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Design and Synthesis of Novel *N*-(2-aminophenyl)benzamide Derivatives as Histone Deacetylase Inhibitors and Their Antitumor Activity Study

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Histone deacetylases (HDACs) are promising therapeutic targets for cancer therapy because inhibition of HDACs triggers growth arrest or apoptosis of tumor cells. In the present study, a new series of fluorinated *N*-(2-aminophenyl)benzamide derivatives were synthesized to investigate potential inhibition of HDACs and associated anticancer activity. Among the synthesized derivatives, compound **24a** showed potent inhibitory activity of HDACs and higher antitumor efficacy in human cancer cell lines (HCT-116, MCF-7, and A549) compared with SAHA. Moreover, animal studies demonstrated that compound **24a** showed potent *in vivo* antitumor efficacy in an HCT-116 colon cancer xenograft mouse model.

Keywords: Histone deacetylases, HDAC inhibitor, Antitumor effect, Colon cancer

Many cancers are associated with epigenetic mechanisms.[1–3] Post-translational modifications of histones regulate gene transcription have been widely recognized to have a large impact on genetic diseases.[4–6] Histone deacetylases (HDACs) play crucial roles in epigenetic regulation of genes.[7,8] It also has been reported that HDACs function as control of the apoptosis and tumorigenesis.[9,10] Thus, HDAC enzymes have received significant attention as promising drug targets.[11] Isoforms of HDACs are divided into four families. Class I (HDAC 1,2,3,8), class II (class IIa HDAC 4,5,7,9 and class IIb HDAC 6,10), and class IV (HDAC 11) are zinc-dependent HDACs, while class III (SIRT 1–7) is NAD^+-dependent HDACs.[12,13] Isoforms of class I HDAC isoenzymes are associated with tumor cell proliferation. Inhibition of isoform of class I HDACs resulted in cancer apoptosis.[14,15] In addition, HDAC 1 and HDAC 2 have crucial roles in various cancers.[16–18] Therefore, inhibition of HDAC 1 and HDAC 2 could provide benefits for treatment of cancers.[19]

HDAC inhibitors (HDACIs) consists of a cap, a zinc binding group (ZBG), and a linker. Various modifications of these three parts have resulted in chemically different HDAC inhibitors. To date, several HDAC inhibitors types have been developed.[20,21] Hydroxamic acid type HDAC inhibitors were the first compounds used in clinical studies. Vorinostat (SAHA), belinostat (PXD101), and panobinostat (LBH589) have been approved by the US FDA (United States Food and Drug Administration) for treatment of T-cell lymphoma and other cancers.[22–25] However, hydroxamic acid type HDAC inhibitors exhibit unselective HDAC inhibition and generate several side effects.[26,27] Another inhibitor type is the benzamide type HDAC inhibitor. Among these, entinostat (MS-275) has been reported to inhibit several solid cancer cell lines.[28–30] Compared to hydroxamic acid type compounds, benzamide type inhibitors possess selective class I HDAC inhibition (especially HDAC 1 and HDAC 2), with reduced side effects.[31] Development of potent HDAC inhibitors revealed that there is a long internal cavity adjacent to the zinc-binding group.[32,33] Thus, we expected that modifications of the enzyme internal cavity group would increase the binding potency. Besides, it was known that several FDA-approved drugs have fluorinated moiety in their structures.[34,35] and it was reported that incorporation of fluorine into organic molecules could give improvement of drug potency: physicochemical and biological properties such as adsorption, metabolic stability, lipophilicity.[35,36] In the present study, we describe the synthesis of novel fluorinated 2-(aminophenyl)benzamide compounds and evaluation of their potential as novel HDAC inhibitors (Figure 1).

The synthetic route to the novel HDAC inhibitors is shown in Schemes 1 and 2. First, we synthesized the 2-(aminophenyl) benzamide scaffold for the ZBG and linker part using the previously reported procedure.[37] The amine of 4-bromo-2-nitroaniline **1** was protected with a *tert*-butyloxycarbonyl (Boc) protecting group. A Suzuki cross-coupling reaction between the Boc-protected aryl bromide **2** and thiophene-2-boronic acid or benzeneboronic acid gave the two biaryl compounds **3a** and **3b** respectively. Reductive hydrogenation using catalytic palladium on carbon converted the nitro group to an amine group to yield

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compounds **4a** and **4b**. Compounds **4a** and **4b** were condensed with 4-(chloromethyl)benzoyl chloride to give compounds **5a** and **5b**. The Gabriel synthesis was used to transform the chloride group of compounds **5a** and **5b** into a free amine. In this step, nucleophilic addition with potassium phthalimide produced the *N*-alkylphthalimide intermediates, which were reacted with hydrazine to give 4-(aminomethyl)benzamide compounds **6a** and **6b**.

Next, for synthesis of the cap containing a fluorine moiety, 4-(hydroxymethyl)piperdine and *N*-methylethanolamine were first protected using benzylchloroformate to yield compounds **8** and **12**.[38] Conversion of the hydroxyl group to a fluoride group through tosylate intermediates was performed to give compounds **9** and **13**.[39] Hydrogenation afforded fluoroamine compounds **10** and **14**. In the final part of the synthesis, treatment of compounds **10** and **14** with the benzamide scaffolds **5a** and **5b** yielded the tertiary amine compounds **15a**, **15b**, **16a**, and **16b**. Removal of the Boc protecting group with CF_3COOH gave the final products. Additionally, for reaction with compounds **6a** and **6b**, other cap structures were synthesized using a similar protocol. Protection of the carboxylic acid group of methyl hydroxypivalate **19** with benzyl bromide gave compound **20**. Fluorination of compound **20** through intermediate triflate, followed by cleavage of the benzyl group by reductive hydrogenation gave the fluorocarboxylic acid **22**. EDC coupling of compound **22** with the corresponding benzamide scaffolds **6a** and **6b** gave amide compounds **23a** and **23b**, respectively. Deprotection of the Boc protecting group with CF_3COOH yielded the final products (**24a** and **24b**).

Inhibitory activities of all novel compounds against HDAC1 and HDAC2 were evaluated, and structure–activity relationship (SAR) was also investigated. SAHA is a popular HDAC inhibitor, and data from newly prepared benzamide derivatives were compared with those of SAHA in several previously reported studies.[31,40] Thus, in this study, SAHA was employed as a control. As shown in Table 1, *in vitro*

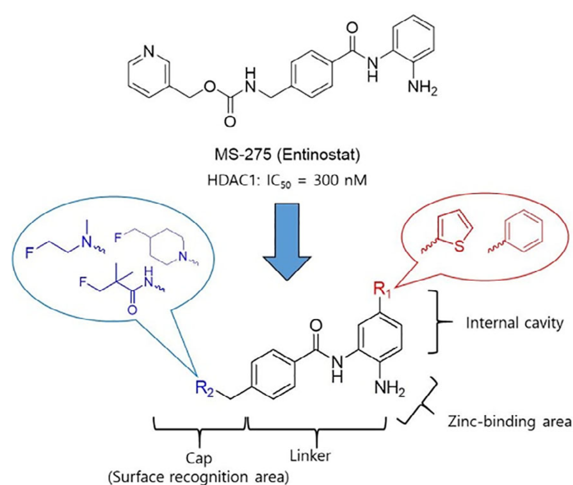
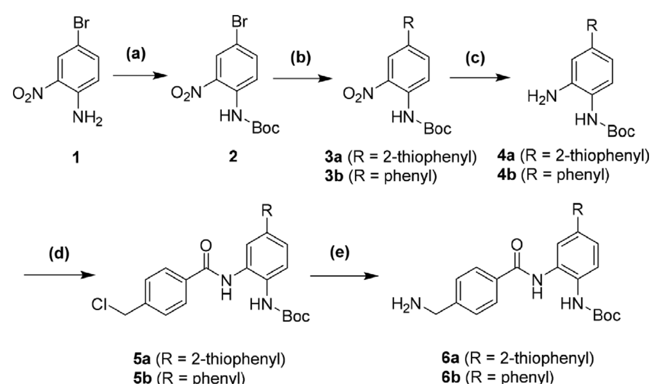
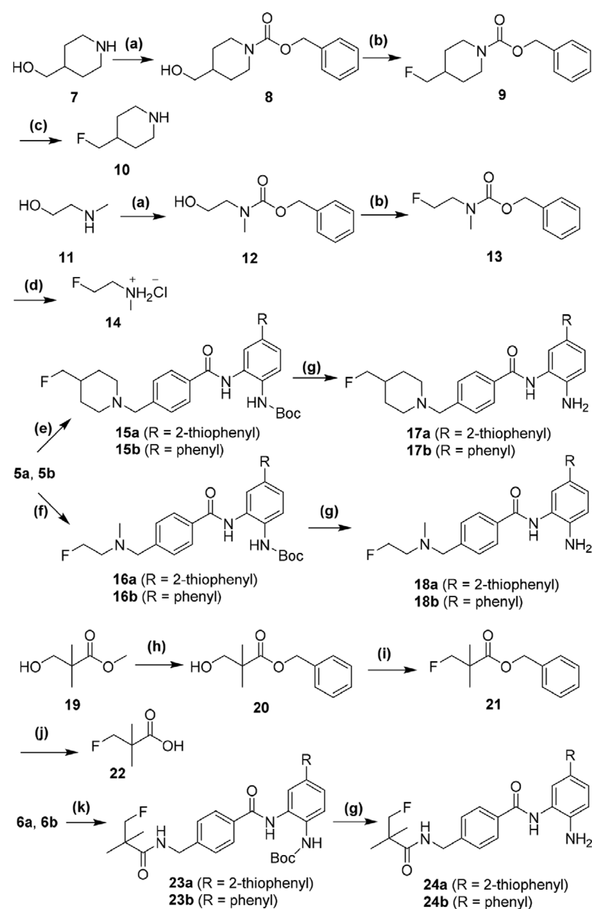


Figure 1. Strategy for development of new fluorinated *N*-(2-aminophenyl)benzamide derivatives as HDAC inhibitors.



Scheme 1. Synthetic scheme of benzamide scaffold. Reaction condition: (a) $(Boc)_2O$, Et_3N, CH_2Cl_2, r.t., 15 h; (b) thiophene-2-boronic acid (**3a**) or benzene-2-boronic acid (**3b**), $Pd(PPh_3)_4$, K_2CO_3, THF, reflux, 15 h; (c) H_2, Pd/C, methanol, r.t., 15 h; (d) 4-(chloromethyl) benzoyl chloride, Et_3N, CH_2Cl_2, r.t., 3 h; (e) 1) potassium phthalimide, DMF, 80°C, 3 h; 2) N_2H_4. H_2O, MeOH, 80°C, 1 h.



Scheme 2. Synthetic scheme for HDAC inhibitors. Reaction condition: (a) benzyl chloroformate, DCM, r.t., 15 h; (b) 1) TsCl, Et_3N, DCM, r.t., 18 h; 2) TBAF, *t*-amylalcohol, 80°C, 8 h; (c) H_2, Pd/C, MeOH, r.t., 15 h; (d) H_2, Pd/C, MeOH/HCl, r.t., 15 h; (e) compound **10**, THF, 70°C, 15 h; (f) compound **14**, MeOH, THF, $NaHCO_3$, 70°C, 15 h; (g) CF_3COOH, CH_2Cl_2, r.t., 5 h; (h) 1) LiOH/MeOH, r.t., 1 h; 2) benzyl bromide, K_2CO_3, DMF, r.t., 18 h; (i) 1) Tf_2O, CH_2Cl_2, r.t., 30 min; 2) TBAF, CH_2Cl_2, r.t., 15 h; (j) H_2, Pd/C, MeOH, r.t., 15 h; (k) compound **22**, EDC, DMAP, CH_2Cl_2, r.t., 15 h.

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HDAC inhibitory assay indicated all synthesized compounds showed nanomolar HDAC1 and HDAC2 inhibition activity. In particular, compound **24a** displayed the most potent anti-HDAC activity against HDAC1 and HDAC2 (IC_{50} = 28.1 nM for HDAC1 and 158 nM for HDAC2). In additions, introduction of the thiophenyl moiety (**17a**, **18a**, and **24a**) into the internal cavity increased HDAC inhibitory activity (1.2- to 1.5-fold) compared to a phenyl group (**17b**, **18b**, and **24b**), which suggests that the thiophenyl group provides favorable interactions with the HDAC enzymes due to more suitable fit with the internal cavity than does the phenyl group. The incorporation of *tert*-butyl amide moiety at cap position (compounds **24a** and **24b**) showed better HDAC inhibition potency (1.5- to 1.9-fold) than other tertiary amine scaffolds (compounds **17a**, **17b**, **18a**, and **18b**). The assay results showed that all compounds had better HDAC1 inhibitory activity than that against HDAC2. In particular, compounds **24a** and **24b** showed around 9-fold and 5-fold selectivity, respectively, for HDAC 1 over HDAC 2. The new compounds were further tested against HDAC 4 and HDAC 6. All tested compounds showed significantly lower HDAC 4 and HDAC 6 inhibition compared to HDAC 1 and HDAC 2 activity, suggesting that the newly prepared compounds exhibited high HDAC selectivity.

Antitumor activities of the newly prepared compounds on human cancer cell lines including HCT-116, MDA-MB-231, MCF-7, and A549 cell lines were determined by MTT assay, and the results are summarized in Table 2. Among these compounds, **24a** displayed the most potent antitumor activity against most of the cancer cell lines, with IC_{50} values of 1.32, 2.07, and 2.62 μM, against HCT-116, MCF-7, and A549, respectively, which was better cancer inhibitory activities than the positive control SAHA in HCT-116, MCF-7, and A549 cell lines. Compounds **17a** and **18a** bearing tertiary amine structure also exhibited potent antitumor activities, with IC_{50} values of 1.53 and 1.61 μM in HCT116 cancer cells and 2.45 and 3.27 μM in MCF-7 cancer cells, respectively. In addition, incorporating thiophenyl group into the terminal of internal cavity led to better antitumor activity than phenyl group.

Based on the result of the HDAC inhibitory assay and the *in vitro* antiproliferation assay, compound **24a** was evaluated for *in vivo* antitumor activity in the colon carcinoma xenograft HCT116. Compound **24a** and SAHA were administered orally to mice at a dose of 100 mg/kg/day.[42] As shown in Figure S1, compound **24a** provided significant antitumor activity and suppressed HCT-116 tumor growth of the xenografts compared to SAHA.

Table 1. *In vitro* HDAC inhibition of **17a-b**, **18a-b**, and **24a-b** [a].

Compound	IC_{50} (nM)			
	HDAC1	HDAC2	HDAC4	HDAC6
17a	49.6 ± 3.12	231 ± 34.65	>20 000	>20 000
17b	63.4 ± 4.05	225 ± 30.69	>20 000	>20 000
18a	53.5 ± 3.87	220 ± 29.18	>20 000	>20 000
18b	66.1 ± 4.36	254 ± 48.86	>20 000	>20 000
24a	28.1 ± 2.74	158 ± 16.12	>20 000	>20 000
24b	42.3 ± 3.26	208 ± 28.34	>20 000	>20 000
SAHA[b]	115.9 ± 5.51	162.9 ± 2.05	>20 000	78.3 ± 8.96

[a] All experiments were independently performed in replicated (N = 3).
[b] Data from reference 41.

Table 2. Effect of **17a-b**, **18a-b**, and **24a-b** on cytotoxicity of cancer cell lines[a].

Compound	IC_{50} (μM)			
	HCT116	MDA-MB-231	MCF-7	A549
17a	1.53 ± 0.22	2.53 ± 0.23	2.45 ± 0.28	3.96 ± 0.35
17b	2.16 ± 0.30	4.67 ± 0.30	3.84 ± 0.39	5.91 ± 0.88
18a	1.61 ± 0.25	3.18 ± 0.24	3.27 ± 0.40	3.92 ± 0.31
18b	2.38 ± 0.32	4.78 ± 0.46	3.59 ± 0.36	5.83 ± 0.79
24a	1.32 ± 0.24	2.27 ± 0.21	2.07 ± 0.18	2.62 ± 0.27
24b	1.63 ± 0.27	2.62 ± 0.23	2.64 ± 0.29	3.67 ± 0.58
SAHA[b]	2.81	1.72	3.78	3.9

[a] All experiments were independently performed in replicated (N = 3).
[b] Data from reference 42.

To predict the binding mode of HDACs inhibitor, compound **24a** was utilized for molecular docking study, Several HDACs models were employed such as HDAC1 (PDB: 4BKX) and HDAC2 (PDB: 4LY1)[43,44] As shown in Figure S2 and S3 there were several interactions between enzymes and ligand; for examples, Hie140, Hie178, Gly149, Tyr303, and Zn600 of HDAC1; Phe155, Hid145, Gly154, Zn401, and water molecules of HDAC2. The carbonyl group of the zinc-binding group (ZBG) of the ligand coordinated with a Zn^{2+} atom (Zn600 of HDAC1 and Zn401 of HDAC2). In addition, π–π stacking was found between the aromatic ring of the linker and Hie178 (HDAC1) and Phe155 (HDAC2). Subsequently, hydrogen bonds were observed between the amide NH of the ZBG and Gly149 (HDAC1) or Gly154 (HDAC2); the carbonyl group of the ZBG and OH-Tyr303 (HDAC1); the NH_2 aromatic ring and Hid145 (HDAC1); and the carbonyl group of the cap group with two water molecules (HDAC2). HDAC1 and HDAC2 binding sites seemed to be fitted with ligand. HDAC1 and HDAC2 specifically contain the 14 Å internal cavity that the thiophene group can occupy and maintain the ZBG rigidly to coordinate with the Zn^{2+} atom.

In conclusion, a series of fluorinated *N*-(2-aminophenyl)-benzamide derivatives were synthesized, and evaluated for antitumor activities. Most of the synthesized compounds exhibited selective inhibition of HDAC 1 and 2. Among the synthesized compounds, Compound **24a** displayed the highest potency for inhibition of HDACs 1 and 2 and antitumor activities in HCT-116, MCF-7, and A549 cancer cell lines. Furthermore, *in vivo* animal studies demonstrated that compound **24a** displayed more potent antitumor efficacy in a HCT-116 colon cancer xenograft mouse model than did SAHA.

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Supporting Information. Additional supporting information may be found online in the Supporting Information section at the end of the article.

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