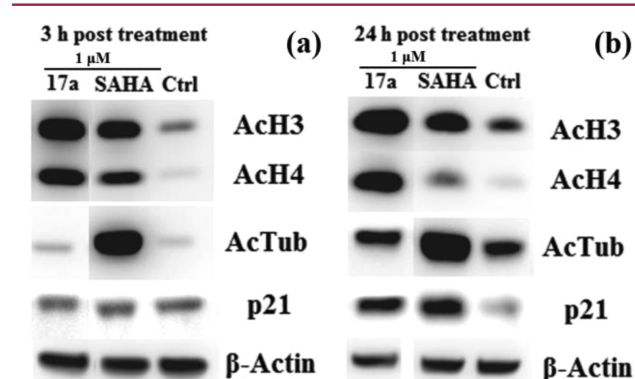
To characterize HDACs isoform selectivity of these analogues, representative compound **17a** with moderate total HDACs inhibitory activity was tested against HDAC1, HDAC2, HDAC3, and HDAC6 using acetylated substrate. Besides, the class IIa inhibitory activity was evaluated against MDA-MB-231 cell lysate using class IIa-specific trifluoroacetylated substrate.<sup>16</sup> Results in Table 2 showed that **17a** displayed modest preference for HDAC1 and HDAC3 over HDAC2 and HDAC6 but exhibited no obvious inhibition against class IIa HDACs up to  $10 \mu\text{M}$ . The overall selectivity profile of **17a** was

Table 2. HDACs Isoform Selectivity of 17a and SAHA<sup>a</sup>

compd	class I			class IIb	class IIa
	HDAC1	HDAC2	HDAC3	HDAC6	cell lysate
17a	0.39 ± 0.12	1.42 ± 0.06	0.28 ± 0.13	0.94 ± 0.14	NA <sup>b</sup>
SAHA	0.076 ± 0.011	0.256 ± 0.003	0.028 ± 0.011	0.118 ± 0.012	NA <sup>b</sup>

<sup>a</sup>Assays were performed in replicate ( $n \geq 2$ ). IC<sub>50</sub> ( $\mu\text{M}$ ) values are shown as means  $\pm$  SDs. <sup>b</sup>NA, not active at 10  $\mu\text{M}$ .

similar to that of SAHA, which was in line with literature information.<sup>17</sup>



**Figure 2.** Western blot analysis of acetylated tubulin, acetylated histone H3, acetylated histone H4, and p21 in MDA-MB-231 cell lines after 3 (a) and 24 h (b) of treatment with compounds at 1  $\mu\text{M}$ .  $\beta$ -Actin was used as a loading control.

Compound 17a was confirmed to be cell permeable and able to inhibit intracellular and even nuclear HDACs by monitoring the acetylation levels of tubulin (target of HDAC6) and histones H3 and H4 (targets of HDAC1 and HDAC2) in the MDA-MB-231 cell line. Moreover, the effect on the expression level of the cyclin-dependent kinase (CDK) inhibitor p21 was also investigated (Figure 2). Although the inhibition against class I HDACs of 17a was inferior to that of SAHA (Table 2), its effects on the levels of histone acetylation and p21 expression at the concentration of 1  $\mu\text{M}$  were comparable and even superior to SAHA, especially after 24 h of treatment. However, 17a had almost no effect on acetylated tubulin level as compared with SAHA at both time points. There was a possible explanation that the intracellular amide hydrolysis of 17a could release compound 15 and valproic acid (VPA), which is a clinical HDACI with class I selectivity.<sup>18</sup> Therefore, 17a and its intracellular metabolites (15 and VPA) could exhibit synergistic inhibition against class I HDACs but not HDAC6. It was noteworthy that the effect on histone H4 acetylation of SAHA decreased with time, while the effect on histone H4 acetylation of 17a was maintained and even increased. This indicated that 17a might be promising in tumor prevention and treatment because it has been shown that global hypoacetylation of histone H4 is a common hallmark of cancer and changes in H4 acetylation happen early in tumorigenesis.<sup>19</sup> The exact intracellular mechanism of 17a needs further research.

Compound 17a and five other analogues with comparable total HDACs inhibitory activity to SAHA were selected to test their effects on tumor cell viability. The results in Table 3 showed that the antiproliferative activities of these derivatives were similar to those of SAHA. It was intriguing that although total HDACs inhibition of 17a was inferior, its cellular potency

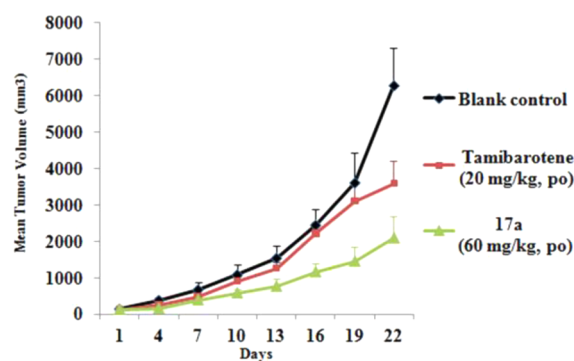
Table 3. In Vitro Antiproliferative Activity of Representative Compounds

compd	IC <sub>50</sub> ( $\mu\text{M}$ ) <sup>a</sup>					
	U937	PC-3	A549	ES-2	MDA-MB-231	HCT116
17a	1.8	3.7	4.4	5.4	3.1	5.5
17c	3.1	10.5	11.8	29.2	7.2	6.0
17g	2.2	10.4	4.2	25.1	4.5	3.8
17h	2.2	5.8	1.6	4.4	6.8	5.9
17j	2.7	5.4	7.0	8.9	7.2	2.4
17k	3.9	8.2	13.5	9.5	11.7	9.4
SAHA	2.3	9.9	3.8	12.7	5.6	6.0

<sup>a</sup>Values are the means of at least two experiments. The SD values are <20% of the mean.

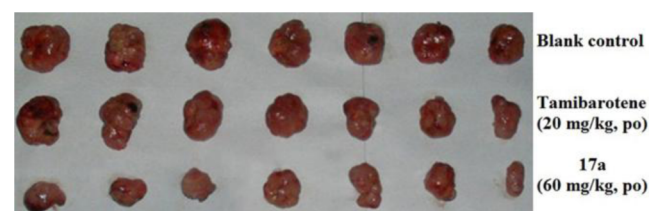
was comparable and even superior, which was in line with our Western blot analysis results.

Among these tested tumor cell lines, human leukemic monocyte lymphoma (U937) was the most sensitive to our HDACI. To preliminarily investigate if our newly designed compounds were active in vivo, representative compound 17a was progressed to a U937 xenograft model using Tamibarotene as a positive control. Mice were treated once daily by oral gavage for 3 weeks. The tumor growth curve depicted in Figure 3 and the final tumor tissue size visualized in Figure 4 explicitly



**Figure 3.** Growth curve of implanted U937 xenograft in nude mice (seven mice per group). Data are expressed as the mean  $\pm$  SD.

showed that compound 17a exhibited potent oral antitumor activity. Tumor growth inhibition (TGI) and relative increment



**Figure 4.** Picture of dissected U937 tumor tissues.

ratio (T/C) were used as the indicators to evaluate the antitumor effects in tumor weight and tumor volume, respectively. Our calculated results revealed that the *in vivo* antitumor activity of **17a** (TGI = 53%, T/C = 42%) was statistically significant ( $P < 0.05$ ), while the potency of tamibarotene (TGI = 33%, T/C = 85%) was not statistically significant. In the mice group treated by **17a**, no significant body weight loss and no evident toxic signs in liver and spleen were detected.

In conclusion, we designed and synthesized a novel series of *N*-hydroxycinnamide-based HDACs with an indole-containing cap group, among which compounds **17c**, **17g**, **17h**, **17j**, and **17k** exhibited similar HDACs inhibition and *in vitro* antitumor potency to SAHA. Our further research focused on **17a** revealed several interesting results, which deserve a detailed mechanism study. Importantly, although its HDACs inhibitory activity was moderate among these analogues, **17a** exhibited potent *in vitro* and *in vivo* antitumor activity. Currently, a detailed activity evaluation and mechanism study of **17a** and other more potent analogues are underway in our laboratory.

## ■ ASSOCIATED CONTENT

### ● Supporting Information

Experimental procedures for compound synthesis, HDACs inhibition fluorescence assay, *in vitro* antiproliferative assay, Western blot analysis, *in vivo* antitumor assay, and characterization data for new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Author Contributions

<sup>†</sup>These two authors contributed equally.

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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

HDACs, histone deacetylases; HDACIs, histone deacetylase inhibitors; SAR, structure–activity relationship; Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; THF, tetrahydrofuran; PTSA, *p*-toluenesulfonic acid; DEAD, diethyl azodicarboxylate; TFA, trifluoroacetic acid; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; CDK, cyclin-dependent kinase; VPA, valproic acid

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