

Synthesis and Biological Evaluation of *N*-(Aminopyridine) Benzamide Analogues as Histone Deacetylase Inhibitors

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A series of benzamide-based histone deacetylases (HDACs) inhibitors possessing *N*-(aminopyridine) residue as the zinc binding site of HDAC were synthesized and evaluated. Among these derivatives, compounds with *N*-(2-amino-4-pyridine) benzamide moiety have been found as the most potent ones. Moreover, introduction of appropriate substituents on the terminal aryl group acting as the surface-recognition domain could significantly improve the antiproliferative activity. In particular, the compound **4k** possessed favorable pharmacokinetic characteristics and exhibited potent antitumor activity on xenograft model in mice at well tolerated doses, thus suggesting a good therapeutic index.

Key Words : *N*-(Aminopyridine) benzamide, HDAC inhibition, Anticancer activity

Introduction

Histone deacetylases (HDACs) catalyze removal of acetyl groups from lysine residues of the core nucleosomal histones.¹⁻³ The acetylation of the core histones closely correlates with transcriptional activity of certain genes, and deacetylation by HDACs is generally associated with transcriptional repression.⁴⁻⁶ Overexpression of HDACs leads to epigenetic inactivation of apoptotic signaling pathways, cell cycle regulators and tumor suppressor genes.^{7,8} These observations suggest that HDACs inhibition provides an opportunity to reverse epigenetic defects that lead to disease and represents a promising new strategy for the treatment of cancer.^{9,10}

There are 11 zinc-dependent HDAC enzymes characterized to date, divided into three classes, class I (HDACs 1-3 and 8), class II a/b (HDACs 4-7,9 and 10) and class IV (HDAC 11).^{11,12} While the biological functions of many HDACs subtypes are still being defined, there is compelling evidence that class I enzymes regulate cell proliferation and therefore are viable targets for cancer therapeutics.¹³⁻²⁰

In late 2006, SAHA (vorinostat, ZolinzaTM) became the first HDACs inhibitor to gain FDA approval and is used for the treatment of the cutaneous manifestations of T-cell lymphoma (Fig. 1).²¹ Like many inhibitors using hydroxamate as the zinc-binding moiety, SAHA is not selective among different HDACs. In comparison, HDACs inhibitors containing an aminobenzamide as the zinc-binding group, such as MS-275 (SNDX-275),²² MGCD0103²³ and Chidamide²⁴ (Fig. 1), generally have less HDAC inhibitory activity but with higher class I selectivity. Some of them are under clinical investigation and have been demonstrated *in vivo* antitumor efficacy.

HDACs inhibitors are usually characterized by a general three-piece pharmacophore model, consisting of a zinc binding group and a surface-recognition domain attached to each other *via* a hydrophobic spacer, as shown by Chidamide in Figure 1.^{25,26} To better understand the structure-activity relationship (SAR) and discover novel HDACs inhibitors with high potency and good safety profiles, we embarked on exploration of both the surface-recognition domain and the zinc binding group and on design and synthesis of *N*-(aminopyridine) benzamide analogues. We

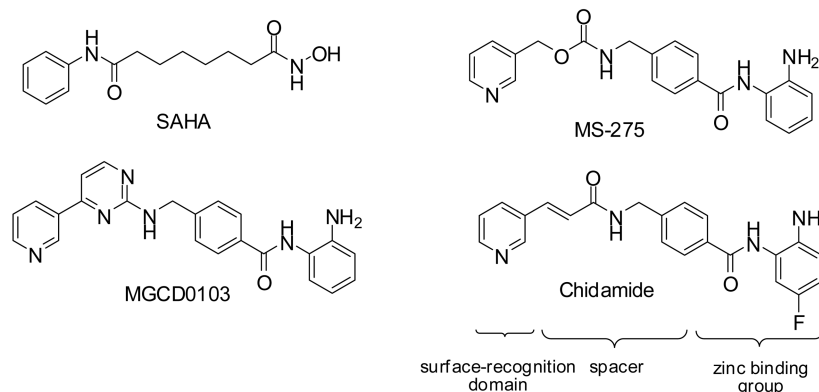
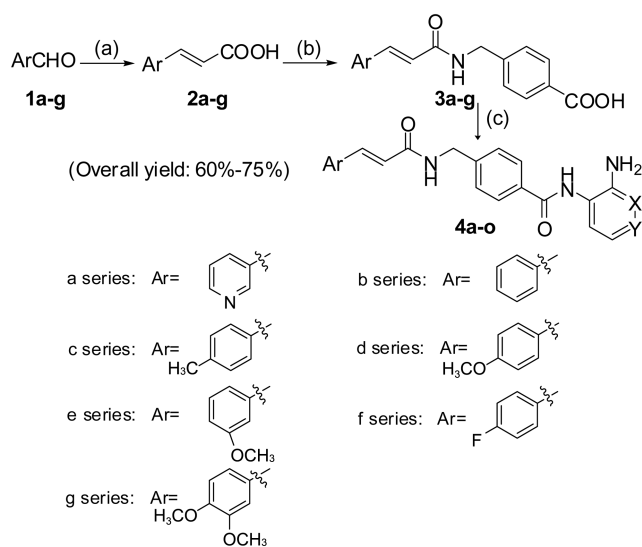


Figure 1. SAHA, MS-275, MGCD0103 and Chidamide.



Scheme 1. Synthesis of the target compounds **4a-o**.

report here the SARs, the anti-proliferative activity and the *in vivo* efficacy of newly synthesized HDAC inhibitors.

Results and Discussion

Chemistry. The synthetic routes of the target compounds are shown in Scheme 1. Aromatic cinnamic acids **2a-g** were condensed from appropriate aromatic aldehydes **1a-g** by Knoevenagel reaction.²⁷ Then, condensation of **2a-g** and 4-(aminomethyl)benzoic acid were performed in THF in the presence of *N,N'*-carbonyldiimidazole (CDI) to give **3a-g** in 74-88% yields. Finally, compounds **3a-g** were treated with CDI at 55-60 °C in THF to provide various imidazolide intermediates, which were subsequently further reacted *in situ* with pyridine-2,3-diamine or pyridine-3,4-diamine in the presence of trifluoroacetic acid (TFA) at room temperature to afford the target compounds **4a-o** (Table 1). The overall yield ranged from 60% to 75%. The final compounds were characterized by ¹H-NMR, mass spectroscopy and element analysis, which were explained in experimental section.

Inhibition of HDAC1. All the newly synthesized compounds were evaluated for their ability to inhibit recombinant human HDAC1 (Table 1). In general, most of these compounds showed their potency as IC₅₀ values close to but lower than Chidamide. For the position of nitrogen atom in the diaminopyridyl group, pyridyl-4-yl derivatives (compounds **4h-n**) were more potent than the corresponding pyridyl-3-yl derivatives (compounds **4a-g**) with the same substitution in the other parts of the molecule. The results in the present manuscript represented that the nitrogen atom at 3- position, in comparison with at 4- position, on the pyridyl ring showed more strong induce effect on the *ortho*-NH₂ group, which led to reduce binding affinity to HDAC1. On the other hand, the electrostatic properties of the substitutions on the aryl moiety as the surface-recognition domain showed great effect on the histone deacetylation activities. For instance,

Table 1. Newly synthesized HDAC inhibitors and their inhibitory activity on HDAC1

Compounds	Ar	X	Y	HDAC1 inhibition IC ₅₀ (μM)
Chidamide				2.7
4a		N	C	54
4b		N	C	40
4c		N	C	6.9
4d		N	C	7.2
4e		N	C	32
4f		N	C	9.8
4g		N	C	24
4h		C	N	18
4i		C	N	33
4j		C	N	4.0
4k		C	N	3.3
4l		C	N	7.8
4m		C	N	7.8
4n		C	N	6.5

compared with the phenyl group, introduction of electron-donating methyl or methoxyl group on the phenyl ring at the C4 position could significantly improve the inhibitory activity against HDAC1 (compounds **4c**, **4d**, **4f** vs **4b** and **4j**, **4k**, **4m** vs **4i**). Meanwhile, a *para*-substituent, in comparison with a *meta*-substituent, on the phenyl ring enhanced (compare **4d** with **4e**, and **4k** with **4l**) the HDAC1 inhibition. Moreover, the size of the substituents had little effect on

Table 2. Effect of compounds **4j-n** on cytotoxicity of human cancer cell lines

Compounds	IC ₅₀ (μM)				
	PC-3	MDA-MB-435S	Hut78	K562	Jurkat E6-1
4j	4.3	5.7	2.1	24	2.2
4k	1.1	6.3	2.3	8.6	1.7
4l	2.3	15	3.5	17	2.1
4m	1.2	13	4.1	20	2.5
4n	6.4	> 30	8.9	> 30	9.3

HDAC1 inhibitory activity. Analogs with smaller substituents on the 3-position of the phenyl group generally exhibited comparable inhibitory activity as those with larger substituents (compare **4e** with **4g** and **4l** with **4n**). Interestingly, analogs containing 3,4-diaminopyridyl moiety stood out as the potent HDAC1 inhibitors with their IC₅₀ ranging from 3.3 μM (**4k**) to 33 μM (**4i**).

Cell Proliferation Study. In our efforts in discovery of anticancer drugs, we targeted HDAC1 in our design strategy because of the linkage between inhibition of this enzyme and histone hyperacetylation and cell proliferation. Thus, we selected the more potent compounds **4j-n** containing 3,4-diaminopyridyl residue and evaluated their inhibitory effect on cell proliferation against a panel of human cancer cells: prostate carcinoma (PC-3), breast carcinoma (MDA-MB-435S), and leukemia (Hut78, K562 and Jurkat E6-1). As shown in Table 2, the *N*-(2-amino-4-pyridine) benzamide series compounds **4j-n** had the significant potency on cell proliferation against PC-3 cells, Hut78 cells and Jurkat E6-1 cells with low IC₅₀ values, ranging from 1.1 μM (**4k**, PC-3) to 9.3 μM (**4n**, Jurkat E6-1).

Installation of methoxyl group on the phenyl ring of the surface-recognition domain at meta-position (compare **4k** with **4l**) led to a little loss in potency, while substitution with fluorine or methyl group was allowed with little penalty (compounds **4k** vs **4j**, **4m**). In contrast with previously noted SARs with little effect on enzymatic inhibition, introduction of larger substituents led to a significant drop in antiproliferative potency (compounds **4j-m** vs **4n**). Among them, compound **4k** exhibited good antiproliferative activity against the growth of human prostate carcinoma cell line PC-3 (IC₅₀ = 1.1 μM) and acute T cell leukemia cell line Jurkat E6-1 (IC₅₀ = 1.7 μM). Therefore, **4k** was selected for further evaluation.

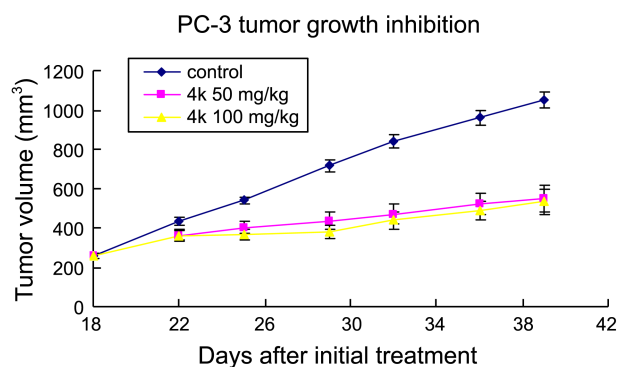
Pharmacokinetic Study of Lead 4k. The evaluation of compound **4k** were carried out using female Sprague Dawley rats after single iv (2 mg/kg) and po (10 mg/kg) administration. The pharmacokinetic parameters are shown in Table 3. The terminal phase half-life after iv dosing was 1.27 h in rats, and the clearance was found to be at 1.18 L/h/kg. The compound was quickly absorbed after oral dosing, with a T_{max} of 0.5 h. The oral bioavailability was found to be 28.6% in rats.

Antitumor Study on Lead 4k. Given these promising pharmacokinetic parameters, compound **4k** was evaluated *in*

Table 3. Pharmacokinetic evaluation of compound **4k**^a

Compound	T _{1/2} iv (h)	CL iv (L/h/kg)	T _{max} po (h)	C _{max} po (μg/L)	AUC po (h*μg/L)	F (%)
4k	1.27	1.18	0.50	1756	7495	28.6

^aFor pharmacokinetic study, blood was collected from rats at various time points up to 6 h, and plasma samples were analyzed using an Agilent 1200 HPLC system coupled with Agilent 6410 B triple quadrupole mass spectrometer. A solution of 0.05 N HCl in saline was used as the vehicle for both iv and po dosing.

**Figure 2.** Antitumor efficacy of compound **4k** in the PC-3 xenograft models.

in vivo in PC-3 mouse xenograft model of cancer (Fig. 2). At a dose of 50 mg/kg administered ig, the inhibitor was well tolerated, and a 37% tumor growth inhibition (TGI) was observed without significant weight loss. As the dose of 100 mg/kg ig, we found that compound **4k** showed no statistically different effect on tumor growth (TGI=39%). However, compound **4k** was still well tolerated at the higher dose and showed no significant loss of body weight in this xenograft models (data not shown).

In conclusion, several HDAC1 inhibitors with *N*-(aminopyridine) benzamide moiety was designed and synthesized. Some of these compounds exhibited *in vitro* antiproliferative activities in numerous human cancer cells. The present study indicates that the zinc binding group and the surface-recognition domain are two pharmacophores which are sensitive to changes and substitutions. Especially, the steric hindrance and electrophilic nature of the surface-recognition domain constitute the most important factors to be taken into consideration during the design of potent benzamide-derived analogues. As the most promising HDAC1 inhibitor among the newly synthesized, compound **4k** displayed good *in vitro* HDAC1 inhibition prole and possessed favorable pharmacokinetic characteristics. Further more, compound **4k** shows significant antitumor activity in the human cancer (PC-3) xenograft model without signs of adverse effects. These results represent an important step toward the development of novel HDAC1 inhibitors with favorable drug-like characteristics as cancer therapeutics.

Experimental Section

Melting points were determined on a Yanaco MP-S3

micro-melting point apparatus and were not corrected. Mass spectra were recorded on a Finnign-MAT 212 spectrometer. $^1\text{H-NMR}$ spectra were recorded on a Varian INOVA 400 (400 MHz) spectrometers with tetramethylsilane as an internal standard. Elemental analyses were obtained with a Carlo Erba EA 1108 instrument. All the solvents were purified before use by routine techniques.

General Synthesis of Aromatic Cinnamic Acids 2a-g from Appropriate Aromatic Aldehydes 1a-g. Appropriate aromatic aldehydes **1a-g** (10 mmol) and malonic acid (1.04 g, 10 mmol) were dissolved in pyridine (0.79 g, 10 mmol), and piperidine (0.1 mL) was added. The mixture was reuxed for 2-2.5 h. After cooling, the resultant mixture was acidified with 1 N HCl to pH 6. Then the precipitate was ltered, dried and recrystallized from ethanol to give the corresponding cinnamic acid **2a-g**.

General Procedure for Compounds 3a-g from Appropriate Aromatic Cinnamic Acids 2a-g. To a suspension of CDI (1.30 g, 8 mmol) in THF (5 mL) was added the corresponding **2a-g** (8.0 mmol) in THF (5 mL), and the mixture was stirred for 1.5 h at rt. The resulting solution was added to a solution of NaOH (0.32 g, 8 mmol) and 4-(aminomethyl)benzoic acid (1.21 g, 8 mmol) in water (100 mL). After stirring for 4 h at rt, the solution was acidied with HCl (pH 5) to precipitate a white solid which was collected by ltration, washed with water (100 mL) and methanol (50 mL), respectively, and dried to give the pure benzoic acid derivative **3a-g**.

Preparation of (E)-N-(2-Aminophenyl)-4-((3-(pyridin-3-yl)acrylamido)methyl)-benzamide (4a). To a suspension of **3a** (1.41 g, 5 mmol) in THF (10 mL) was added CDI (0.97 g, 6 mmol), and the mixture was stirred for 1.5 h at 60 °C. After formation of acylimidazole the clear solution was cooled to rt. To this were added pyridine-2,3-diamine (1.20 g, 11 mmol) and TFA (0.35 mL, 4.4 mmol) and then stirred for 16 h. The reaction mixture was evaporated to remove THF and the crude product was stirred in hexane (10 mL) for 1 h and ltered and dried. The residue was triturated in dichloromethane twice to afford pure **4a** as white powder 1.56 g, 84% yield: mp 165-167 °C. ESI-MS m/z 373.9 $[\text{M}+\text{H}]^+$. $^1\text{H-NMR}$ (400 MHz, DMSO- d_6) δ 4.49 (d, 2H, $J = 6.0$ Hz, NHCH_2), 5.71 (s, 2H, PyNH_2), 6.62 (dd, 1H, $J = 4.8, 7.6$ Hz, Ar-H), 6.82 (d, 1H, $J = 16.0$ Hz, CHCHCO), 7.42-7.46 (m, 3H, Ar-H), 7.52 (d, 1H, $J = 16.0$ Hz, CHCHCO), 7.56 (m, 1H, Ar-H), 7.86 (m, 2H, Ar-H), 7.95 (d, 1H, $J = 8.4$ Hz, Ar-H), 8.00 (m, 1H, Ar-H), 8.55 (dd, 1H, $J = 1.2, 4.8$ Hz, Ar-H), 8.71 (t, 1H, $J = 6.0$ Hz, NHCH_2), 8.77 (br s, 1H, Ar-H), 9.55 (s, 1H, CONH). Anal. calcd. for $\text{C}_{21}\text{H}_{19}\text{N}_5\text{O}_2$: C, 67.55; H, 5.13. found: C, 67.52; H, 5.11.

Preparation of (E)-N-(2-Aminopyridin-3-yl)-4-(cinnamamidomethyl)benzamide (4b). Compound **4b** was synthesized by a procedure similar to that described for **4a**: yield 79%. mp 195-196 °C. ESI-MS m/z 411.1 $[\text{M}+\text{K}]^+$. $^1\text{H-NMR}$ (400 MHz, DMSO- d_6) δ 4.48 (d, 2H, $J = 6.0$ Hz, NHCH_2), 5.69 (s, 2H, PyNH_2), 6.60 (dd, 1H, $J = 4.8, 7.6$ Hz), 6.71 (d, 1H, $J = 15.6$ Hz, CHCHCO), 7.37-7.44 (m, 5H, Ar-H), 7.48 (d, 1H, $J = 15.6$ Hz, CHCHCO), 7.56-7.59 (m, 3H, Ar-H),

7.83 (dd, 1H, $J = 1.2, 5.2$ Hz, Ar-H), 7.95 (d, 2H, $J = 8.0$ Hz, Ar-H), 8.66 (t, 1H, $J = 5.6$ Hz, NHCH_2), 9.56 (s, 1H, CONH). Anal. calcd. for $\text{C}_{22}\text{H}_{20}\text{N}_4\text{O}_2$: C, 70.95; H, 5.41. found: C, 70.92; H, 5.38.

Preparation of (E)-N-(2-Aminopyridin-3-yl)-4-((3-*p*-tolylacrylamido)methyl)benzamide (4c). Compound **4c** was synthesized by a procedure similar to that described for **4a**: yield 81%. mp 205-206 °C. ESI-MS m/z 385.1 $[\text{M}+\text{H}]^+$. $^1\text{H-NMR}$ (400 MHz, DMSO- d_6) δ 2.33 (s, 3H, PhCH_3), 4.48 (d, 2H, $J = 5.6$ Hz, NHCH_2), 5.71 (s, 2H, PyNH_2), 6.62 (m, 1H, Ar-H), 6.66 (d, 1H, $J = 15.6$ Hz, CHCHCO), 7.23 (d, 2H, $J = 8.0$ Hz, Ar-H), 7.41-7.48 (m, 5H), 7.57 (d, 1H, $J = 8.0$ Hz, Ar-H), 7.86 (d, 1H, $J = 3.2$ Hz, Ar-H), 7.95 (d, 2H, $J = 7.6$ Hz, Ar-H), 8.60 (t, 1H, $J = 6.0$ Hz, NHCH_2), 9.56 (s, 1H, CONH). Anal. calcd. for $\text{C}_{23}\text{H}_{22}\text{N}_4\text{O}_2$: C, 71.48; H, 5.74. found: C, 71.42; H, 5.71.

Preparation of (E)-N-(2-Aminopyridin-3-yl)-4-((3-(4-methoxyphenyl)acrylamido)-methyl)benzamide (4d). Compound **4d** was synthesized by a procedure similar to that described for **4a**: yield 78%. mp 165-166 °C. ESI-MS m/z 403.4 $[\text{M}+\text{H}]^+$. $^1\text{H-NMR}$ (400 MHz, DMSO- d_6) δ 3.79 (s, 3H, PhOCH_3), 4.47 (d, 2H, $J = 5.6$ Hz, NHCH_2), 5.70 (s, 2H, PyNH_2), 6.56 (d, 1H, $J = 15.6$ Hz, CHCHCO), 6.62 (dd, 1H, $J = 4.8, 7.6$ Hz, Ar-H), 6.98 (d, 1H, $J = 8.8$ Hz, Ar-H), 7.41-7.46 (m, 5H), 7.51-7.57 (m, 3H, Ar-H), 7.85 (dd, 1H, $J = 1.2, 4.8$ Hz, Ar-H), 7.94 (d, 1H, $J = 8.4$ Hz, Ar-H), 8.55 (t, 1H, $J = 5.6$ Hz, NHCH_2), 9.56 (s, 1H, CONH). Anal. calcd. for $\text{C}_{23}\text{H}_{22}\text{N}_4\text{O}_3$: C, 68.64; H, 5.51. found: C, 68.62; H, 5.48.

Preparation of (E)-N-(2-Aminopyridin-3-yl)-4-((3-(3-methoxyphenyl)acrylamido)-methyl)benzamide (4e). Compound **4e** was synthesized by a procedure similar to that described for **4a**: yield 80%. mp 177-178 °C. ESI-MS m/z 402.8 $[\text{M}+\text{H}]^+$. $^1\text{H-NMR}$ (400 MHz, DMSO- d_6) δ 3.79 (s, 3H, PhOCH_3), 4.47 (d, 2H, $J = 6.0$ Hz, NHCH_2), 5.70 (s, 2H, PyNH_2), 6.62 (dd, 1H, $J = 4.8, 7.6$ Hz, Ar-H), 6.71 (d, 1H, $J = 16.0$ Hz, CHCHCO), 6.95 (dd, 1H, $J = 2.4, 8.0$ Hz, Ar-H), 7.13-7.16 (m, 2H, Ar-H), 7.33 (t, 1H, $J = 8.0$ Hz, Ar-H), 7.41 (m, 3H, Ar-H), 7.45 (d, 1H, $J = 16.0$ Hz, CHCHCO), 7.55 (d, 1H, $J = 6.0$ Hz, Ar-H), 7.85 (dd, 1H, $J = 1.6, 4.8$ Hz, Ar-H), 7.94 (d, 1H, $J = 7.6$ Hz, Ar-H), 8.63 (t, 1H, $J = 6.0$ Hz, NHCH_2), 9.55 (s, 1H, CONH). Anal. calcd. for $\text{C}_{23}\text{H}_{22}\text{N}_4\text{O}_3$: C, 68.64; H, 5.51. found: C, 68.60; H, 5.49.

Preparation of (E)-N-(2-Aminopyridin-3-yl)-4-((3-(4-fluorophenyl)acrylamido)-methyl)benzamide (4f). Compound **4f** was synthesized by a procedure similar to that described for **4a**: yield 86%. mp 192-193 °C. ESI-MS m/z 391.2 $[\text{M}+\text{H}]^+$. $^1\text{H-NMR}$ (400 MHz, DMSO- d_6) δ 4.48 (d, 2H, $J = 5.6$ Hz, NHCH_2), 5.70 (s, 2H, PyNH_2), 6.60 (m, 1H, Ar-H), 6.65 (d, 1H, $J = 16.0$ Hz, CHCHCO), 7.24 (m, 2H, Ar-H), 7.41 (d, 2H, $J = 8.0$ Hz, Ar-H), 7.48 (d, 1H, $J = 16.0$ Hz, CHCHCO), 7.55 (d, 1H, $J = 7.6$ Hz, Ar-H), 7.64 (m, 3H, Ar-H), 7.85 (d, 1H, $J = 4.8$ Hz, Ar-H), 7.94 (d, 1H, $J = 8.0$ Hz, Ar-H), 8.63 (t, 1H, $J = 6.0$ Hz, NHCH_2), 9.55 (s, 1H, CONH). Anal. calcd. for $\text{C}_{22}\text{H}_{19}\text{FN}_4\text{O}_2$: C, 67.68; H, 4.91. found: C, 67.64; H, 4.89.

Preparation of (E)-N-(2-Aminopyridin-3-yl)-4-((3-(3,4-dimethoxyphenyl)acrylamido)methyl)benzamide (4g).

Compound **4g** was synthesized by a procedure similar to that described for **4a**: yield 78%. mp 221-223 °C. ESI-MS m/z 470.9 [M+K]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 3.79 (s, 3H, PhOCH₃), 3.81 (s, 3H, PhOCH₃), 4.47 (d, 2H, *J* = 6.0 Hz, NHCH₂), 5.73 (s, 2H, PyNH₂), 6.59-6.64 (m, 2H, CHCHCO, Ar-H), 6.93-7.01 (m, 2H, Ar-H), 7.12-7.18 (m, 2H, Ar-H), 7.40-7.44 (m, 2H, CHCHCO, Ar-H), 7.57 (dd, 1H, *J* = 1.6, 8.0 Hz, Ar-H), 7.85 (dd, 1H, *J* = 1.6, 4.8 Hz, Ar-H), 7.95 (d, 2H, *J* = 8.0 Hz, Ar-H), 8.62 (t, 1H, *J* = 6.4 Hz, NHCH₂), 9.66 (s, 1H, CONH). Anal. calcd. for C₂₄H₂₄N₄O₄: C, 66.65; H, 5.59. found: C, 66.60; H, 5.55.

Preparation of (E)-N-(3-Aminopyridin-4-yl)-4-((3-pyridin-3-yl)acrylamido)methyl)benzamide (4h). Compounds **3a** were treated with CDI and pyridine-3,4-diamine in the presence of TFA by a procedure similar to that described for **4a** to afford the target compound **4h**: yield 82%. mp 187-188 °C. ESI-MS m/z 373.8 [M+H]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 4.49 (d, 2H, *J* = 6.0 Hz, NHCH₂), 5.11 (s, 2H, PyNH₂), 6.81 (d, 1H, *J* = 15.6 Hz, CHCHCO), 7.42-7.45 (m, 4H, Ar-H), 7.52 (d, 1H, *J* = 16.0 Hz, CHCHCO), 7.80 (d, 1H, *J* = 5.2 Hz, Ar-H), 7.93 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.99 (d, 1H, *J* = 8.0 Hz, Ar-H), 8.09 (s, 1H, Ar-H), 8.55 (d, 1H, *J* = 4.8 Hz, Ar-H), 8.62 (t, 1H, *J* = 6.0 Hz, NHCH₂), 8.77 (s, 1H, Ar-H), 9.66 (s, 1H, CONH). Anal. calcd. for C₂₁H₁₉N₅O₂: C, 67.55; H, 5.13. found: C, 67.51; H, 5.09.

Preparation of (E)-N-(3-Aminopyridin-4-yl)-4-(cinnamamidomethyl)benzamide (4i). Compound **4i** was synthesized by a procedure similar to that described for **4h**: yield 81%. mp > 250 °C. ESI-MS m/z 373.1 [M+H]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 4.49 (d, 2H, *J* = 6.0 Hz, NHCH₂), 5.11 (s, 2H, PyNH₂), 6.70 (d, 1H, *J* = 16.0 Hz, CHCHCO), 7.37-7.50 (m, 7H, Ar-H), 7.57 (d, 2H, *J* = 16.0 Hz, CHCHCO), 7.79 (d, 1H, *J* = 5.2 Hz, Ar-H), 7.93 (d, 2H, *J* = 8.4 Hz, Ar-H), 8.10 (s, 1H, Ar-H), 8.66 (t, 1H, *J* = 6.0 Hz, NHCH₂), 9.66 (s, 1H, CONH). Anal. calcd. for C₂₂H₂₀N₄O₂: C, 70.95; H, 5.41. found: C, 70.90; H, 5.37.

Preparation of (E)-N-(3-Aminopyridin-4-yl)-4-((3-*p*-tolylacrylamido)methyl)benzamide (4j). Compound **4j** was synthesized by a procedure similar to that described for **4h**: yield 85%. mp 207-208 °C. ESI-MS m/z 425.1 [M+K]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 2.33 (s, 3H, PhCH₃), 4.49 (d, 2H, *J* = 5.6 Hz, NHCH₂), 5.12 (s, 2H, PyNH₂), 6.66 (d, 1H, *J* = 16.0 Hz, CHCHCO), 7.23 (d, 2H, *J* = 8.0 Hz, Ar-H), 7.43-7.48 (m, 5H), 7.80 (d, 2H, *J* = 4.8 Hz, Ar-H), 7.94 (d, 2H, *J* = 8.0 Hz, Ar-H), 8.09 (s, 1H, Ar-H), 8.62 (t, 1H, *J* = 6.0 Hz, NHCH₂), 9.66 (s, 1H, CONH). Anal. calcd. for C₂₃H₂₂N₄O₂: C, 71.48; H, 5.74. found: C, 71.43; H, 5.70.

Preparation of (E)-N-(3-Aminopyridin-4-yl)-4-((3-(4-methoxyphenyl)acrylamido)methyl)benzamide (4k). Compound **4k** was synthesized by a procedure similar to that described for **4h**: yield 88%. mp 234-235 °C. ESI-MS m/z 403.4 [M+H]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 3.79 (s, 3H, PhOCH₃), 4.48 (d, 2H, *J* = 6.4 Hz, NHCH₂), 5.12 (s, 2H, PyNH₂), 6.56 (d, 1H, *J* = 15.6 Hz, CHCHCO), 6.99 (d, 2H, *J* = 8.8 Hz, Ar-H), 7.42-7.45 (m, 4H), 7.52 (d, 2H, *J* = 8.0 Hz, Ar-H), 7.80 (d, 1H, *J* = 5.2 Hz, Ar-H), 7.93 (d, 2H, *J* = 8.4 Hz, Ar-H), 8.10 (s, 1H, Ar-H), 8.56 (t, 1H, *J* = 5.6 Hz,

NHCH₂), 9.65 (s, 1H, CONH). Anal. calcd. for C₂₃H₂₂N₄O₃: C, 68.64; H, 5.51. found: C, 68.61; H, 5.49.

Preparation of (E)-N-(3-Aminopyridin-4-yl)-4-((3-(3-methoxyphenyl)acrylamido)methyl)benzamide (4l). Compound **4l** was synthesized by a procedure similar to that described for **4h**: yield 76%. mp 200-201 °C. ESI-MS m/z 402.9 [M+H]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 3.80 (s, 3H, PhOCH₃), 4.50 (d, 2H, *J* = 6.0 Hz, NHCH₂), 5.13 (s, 2H, PyNH₂), 6.72 (d, 1H, *J* = 15.6 Hz, CHCHCO), 6.96 (m, 1H, Ar-H), 7.15 (m, 2H, Ar-H), 7.34 (m, 1H, Ar-H), 7.43-7.48 (m, 4H), 7.81 (d, 1H, *J* = 5.2 Hz, Ar-H), 7.94 (d, 2H, *J* = 8.0 Hz, Ar-H), 8.11 (s, 1H, Ar-H), 8.65 (t, 1H, *J* = 5.6 Hz, NHCH₂), 9.67 (s, 1H, CONH). Anal. calcd. for C₂₃H₂₂N₄O₃: C, 68.64; H, 5.51. found: C, 68.62; H, 5.48.

Preparation of (E)-N-(3-Aminopyridin-4-yl)-4-((3-(4-fluorophenyl)acrylamido)methyl)benzamide (4m). Compound **4m** was synthesized by a procedure similar to that described for **4h**: yield 89%. mp > 250 °C. ESI-MS m/z 390.9 [M+H]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 4.49 (d, 2H, *J* = 6.0 Hz, NHCH₂), 5.12 (s, 2H, PyNH₂), 6.66 (d, 1H, *J* = 16.0 Hz, CHCHCO), 7.25 (t, 2H, *J* = 8.8 Hz, Ar-H), 7.43-7.50 (m, 5H), 7.64 (m, 2H, Ar-H), 7.80 (d, 1H, *J* = 5.2 Hz, Ar-H), 7.93 (d, 1H, *J* = 8.0 Hz, Ar-H), 8.10 (s, 1H, Ar-H), 8.64 (t, 1H, *J* = 5.6 Hz, NHCH₂), 9.66 (s, 1H, CONH). Anal. calcd. for C₂₂H₁₉FN₄O₂: C, 67.68; H, 4.91. found: C, 67.62; H, 4.86.

Preparation of (E)-N-(3-Aminopyridin-4-yl)-4-((3-(3,4-dimethoxyphenyl)acrylamido)methyl)benzamide (4n). Compound **4n** was synthesized by a procedure similar to that described for **4h**: yield 89%. mp 195-197 °C. ESI-MS m/z 432.9 [M+H]⁺. ¹H-NMR (400 MHz, DMSO-*d*₆) δ 3.70 (m, 6H, PhOCH₃), 4.45 (d, 2H, *J* = 5.6 Hz, NHCH₂), 5.12 (s, 2H, PyNH₂), 6.59 (d, 1H, *J* = 15.6 Hz, CHCHCO), 6.99 (d, 1H, *J* = 8.0 Hz, Ar-H), 7.13 (d, 1H, *J* = 15.6 Hz, CHCHCO), 7.17 (s, 1H, Ar-H), 7.40-7.44 (m, 4H, Ar-H), 7.80 (d, 1H, *J* = 5.2 Hz, Ar-H), 7.93 (d, 2H, *J* = 8.4 Hz, Ar-H), 8.10 (s, 1H, Ar-H), 8.54 (t, 1H, *J* = 6.0 Hz, NHCH₂), 9.66 (s, 1H, CONH). Anal. calcd. for C₂₄H₂₄N₄O₄: C, 66.65; H, 5.59. found: C, 66.61; H, 5.52.

Assay of HDAC1 Enzyme Activity. HDAC inhibitors were screened against a cloned recombinant human HDAC1 enzyme expressed and purified from a Baculovirus insect cell expression system. For deacetylase assays, 20,000 cpm of the [³H]-metabolically labeled acetylated histone substrate was incubated with 30 μg of the cloned recombinant hHDAC1 for 10 minutes at 37 °C. The reaction was stopped by adding acetic acid (0.04 M, final concentration) and HCl (250 mM, final concentration). The mixture was extracted with ethyl acetate and the released [³H]-acetic acid was quantified by scintillation counting. For inhibition studies, the enzyme was preincubated with compounds at 4 °C for 30 minutes prior to initiation of the enzymatic assay. IC₅₀ values for HDAC enzyme inhibitors were determined by performing dose response curves with individual compounds and determining the concentration of inhibitor producing fifty percent of the maximal inhibition. IC₅₀ values for target compounds are presented in the fifth column of Table 1.

Cell Proliferation Assay. Cells (2000/well) were plated in 96-well tissue culture plates one day before compound treatment. Compounds at various concentrations were added to the cells. The cells were incubated for 72 hours at 37 °C in 5% CO₂ incubator. Thiazolyl Blue Tetrazolium Bromide (MTT; Sigma) was added at a final concentration of 0.5 mg/ml and incubated with the cells for 4 hours before one volume of solubilization buffer (50% *N,N*-dimethylformamide, 20% SDS, pH 4.7) was added into the cultured cells. After overnight incubation, solubilized dye was quantified by colorimetric reading at 570 nM using a reference at 630 nM using an MR700 plate reader (Dynatech Laboratories Inc.). OD values were converted to cell numbers according to a standard growth curve of the relevant cell line. The concentration which reduces cell numbers to 50% of that of solvent treated cells is determined as MTT IC₅₀. IC₅₀ values for representative compounds are presented in Table 2.

In Vivo Antitumor Activity Assay. Animal experiments were performed according to institutional ethical guidelines of animal care. The cells at density of 4×10^7 in 120 μ L rslty implanted sc into the right ank of each nude mice and then allowing to grow to 100-300 mm³. After that, tumor-bearing nude mice were randomly divided in groups (8 mice per group). Control groups were given vehicle alone, and treatment groups received compound **4k** as indicated doses *via ig* administration 7 days per week for 3 weeks. The sizes of the tumors were measured twice per week using microcaliper. The tumor volume (TV) was calculated as: $TV = (\text{length} \times \text{width}^2)/2$. Tumor volume was shown on indicated days as the median tumor volume \pm SE indicated for groups of mice. Percent (%) inhibition values were measured on the nal day of study for drug-treated compared with vehicle-treated mice and are calculated as $100 \times \{1 - [(\text{treated nal day-treated day 1}) / (\text{control nal day-control day 1})]\}$. Signicant differences between the treated versus the control groups ($P \leq 0.001$) were determined using Student's *t*-test.

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