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New orally bioavailable 2-aminobenzamide-type histone deacetylase inhibitor possessing a (2-hydroxyethyl)(4-(thiophen-2-yl)benzyl)amino group

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ABSTRACT

New 2-aminobenzamide-type histone deacetylase (HDAC) inhibitors were synthesized. They feature a sulfur-containing bicyclic arylmethyl moiety—a surface recognition domain introduced to increase in cellular uptake—and a substituted *tert*-amino group which affects physicochemical properties such as aqueous solubility. Compound **22** with a (2-hydroxyethyl)(4-(thiophen-2-yl)benzyl)amino group reduced the volume of human colon cancer HCT116 xenografts in nude mice to T/C 67% by oral administration at 45 mg/kg, which was comparable to the rate (T/C 62%) for a positive control, MS-275. Western blot analyses as well as cell cycle and TUNEL assays by flow cytometry suggested that the two compounds inhibited the growth of cancer cells via similar mechanisms.

1. Introduction

Histone deacetylase (HDAC) inhibitors induce gene expression, differentiation, growth arrest, and apoptosis in cancer cells, and represent a new, less toxic anti-cancer agent. A variety of HDAC inhibitors possessing a hydroxamic acid or non-hydroxamic acid moiety such as a 2-aminobenzamide group for binding a zinc ion have been reported. Among them, vorinostat,[1,2] romidepsin,[3–5] MS-275,[6–8] CI-994,[9,10] LAQ824,[11,12] PDX101[13,14] and MGCD0103[15] are under clinical trials.[16] Vorinostat and romidepsin are now approved by US FDA for use in patients with relapsed cutaneous T-cell lymphomas.[17]

In the present study, we have designed new orally active HDAC inhibitors with a 2-aminobenzamide group as a Zn^{2+}-chelating unit and a 1,4-phenylene as a linker as well as a sulfur-containing (bicyclic arylmethyl)(2-hydroxyethyl)amino, an aryl sulfide, or an arylmethyl sulfide moiety as a surface recognition domain for the following reasons: first, the 2-aminobenzamide group is metabolically more stable than the hydroxamic acid group[18] and renders a molecule more orally-bioavailable[6–8] and HDAC class I-selective.[19,20] Second, in our previous study, replacement of a hydrocarbon chain with the 1,4-phenylene as a linker improved plasma stability and HDAC inhibition.[18] Third, insertion of a sulfur atom in the surface recognition domain (a specificity element in the recognition of HDACs)[21] could induce a change in the pharmacological profile by, for example, enhancing cellular uptake.[21] Additionally, we expected the introduction of a 2-hydroxyethyl group at a *sec*-amino group to improve physicochemical properties including the aqueous solubility of the compound. Among the inhibitors synthesized, **22** having a (2-hydroxyethyl)(4-(thiophen-2-yl)benzyl)amino moiety as a surface recognition domain showed marked growth inhibition of human colorectal carcinoma HCT116 cells and good solubility (Fig. 1). Anti-tumor tests with a HCT116 xenograft model showed that **22** reduced the growth of tumors by 33% (T/C, 67%) at 45 mg/kg/day by oral administration, which was similar to the value (T/C, 62%) for a positive control, MS-275. Furthermore, the administration of MS-275 induced an increase in running behavior as well as a slight loss of weight, neither of which was observed in the mice treated with **22**. The two compounds accumulated acetylated-histone H3, stimulated p21/WAF1 protein expression and induced G_1-phase arrest and apoptosis to nearly the same extent. Thus, **22** appears to inhibit the growth of cancer cells via mechanisms similar to MS-275.[6,7]

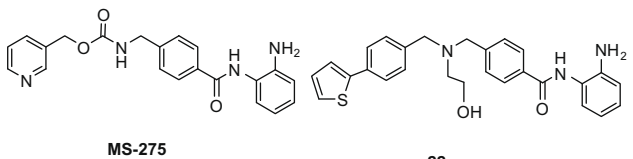

Figure 1. Structures of MS-275 and **22**.

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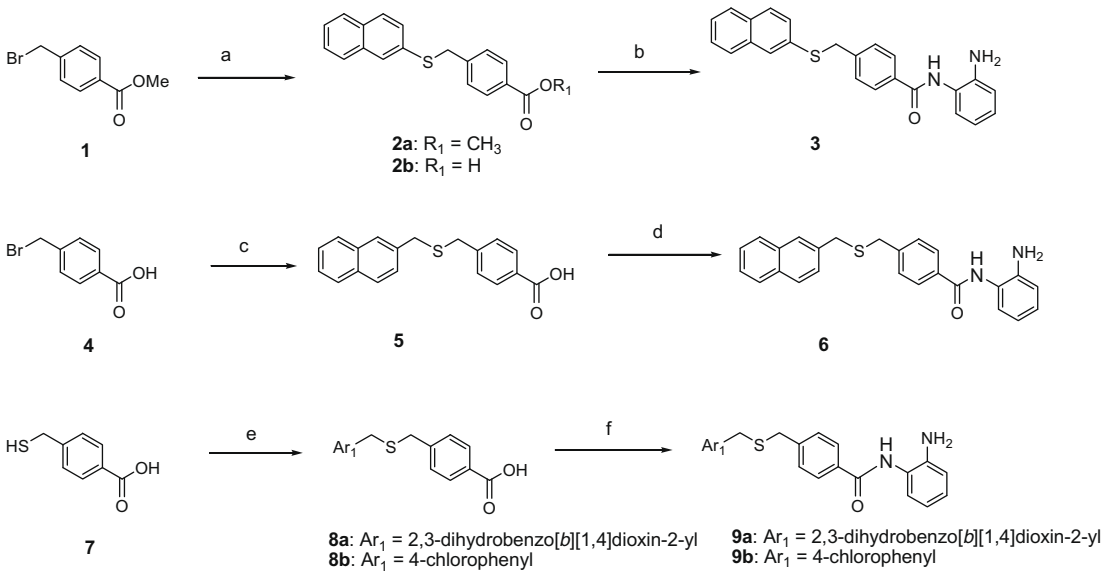
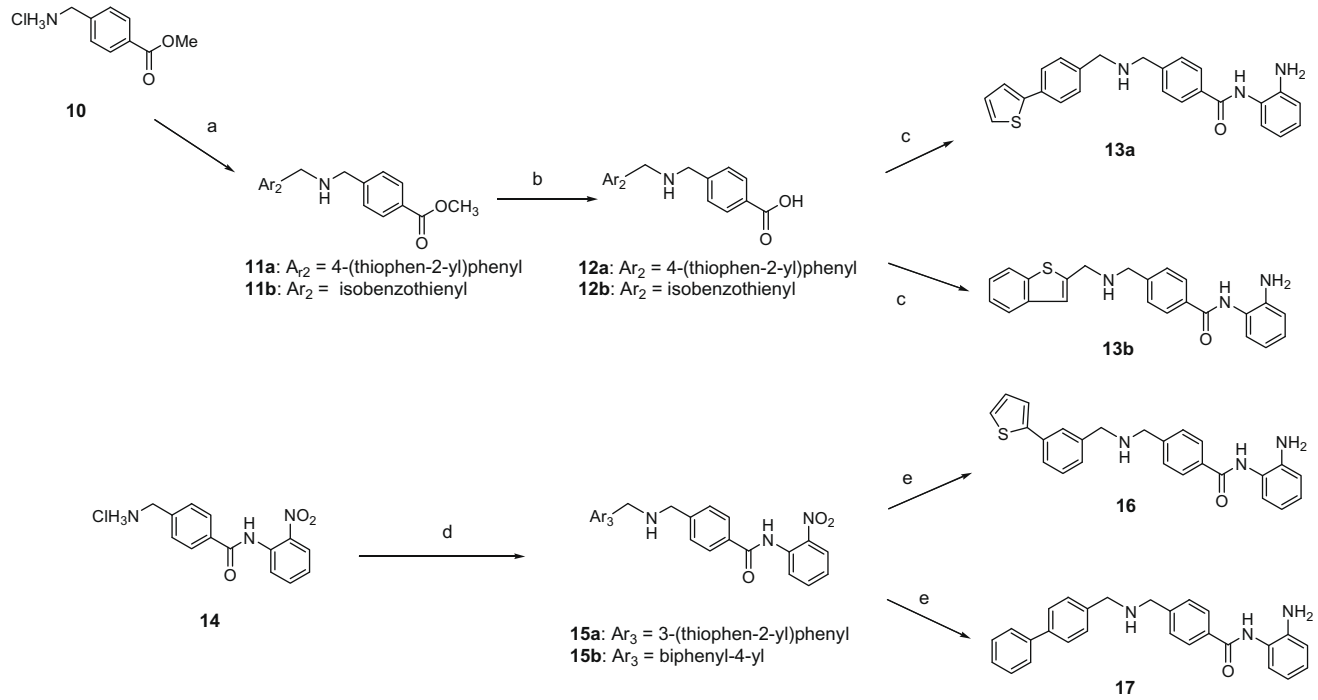
2. Chemistry

Sulfides **3**, **6**, **9a** and **9b** were synthesized as illustrated in Scheme 1. Methyl 4-(bromomethyl)benzoate (**1**) was reacted with naphthalene-2-thiol using K_2CO_3 to yield the thioether **2a**, which was hydrolyzed to give the carboxylic acid **2b**. The coupling of **2b** to benzene-1,2-diamine utilizing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) and 1-hydroxybenzotriazole (HOBt) afforded (naphthalene-2-yl) sulfide **3**. Arylmethyl sulfides **6**, **9a** and **9b** were prepared in two ways: bromomethylbenzoic acid (**4**) was treated with naphthalene-2-ylmethanethiol in the presence of NaOH to give the carboxylic acid **5**, which was condensed to benzene-1,2-diamine in the same way as for **3**, yielding (naphthalene-2-yl)methyl sulfide **6**. On the other hand, **9a** and **9b** were prepared starting with the formation of a thioether bond between mercaptomethylbenzoic acid (**7**) and arylmethybromide followed by condensation of the resulting products **8a** and **8b** with benzene-1,2-diamine. Compounds **13a**, **13b**, **16** and **17**, all having a bicyclic arylmethyl-*sec*-amino group, were synthesized starting from methyl 4-(aminomethyl)benzoate hydrochloride (**10**) or 4-(aminomethyl)-*N*-(2-nitorophenyl))benzamide hydrochloride (**14**) (Scheme 2). Thus, **10** was subjected to reductive



Scheme 1. Reagents and conditions: (a) (1) naphthalene-2-thiol, K_2CO_3, MeCN, 92% (2) LiOH, THF–H_2O, 94%; (b) benzene-1,2-diamine, HOBt, WSC, DMF, 51%; (c) naphthalene-2-ylmethanethiol, NaOH, EtOH, 75%; (d) benzene-1,2-diamine, HOBt, WSC, DMF, 20%; (e) Ar_1CH_2Br, NaOH, EtOH, **8a**, 28%: **8b**, 46%; (f) benzene-1,2-diamine, HOBt, WSC, DMF, **9a**, 84%: **9b**, 82%.



Scheme 2. Reagents and conditions: (a) Ar_2CHO, $NaBH(OAc)_3$, TEA, THF, **11a**, 56%: **11b**, 22%; (b) LiOH, THF–H_2O, **12a**, 95%: **12b**, 89%; (c) benzene-1,2-diamine, HOBt, WSC, DMF, **13a**, 84%: **13b**, 79%; (d) Ar_3CHO, $NaBH(OAc)_3$, THF, **15a**, 51%: **15b**, 40%; (e) $SnCl_2$, NH_4OAc, MeOH, **16**, 32%: **17**, 29%.

amination with arylaldehyde in the presence of $NaBH(OAc)_3$ and triethylamine (TEA) to give **11a** or **11b**. These compounds, after their conversion into the carboxylic acids **12a** and **12b**, were condensed with benzene-1,2-diamine using WSC and HOBt to yield **13a** and **13b**, respectively.

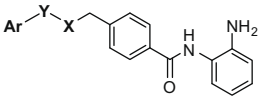
Meanwhile, **14** was subjected to reductive amination with arylaldehyde in the presence of $NaBH(OAc)_3$ and TEA to give **15a** or **15b**. The reduction of these products with $SnCl_2$ and NH_4OAc, respectively, gave **16** and **17**. Compounds **19**, **20**, **21**, **22** and **23**, all possessing a bicyclic arylmethyl-*tert*-amino group, were synthesized from **14** (Scheme 3). Compound **15b** obtained from **14** was converted to **23** by sequential alkylation with 2-bromoethanol and reduction with $SnCl_2$ and NH_4OAc. Furthermore, compound **18** prepared from **14** in the same way as **15b** was converted to **19**, **20** and **21** by N-methylation, N-methylsulfonylation and *N*-(*N*,*N*-dimethylamino)carbonylation followed by reduction with $SnCl_2$ and NH_4OAc, respectively.

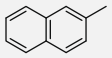
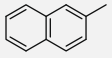
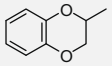
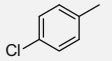
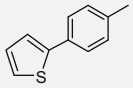
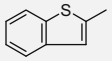
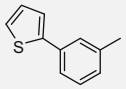
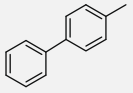
3. Results and discussion

The sulfides **3**, **6**, **9a** and **9b** inhibited significantly the growth of HCT116 cells (IC_{50}s between 2.6 and 3.9 μM) (Table 1). However, they showed greater IC_{50} values (18 to >100 μM) against HDAC1 than the other compounds synthesized as well as MS-275. In contrast, **13a** and **13b** possessing a S-containing bicyclic aryl group exhibited lower IC_{50} rates both for the growth of HCT116 cells (2.3 and 3.4 μM) and for HDAC1 (1.0 and 1.1 μM), respectively. Compound **13a**, having a (4-(thiophen-2-yl)benzyl)amino moiety as a surface recognition domain, seemed especially promising. Its regioisomer **16**, possessing a (3-(thiophen-2-yl)benzyl)amino group, as well as non-sulfur-containing counterpart **17**, possessing a 4-phenylbenzylamino group, were inferior to **13a** in terms of inhibiting HCT116 and/or HDAC1. Thus, **13a** was subjected to further structural optimization by including a methyl (**19**), methylsulfonyl (**20**), *N*,*N*-dimethylaminocarbonyl (**21**), or hydroxyethyl (**22**) group at the secondary amine.

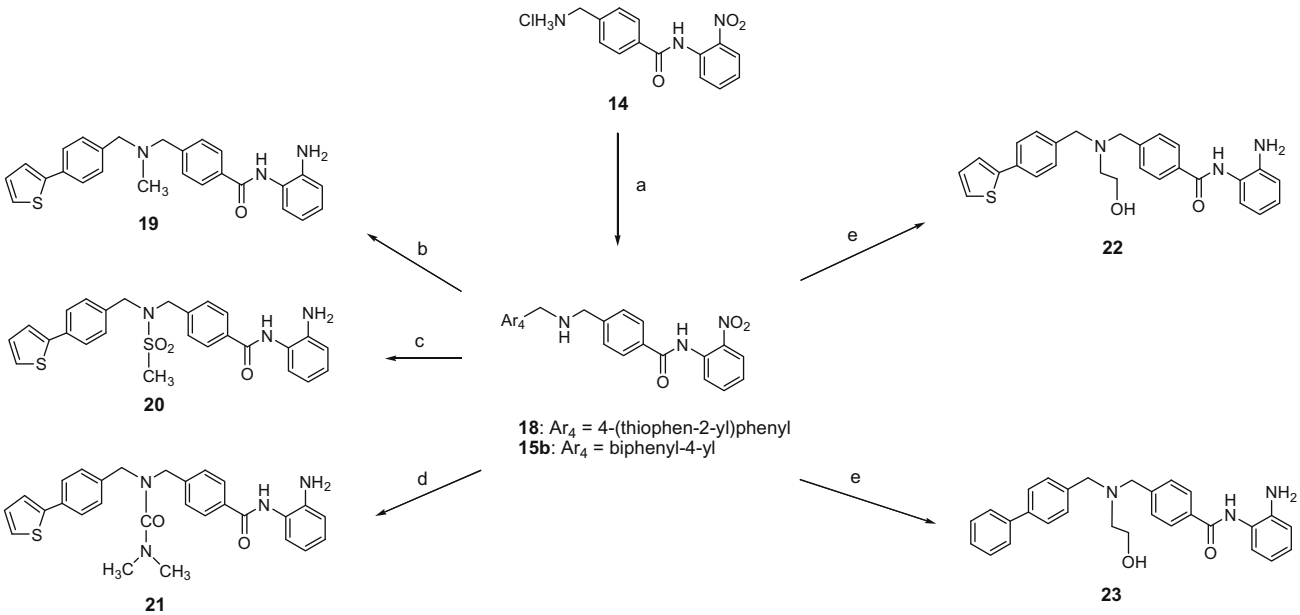
As shown in Table 2, introduction of the *N*,*N*-dimethylaminocarbonyl or hydroxyethyl group improved solubility. The latter group was previously utilized for the design of LAQ824.[11] Compound **22** showed the most promising profile in terms of inhibiting the growth of HCT116 cells (IC_{50} 0.7 μM) and HDAC1 (IC_{50} 0.8 μM), being comparable to MS-275 (0.8 and 0.5 μM, respectively).

Table 1
Effect of HDAC inhibitors on HCT116 cell growth and HDAC1



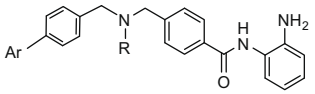
Compd	Ar	X	Y	HCT116 IC_{50}[a,b] (μM)	HDAC1 IC_{50}[c] (μM)
3		s	—	2.6	>100
6		s	CH_2	3.6	>100
9a		s	CH_2	3.1	18
9b		s	CH_2	3.9	19
13a		NH	CH_2	2.3	1.0
13b		NH	CH_2	3.4	1.1
16		NH	CH_2	5.0	3.3
17		NH	CH_2	3.9	0.9
MS-275				0.8	0.5

[a] Measured after a 3-day incubation of test compounds with cells.
[b] Assays were performed in triplicate.
[c] Assays were performed in duplicate.



Scheme 3. Reagents and conditions: (a) Ar_4CHO, $NaBH(OAc)_3$, TEA, THF, **18**, 54%; (b) (1) HCHO, AcOH, $NaBH(OAc)_3$, CH_2Cl_2, 82% (2) $SnCl_2$, NH_4OAc, MeOH, 19%; (c) (1) $MeSO_2Cl$, TEA, CH_2Cl_2, 78% (2) $SnCl_2$, NH_4OAc, MeOH, 24%; (d) (1) $(Me)_2NCOCl$, TEA, CH_2Cl_2, 94% (2) $SnCl_2$, NH_4OAc, MeOH, 38%; (e) (1) 2-bromoethanol, TEA, MeCN (2) $SnCl_2$, NH_4OAc, MeOH, **22**, 24%: **23**, 20%.

Table 2
Solubility and effects on HCT116 cell growth and HDAC1 of HDAC inhibitors



Compd	Ar	R	Solubility in 10% DMSO/H_2O (mg/mL)	HCT 116 IC_{50}[a,b] (μM)	HDAC1 IC_{50}[c] (μM)
13a		H	0.1	2.3	1.0
19		CH_3	0.2	4.7	>10
20		CH_3–SO_2–	0.1	5.7	1.2
21		$(CH_3)_2NCO$–	0.8	2.0	0.8
22		$HOCH_2CH_2$–	0.5	0.7	0.8
23		$HOCH_2CH_2$–	0.6	2.0	0.7
MS-275			0.8	0.8	0.5
TSA			—	—	0.0099

[a] Measured after a 3-day incubation of test compounds with cells.
[b] Assays were performed in triplicate.
[c] Assays were performed in duplicate.

Furthermore, in view of the lower value for HCT116 (IC_{50} 0.7 μM) of **22** relative to that (IC_{50} 2.0 μM) of **23** having the 4-phenylbenzylamino unit, the thienyl group may contribute to the pharmacological potency of **22**.

Since **22** has a pharmacological profile comparable to MS-275, we conducted comparative anti-tumor tests using xenografts of HCT116 cells. The two compounds were administered orally to mice at a dose of 45 mg/kg/day over 18 days according to the schedules indicated in Figure 2a. Compound **22** suppressed the growth of the xenografts to T/C: 67%, similar to MS-275 (62%). The administration of MS-275 induced an increase in running behavior as well as a slight loss of weight (Fig. 2b), these effects being not observed in the mice treated with **22**.

Figure 3 shows the wet weights of tumor masses removed from the mice at day 18. The average weights of tumor masses with MS-275 and **22** decreased to 61% and 63% of the control values, respectively.

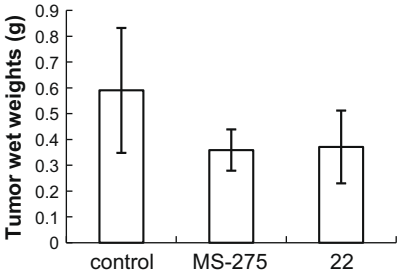


Figure 3. Wet weights of tumor masses of mice fed **22** or MS-275. The cervical spine was dislocated, and tumor masses were removed and weighed. Vertical bars indicate standard errors. Significant differences (p <0.05) were obtained for tumor volume with **22** or MS-275 versus the control.

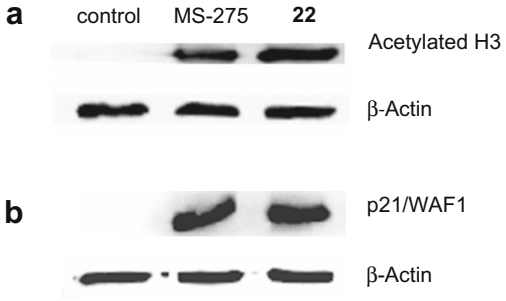


Figure 4. Effect of **22** and MS-275 on nuclear histone (H3) acetylation and p21WAF1 expression. (4a) Lysate (11 μg) of HCT116 cells exposed to **22** or MS-275 at 10 μM or 0.1% DMSO for 24 h was separated by polyacrylamide gel electrophoresis (SDS–PAGE). (4b) Lysate (11 μg) of SKBR3 cells exposed to **22** or MS-275 at 10 μM or 0.1% DMSO for 24 h was examined using SDS–PAGE. The experiment was repeated four times and representative blots are shown. Details are described in Section 5.

Both **22** and MS-275 promoted the hyperacetylation of core histone H3 in the HCT 116 cells as detected by Western blotting (Fig. 4a) and regulated the cells in the G_0/G_1 phase of the cell cycle at 10 μM (Table 3). These compounds also induced apoptosis as suggested by the appearance of a $subG_1$ population, which was confirmed with a TUNEL assay. The percentages of cells with positive TUNEL staining were 28.7% (**22**) and 27.6% (MS-275), respectively (Fig. 5).

Furthermore, **22** induced the expression of p21/WAF1 protein in SKBR-3 cells (p53-mutant) as did MS-275 (Fig. 4b). These findings

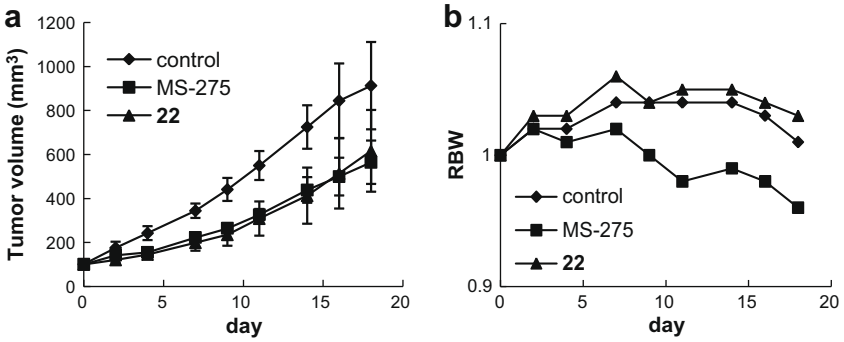


Figure 2. Effects of **22** and MS-275 on HCT116 cell-inoculated xenografts in vivo. The effects of **22** (▲) and MS-275 (■) on tumor volume (2a) as well as relative body weight (RBW) changes (2b) at a dose of 45 mg/kg/day were examined as described in Section 5. (♦) Represents the control group. Compounds were orally administered to mice on days 0, 2, 4, 7, 9, 11, 14 and 16. Vertical bars indicate standard errors. Significant differences (p <0.01) were obtained at all time points except day0 for tumor volume with **22** or MS-275 vs. the control.

Table 3
Effects of **22** and MS-275 on the cell cycle in HCT116 cells

	% of subG$_1$		% of G$_0$/G$_1$		% of S		%of G$_2$/M	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control (0.1% DMSO)	6.2	1.9	81.3	84.9	11.1	15.4	10.2	7.7
22	15.3	20.6	65.0	64.9	6.9	5.1	12.7	9.4
MS-275	4.7	17.4	75.6	76.1	5.8	2.2	13.9	4.1

HCT116 cells (1.0×10^6), after incubating for 24 h, were treated with **22** or MS-275 (each 10 μM) for 24 or 48 h. The cells were then treated with a BD Cycle Test Plus DNA reagent Kit. Data are representative of three independent experiments.

suggested that **22** inhibited the growth of the cancer cells via mechanisms similar to MS-275.

4. Conclusion

A new orally bioavailable HDAC inhibitor, **22**, comprising a (2-hydroxyethyl)(4-(thiophen-2-yl)benzyl)amino group inhibited the growth of HCT116 xenografts in nude mice more or less equivalent to MS-275 (**4**) and showed a promising in vivo profile. Thus, **22** warrants further tests with other human cancer xenografts to identify its effectiveness as an antitumor agent.

5. Experimental

5.1. General

Melting points were determined on a Yanagimoto MP-32 micro-melting point apparatus and are uncorrected. IR spectra were recorded on a Shimadzu FTIR-8400 infrared spectrophotometer. Low-resolution (LR)-FAB-MS spectra were measured on a JEOL JMS-HX 100, and high-resolution (HR)- and LR-FAB-MS spectra, on a JEOL Tandem MStation JMS-700. ^{1}H NMR spectra were recorded on a JEOL EX-400 (400 MHz) using tetramethylsilane as an internal standard. Analytical TLC and PLC were performed using Silica Gel 60 F254 (Merck, 0.25 and 0.5 mm, respectively) glass plates. Column chromatography was performed using Silica Gel 60 (70–230 mesh ASTM). All solvents were dried over Na_2SO_4, and evaporated in vacuo. The HCT 116 cell line was purchased from American Type Culture Collection. The Cycle Test Plus DNA reagent Kit was purchased from Becton Dickinson and Company. The TUNEL assay reagent, DeadEnd™ Fluorometric TUNEL System, was obtained from Promega. Results of flow cytometry were collected with a FACSCant II flow cytometer (Becton Dickinson and Company).

5.2. Chemistry

5.2.1. Synthesis of MS-275

MS-275 was prepared from 4-(((pyridin-3-ylmethoxy)carbonylamino)methyl)benzoic acid (2.0 g, 7.0 mmol) and benzene-1,2-diamine (6.1 g, 8.0 mmol) as reported:[22] (1.8 g, 4.7 mmol, 68%). Mp 155–156 °C. ^{1}H NMR (DMSO-d_6): δ 4.28 (2H, d, J = 6.0 Hz, CH_2), 4.88 (2H, s, NH_2), 5.10 (2H, s, CH_2), 6.60 (1H, t, J = 8.0 Hz, PhH), 6.78 (1H, dd, J = 8.0, 1.2 Hz, PhH), 6.97 (1H, ddd, J = 8.0, 8.0, 1.2 Hz, PhH), 7.17 (1H, d, J = 8.0 Hz, PhH), 7.36–7.43 (3H, m, ArH), 7.79 (1H, d, J = 7.6 Hz, ArH), 7.92–7.97 (3H, m, ArH), 8.54 (1H, d, J = 3.6 Hz, Pyr$H$), 8.60 (1H, s, Pyr$H$), 9.61 (1H, s, CONH).

5.2.2. Methyl 4-((naphthalen-2-ylthio)methyl)benzoate (2a)

To a solution of **1** (0.70 g, 3.1 mmol) in MeCN (20 mL) was added 2-naphthalenethiol (0.49 g, 3.1 mmol) followed by K_2CO_3 (0.21 g, 1.5 mmol), and the mixture stirred at room temperature for 8 h. After the removal of insoluble materials, the solution was concentrated, and the resulting solid was recrystallized from $CHCl_3$–n-hexane to yield **2a** (0.87 g, 2.8 mmol, 92%). Mp 100–101 °C. IR (KBr) 1725, 1434, 1283, 1095, 816 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 3.89 (3H, s, OCH_3), 4.22 (2H, s, CH_2Ph), 7.35–7.73 (9H, m, ArH), 7.93 (2H, d, J = 8.4 Hz, ArH). FAB-MS m/z: 309 (M+H)$^+$. HR-FAB-MS m/z: (M+H)$^+$ calcd for $C_{19}H_{17}O_2S$, 309.0949; found, 309.0942.

5.2.3. 4-((Naphthalen-2-ylthio)methyl)benzoic acid (2b)

1 M LiOH in H_2O (4.5 mL) was added to a solution of **2a** (0.35 g, 1.1 mmol) in tetrahydrofuran (THF) (5 mL), and the mixture stirred at room temperature overnight. After the removal of THF, the mixture was adjusted to pH 3 with 1 M HCl. The resulting precipitate was collected by filtration, giving **2b** (0.31 g, 1.1 mmol, 94%) as a solid. Mp >390 °C. IR (KBr) 3047, 1597, 1551, 1427 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 4.33 (2H, s, SCH_2Ph), 7.27 (2H, d, J = 8.0 Hz, PhH), 7.26–7.50 (3H, m, ArH), 7.75 (2H, d, J = 8.0 Hz, PhH), 7.79–7.85 (4H, m, ArH). FAB-MS m/z: 293 (M−H)$^-$. HR-FAB-MS m/z: (M−H)$^-$ calcd for $C_{18}H_{13}O_2S$, 293.0636; found, 293.0640.

5.2.4. *N*-(2-Aminophenyl)-4-((naphthalen-2-ylthio)ethyl) benzamide (3)

To a solution of benzene-1,2-diamine (0.07 g, 0.68 mmol) in *N*,*N*-dimethylformamide (DMF) (1 mL) was added **2b** (0.10 g, 0.34 mmol), then HOBt (0.069 g, 0.51 mmol) and finally WSC (0.098 g, 0.51 mmol), and the mixture stirred at room temperature overnight. The reaction mixture was diluted with EtOAc, washed with satd. $NaHCO_3$ and brine, dried, and concentrated. The residue was crystallized from $CHCl_3$ to give **3** (0.066 g, 0.17 mmol, 51%) as a solid. Mp 187–189 °C. IR (KBr) 3288, 1643, 1612, 1502, 1454, 1306 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 4.45 (2H, s, SCH_2Ph), 4.87 (2H, s, PhNH_2), 6.58 (1H, ddd, J = 7.6, 7.6, 1.2 Hz, PhH), 6.76 (1H, dd, J = 7.6, 1.2 Hz, PhH), 6.96 (1H, ddd, J = 7.6, 7.6, 1.2 Hz, PhH), 7.13 (1H, d, J = 7.6 Hz, PhH), 7.44–7.54 (5H, m, ArH), 7.81–7.90 (6H, m, ArH), 9.59 (1H, s, CONH). FAB-MS m/z: 385 (M+H)$^+$. HR-FAB-MS m/z: (M+H)$^+$ calcd for, 385.1375; found, 385.1370.

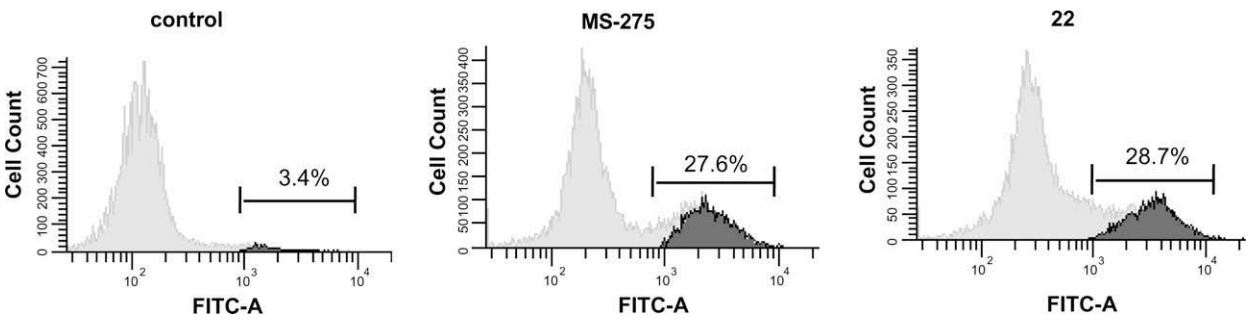


Figure 5. Flow cytometric analysis of FITC-TUNEL staining of HCT116 cells. Cells were incubated with **22**, MS-275 (each 10 μM) or vehicle (no sample) for 24 h and then treated as indicated in Section 5. Percentages of FITC-TUNEL-positive cells are indicated. They are results of one of two independent experiments.

5.2.5. 4-((Naphthalen-2-ylmethylthio)methyl)benzoic acid (5)

To a suspension of **4** (0.25 g, 1.2 mmol) in EtOH (2 mL) were added 2-naphthalenemethanethiol (0.20 g, 1.15 mmol) and a solution of NaOH (0.092 g, 2.3 mmol) in EtOH (3.7 mL), and the mixture was stirred at room temperature over night. The solution, its pH adjusted to 3 with 1 M HCl, was concentrated. The resulting solid was recrystallized from EtOAc–hexane to give **5** (0.27 g, 0.86 mmol, 75%). Mp 154–156 °C. IR (KBr) 3053, 1688, 1427 cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.74 (2H, s, SCH_2Ph), 3.84 (2H, s, ArCH_2S), 7.41 (2H, d, J = 8.4 Hz, PhH), 7.45–7.53 (4H, m, ArH), 7.76 (1H, s, ArH), 7.87–7.90 (4H, m, ArH). FAB-MS m/z: 307 $(M-H)^-$. HR-FAB-MS m/z: $(M-H)^-$ calcd for $C_{19}H_{15}O_2S$, 307.0793; found, 307.0800.

5.2.6. *N*-(2-Aminophenyl)-4-((naphthalen-2-ylmethylthio) methyl)benzamide (6)

The acid **5** (0.10 g, 0.32 mmol) was treated with benzene-1,2-diamine (0.069 g, 0.64 mmol), DMF (1 mL), HOBt (0.065 g, 0.48 mmol) and WSC (0.092 g, 0.48 mmol) as for the preparation of **3**. The resulting solid was purified by PLC ($CHCl_3$/MeOH = 10:1) to give **6** (0.025 g, 0.063 mmol, 20%). Mp 145–147 °C. IR (KBr) 3377, 3250, 1649, 1612, 1456, 1306 cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.76 (2H, s, SCH_2Ph), 3.85 (2H, s, ArCH_2S), 4.90 (2H, s, PhNH_2), 6.60 (1H, t, J = 7.6 Hz, PhH), 6.78 (1H, d, J = 8.0 Hz, PhH), 6.98 (1H, ddd, J = 8.0, 8.0, 1.2 Hz, PhH), 7.17 (1H, d, J = 8.0 Hz, PhH), 7.43 (2H, d, J = 8.4 Hz, PhH), 7.47–7.54 (3H, m, ArH), 7.77 (1H, s, ArH), 7.89–7.91 (3H, m, ArH), 7.95 (2H, d, J = 8.0 Hz, PhH), 9.65 (1H, s, CONH). FAB-MS m/z: 399 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{25}H_{23}N_2OS$, 399.1531; found, 399.1530.

5.2.7. 4-(((2,3-Dihydrobenzo[*b*][1,4]dioxin-2-yl)methylthio) methyl)benzoic acid (8a)

To a suspension of **7** (0.048 g, 0.29 mmol) in EtOH (0.7 mL) was added 2-bromomethyl-1,4-benzodioxane (0.043 mL, 0.29 mmol) and then ethanolic NaOH (0.023 g, 0.58 mmol) (0.25 mL). After being stirred at room temperature overnight, the mixture was adjusted to pH 3 with 1 M HCl and concentrated. The resulting crystal was purified by PLC ($CHCl_3$/MeOH = 10:1), yielding **8a** (0.026 g, 0.082 mmol, 28%). Mp 120–122 °C. IR (KBr) 2918, 1686, 1429, 1267 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.69 (2H, d, J = 6.4 Hz, ArCH_2S), 3.91 (2H, s, SCH_2Ph), 3.93–3.98 (1H, m, CH), 4.25–4.31 (2H, m, OCH_2CH), 6.80–6.88 (4H, m, ArH), 7.43 (2H, d, J = 8.0 Hz, PhH), 7.87 (2H, d, J = 8.0 Hz, PhH). FAB-MS m/z: 315 $(M-H)^-$. HR-FAB-MS m/z: $(M-H)^-$ calcd for $C_{17}H_{15}O_4S$, 315.0691; found, 315.0689.

5.2.8. *N*-(2-Aminophenyl)-4-(((2,3-dihydrobenzo[*b*][1,4]dioxin-2-yl)methylthio) methyl)benzamide (9a)

Compound **8a** (0.09 g, 0.28 mmol) was treated with benzene-1,2-diamine (0.06 g, 0.56 mmol), DMF (1 mL), HOBt (0.057 g, 0.42 mmol) and WSC (0.081 g, 0.42 mmol) in the conventional manner. The product was purified by PLC ($CHCl_3$/MeOH = 10:1) to give **9a** (0.095 g, 0.23 mmol, 84%). Mp 125–127 °C. IR (KBr) 3381, 3217, 1630, 1458, 1304, 1265 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.72 (2H, d, J = 6.0 Hz, ArCH_2S), 3.93 (2H, s, SCH_2Ph), 3.97–4.02 (1H, m, CH), 4.30–4.39 (2H, m, OCH_2CH), 6.60 (1H, t, J = 7.6 Hz, PhH), 6.78 (1H, d, J = 7.6 Hz, PhH), 6.83–6.91 (6H, m, ArH), 6.97 (1H, t, J = 7.6 Hz, PhH), 7.16 (1H, d, J = 7.6 Hz, PhH), 7.45 (2H, d, J = 8.4 Hz, PhH), 7.94 (2H, d, J = 8.4 Hz, PhH), 9.65 (1H, s, CONH). FAB-MS m/z: 407 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{23}H_{23}N_2O_3S$, 407.1429; found, 407.1436.

5.2.9. 4-((4-Chlorobenzylthio)methyl)benzoic acid (8b)

Compound **8b** was prepared from **7** (0.025 g, 0.12 mmol) according to the procedure used for **8a** (0.016 g, 0.055 mmol, 46%). Mp 158–159 °C. IR (KBr) 2939, 1692, 1427 cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.66 (2H, s, SCH_2Ph), 3.72 (2H, s, PhCH_2S), 7.31 (2H, d, J = 8.4 Hz, PhH), 7.37 (2H, d, J = 8.0 Hz, PhH), 7.39 (2H, d, J = 8.0 Hz, PhH), 7.88 (2H, d, J = 8.4 Hz, PhH). FAB-MS m/z: 291 $(M-H)^-$. HR-FAB-MS m/z: $(M-H)^-$ calcd for $C_{15}H_{12}ClO_2S$, 291.0247; found, 291.0250.

5.2.10. *N*-(2-Aminophenyl)-4-((4-chlorobenzylthio) methyl)benzamide (9b)

Compound **9b** was prepared from **8b** (0.14 g, 0.46 mmol) according to the procedure used for **9a** (0.145 g, 0.38 mmol, 82%). Mp 120–122 °C. IR (KBr) 3300, 1632, 1601, 1489, 1230 cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.68 (2H, s, SCH_2Ph), 3.74 (2H, s, PhCH_2S), 4.90 (2H, s, PhNH_2), 6.60 (1H, t, J = 7.2 Hz, PhH), 6.78 (1H, d, J = 8.0 Hz, PhH), 6.97 (1H, t, J = 7.2 Hz, PhH), 7.16 (1H, d, J = 8.0 Hz, PhH), 7.33 (2H, d, J = 8.4 Hz, PhH), 7.39 (2H, d, J = 8.0 Hz, PhH), 7.41 (2H, d, J = 8.0 Hz, PhH), 7.94 (2H, d, J = 8.0 Hz, PhH), 9.64 (1H, s, CONH). FAB-MS m/z: 383 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{21}H_{20}ClN_2OS$, 383.0985; found, 383.0974.

5.2.11. Methyl 4-((4-(thiophen-2-yl)benzylamino) methyl)benzoate (11a)

A solution of **10** (0.21 g, 1.1 mmol) in THF (2 mL) was subjected to reductive amination with 4-(2-thienyl)benzaldehyde (0.20 g, 1.1 mmol), $NaBH(OAc)_3$ (0.34 g, 1.59 mmol) and TEA (0.22 mL, 1.59 mmol) in the conventional manner. The product was purified by PLC ($CHCl_3$) to give **11a** (0.20 g, 0.59 mmol, 56%). Mp 71–72 °C. IR (KBr) 3321, 1711, 1435, 1277 cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.69 (2H, s, NHCH_2), 3.76 (2H, s, CH_2NH), 3.84 (3H, s, OCH_3), 7.13 (1H, dd, J = 5.2, 3.6 Hz, thienyl$H$), 7.38 (2H, d, J = 8.4 Hz, PhH), 7.48–7.53 (4H, m, ArH), 7.61 (2H, d, J = 8.4 Hz, PhH), 7.92 (2H, d, J = 8.4 Hz, PhH). FAB-MS m/z: 338 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{20}H_{20}NO_2S$, 338.1215; found, 338.1216.

5.2.12. 4-((4-(Thiophen-2-yl)benzylamino)methyl)benzoic acid (12a)

A solution of **11a** (0.17 g, 0.52 mmol) in THF (2 mL) was treated with 1 M LiOH in H_2O (2.1 mL) in the conventional manner. The mixture, after removal of THF, was adjusted to pH 3 with 1 M HCl, and the precipitate was collected by filtration to give **12a** (0.16 g, 0.49 mmol, 95%). Mp 254–256 °C. IR (KBr) 2930, 1686, 1425 cm^{-1}. ^1H NMR (DMSO-d_6): δ 4.17 (2H, s, NHCH_2), 4.23 (2H, s, CH_2NH), 7.16 (1H, dd, J = 5.2, 3.6 Hz, thienyl$H$), 7.57 (2H, d, J = 8.4 Hz, PhH), 7.58–7.6 (2H, m, thienyl$H$), 7.66 (2H, d, J = 8.4 Hz, PhH), 7.73 (2H, d, J = 8.4 Hz, ph$H$), 7.99 (2H, d, J = 8.4 Hz, PhH). FAB-MS m/z: 324 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{19}H_{18}NO_2S$, 324.1058; found, 324.1063.

5.2.13. *N*-(2-Aminophenyl)-4-((4-(thiophen-2-yl)benzylamino) methyl)benzamide (13a)

Compound **12a** (0.10 g, 0.31 mmol) was treated with benzene-1,2-diamine (0.067 g, 0.62 mmol), DMF (1 mL), HOBt (0.064 g, 0.47 mmol) and WSC (0.09 g, 0.47 mmol). The product was purified by PLC ($CHCl_3$/MeOH = 20:1) to give **13a** (0.11 g, 0.26 mmol, 82%). Mp 168–169 °C. IR (KBr) 3329, 3233, 1634, 1454, 1306 cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.70 (2H, s, NHCH_2), 3.77 (2H, s, CH_2NH), 4.89 (2H, s, PhNH_2), 6.60 (1H, ddd, J = 7.2, 7.2, 1.2 Hz, PhH), 6.78 (1H, dd, J = 8.0, 1.2 Hz, PhH), 6.97 (1H, ddd, J = 7.2, 7.2, 1.2 Hz, PhH), 7.13 (1H, dd, J = 4.8, 3.2 Hz, thienyl$H$), 7.17 (1H, d, J = 7.2 Hz, PhH), 7.40 (2H, d, J = 8.4 Hz, PhH), 7.48–7.50 (4H, m, ArH), 7.52 (2H, dd, J = 4.8, 1.2 Hz, thienyl$H$), 7.62 (2H, d, J = 8.4 Hz, PhH), 7.95 (2H, d, J = 8.4 Hz, PhH), 9.64 (1H, s, CONH). FAB-MS m/z: 414 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{25}H_{24}N_3OS$, 414.1640; found, 414.1637.

5.2.14. Methyl 4-((benzo[*b*]thiophen-2-ylmethylamino) methyl)benzoate (11b)

Compound **11b** was prepared from **10** (0.25 g, 1.2 mmol) according to the procedure used for **11a** (0.086 g, 0.28 mmol, 22%). Mp 67–68 °C. IR (KBr) 3327, 1715, 1429, 1279 cm^{-1}. ^1H NMR (DMSO-d_6): δ

3.81 (2H, s, NHCH_2), 3.85 (3H, s, CH_3), 3.97 (2H, s, CH_2NH), 7.26 (1H, s, Ar*H*), 7.27–7.35 (2H, m, Ar*H*), 7.52 (2H, d, *J* = 8.4 Hz, Ph*H*), 7.74 (1H, d, *J* = 7.6 Hz, Ar*H*), 7.90 (1H, d, *J* = 7.6 Hz, Ar*H*), 7.93 (2H, d, *J* = 8.4 Hz, Ph*H*). FAB-MS *m/z*: 312 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{18}H_{18}NO_2S$, 312.1058; found, 312.1056.

5.2.15. 4-((Benzo[*b*]thiophen-2-ylmethylamino)methyl)benzoic acid (12b)

Compound **12b** was prepared from **11b** (0.063 g, 0.20 mmol) according to the procedure used for **12a** (0.053 g, 0.18 mmol, 89%). Mp 258–260 °C. IR (KBr) 2932, 1693, 1421 cm^{-1}. ^1H NMR (DMSO-d_6): δ 4.28 (2H, s, NHCH_2), 4.51 (2H, s, CH_2NH), 7.39–7.44 (2H, m, Ar*H*), 7.66 (1H, s, Ar*H*), 7.68 (2H, d, *J* = 8.4 Hz, Ph*H*), 7.90 (1H, t, *J* = 4.4 Hz, Ar*H*), 7.99 (2H, d, *J* = 8.4 Hz, Ph*H*), 8.02 (1H, t, *J* = 4. Hz, Ar*H*). FAB-MS *m/z*: 298 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{17}H_{16}NO_2S$, 298.0902; found, 298.0904.

5.2.16. *N*-(2-Aminophenyl)-4-((benzo[*b*]thiophen-2-ylmethylamino)methyl) benzamide (13b)

Compound **13b** was prepared from **12b** (0.040 g, 0.13 mmol) according to the procedure used for **13a** (0.040 g, 0.10 mmol, 79%). Mp 149–150 °C. IR (KBr) 3250, 1649, 1612, 1454, 1308 cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.82 (2H, s, NHCH_2), 3.98 (2H, s,CH_2NH), 4.90 (2H, s, PhNH_2), 6.60 (1H, t, *J* = 7.2 Hz, Ph*H*), 6.78 (1H, dd, *J* = 7.2, 1.2 Hz, Ph*H*), 6.97 (1H, ddd, *J* = 7.2, 7.2, 1.2 Hz, Ph*H*), 7.17 (1H, d, *J* =7.2 Hz, Ph*H*), 7.28 (1H, s, Ar*H*), 7.29–7.35 (2H, m, Ar*H*), 7.50 (2H, d, *J* = 8.0 Hz, Ar*H*), 7.75 (1H, d, *J* = 8.0 Hz, Ar*H*), 7.91 (1H, d, *J* = 8.0 Hz, Ar*H*), 7.95 (2H, d, *J* = 8.0 Hz, Ph*H*). FAB-MS *m/z*: 388 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{23}H_{22}N_3OS$, 388.1484; found, 388.1474.

5.2.17. *N*-(2-Nitrophenyl)-4-((3-(thiophen-2-yl)benzylamino) methyl)benzamide (15a)

A suspension of **14** (0.47 g, 1.5 mmol) in THF (10 mL) was subjected to reductive amination with 3-(2-thienyl)benzaldehyde (0.29 mL, 1.53 mmol), $NaBH(OAc)_3$ (0.49 g, 2.3 mmol) and TEA (0.29 mL, 2.3 mmol). The product was purified by PLC ($CHCl_3$) to give **15a** (0.34 g, 0.77 mmol, 51%). Mp 75–77 °C. IR (KBr) 3375, 1680, 1502, 1433, 1340 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.86 (2H, s, NHCH_2), 3.92 (2H, s, CH_2NH), 7.08 (1H, t, *J* = 3.6 Hz, thienyl*H*), 7.21–7.35 (6H, m, Ar*H*), 7.51–7.59 (4H, m, Ar*H*), 7.71 (1H, ddd, *J* = 8.4, 7.2, 1.6 Hz, Ph*H*), 7.97 (2H, d, *J* = 8.4 Hz, Ph*H*), 8.28 (1H, dd, *J* = 8.4, 1.6 Hz, Ph*H*), 9.01 (1H, d, *J* = 8.4 Hz, Ph*H*), 11.35 (1H, s, CON*H*). FAB-MS *m/z*: 444 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{25}H_{22}N_3O_3S$, 444.1382; found, 444.1378.

5.2.18. *N*-(2-Aminophenyl)-4-((3-(thiophen-2-yl)benzylamino) methyl)benzamide (16)

Compound **15a** (0.044 g, 0.099 mmol) was reduced with NH_4OAc (0.076 g, 0.99 mmol) and $SnCl_2{\cdot}2H_2O$ (0.13 g, 0.59 mmol) and MeOH (1 mL) at 60 °C for 5 h. The mixture was concentrated to a residue, which was purified with PLC ($CHCl_3$/MeOH = 20:1) to give **16** (0.013 g, 0.031 mmol, 32%). Mp 144–146 °C. IR (KBr) 3321, 3231, 1634, 1456, 1296 cm^{-1}. ^1H NMR (CD_3OD): δ 3.91 (2H, s, NHCH_2), 3.97 (2H, s, CH_2NH), 6.77 (1H, ddd, *J* = 8.0, 8.0, 1.2 Hz, Ph*H*), 6.91 (1H, dd, *J* = 8.0, 1.2 Hz, Ph*H*), 7.06–7.10 (2H, m, Ar*H*), 7.19 (1H, dd, *J* = 8.0, 1.2 Hz, Ph*H*), 7.30 (1H, d, *J* = 8.0 Hz, Ar*H*), 7.37–7.42 (3H, m, Ar*H*), 7.54 (2H, d, *J* = 8.0 Hz, Ar*H*), 7.59 (1H, dt, *J* = 8.0, 1.2, 1.2 Hz, Ar*H*), 7.68 (1H, s, Ph*H*), 7.99 (2H, d, *J* = 8.0 Hz, Ph*H*). FAB-MS *m/z*: 414 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{25}H_{24}N_3OS$, 414.1640; found, 414.1645.

5.2.19. 4-((Biphenyl-4-ylmethylamino)methyl)-*N*-(2-nitrophenyl)benzamide (15b)

Compound **15b** was prepared from **14** (0.41 g, 1.3 mmol) according to the procedure used for **15a** (0.32 g, 0.72 mmol, 54%). Mp 75–77 °C. IR (KBr) 3366, 1666, 1504, 1337 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.86 (2H, s, NCH_2Ph), 3.92 (2H, s, PhCH_2N), 7.18–7.60 (12H, m, Ph*H*), 7.70 (1H, t, *J* = 8.0 Hz, Ph*H*), 7.97 (2H, d, *J* = 7.2 Hz, Ph*H*), 8.26 (1H, d, *J* = 8.8 Hz, Ph*H*), 9.00 (1H, d, *J* = 8.8 Hz, Ph*H*), 11.35 (1H, s, CON*H*). FAB-MS *m/z*: 438 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{27}H_{24}N_3O_3$, 438.1818; found, 438.1821.

5.2.20. *N*-(2-Aminophenyl)-4-((biphenyl-4-ylmethylamino) methyl)benzamide (17)

Compound **17** was prepared from **15b** (0.073 g, 0.17 mmol) according to the procedure used for **16** (0.020 g, 0.049 mmol, 29%). Mp 150–152 °C. IR (KBr) 3371, 3223, 1643, 1614, 1454, 1306 cm^{-1}. ^1H NMR (DMSO-d_6): δ 3.73 (2H, s, NHCH_2), 3.79 (2H, s, CH_2NH), 4.90 (2H, s, PhNH_2), 6.60 (1H, t, *J* = 6.8 Hz, Ph*H*), 6.78 (1H, d, *J* = 7.6 Hz, Ph*H*), 7.17 (1H, d, *J* = 7.6 Hz, Ph*H*), 7.35–7.67 (10H, m, Ph*H*), 7,95 (2H, d, *J* = 7.6 Hz, Ph*H*), 9.64 (1H, s, CON*H*). FAB-MS *m/z*: 408 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{27}H_{26}N_3O$, 408.2076; found, 408.2077.

5.2.21. *N*-(2-Nitrophenyl)-4-((4-(thiophen-2-yl)benzylamino) methyl)benzamide (18)

A suspension of **14** (0.41 g, 1.3 mmol) in THF (5 mL) was subjected to reductive amination with 4-(2-Thienyl)benzaldeyde (0.25 g, 1.33 mmol), $NaBH(OAc)_3$ (0.42 g, 2.0 mmol) and TEA (0.28 mL, 2.0 mmol). The product was purified by PLC ($CHCl_3$) to give **18** (0.32 g, 0.72 mmol, 54%). Mp 119–121 °C. IR (KBr) 3342, 1678, 1501, 1431, 1335 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.84 (2H, s, NHCH_2), 3.92 (2H, s, CH_2NH), 7.08 (1H, dd, *J* = 5.2, 3.6 Hz, thienyl*H*), 7.21–7.31 (3H, m, Ar*H*), 7.37 (2H, d, *J* = 8.4 Hz, Ph*H*), 7.54 (2H, d, *J* = 8.4 Hz, Ph*H*), 7.60 (2H, d, *J* = 8.4 Hz, Ph*H*), 7.72 (1H, t, *J* = 8.4 Hz, Ph*H*), 7.98 (2H, d, *J* = 8.4 Hz, Ph*H*), 8.29 (1H, dd, *J* = 8.4, 1.6 Hz, Ph*H*), 9.02 (1H, d, *J* = 8.4 Hz, Ph*H*), 11.36 (1H, s, CON*H*). FAB-MS *m/z*: 444 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{25}H_{22}N_3O_3S$, 444.1382; found, 444.1386.

5.2.22. *N*-(2-Aminophenyl)-4-((methyl(4-(thiophen-2-yl) benzyl)amino)methyl)-benzamide (19)

A suspension of **18** (0.10 g, 0.23 mmol) in THF (3 mL) was subjected to reductive amination with HCHO in H_2O (36%) (0.024 mL, 0.23 mmol), AcOH (0.020 mL, 0.35 mmol) and $NaBH(OAc)_3$ (0.074 g, 0.35 mmol). The mixture, after purification by PLC ($CHCl_3$) to a solid (0.05 g, 0.11 mmol, 82%), was reduced with NH_4OAc (0.091 g, 1.2 mmol), $SnCl_2{\cdot}2H_2O$ (0.15 g, 0.66 mmol) and MeOH (1 mL) at 50 °C for 5 h. The mixture was concentrated to a residue, which was suspended with EtOAc. After the removal of insoluble materials, the solution was treated with PLC ($CHCl_3$/MeOH = 19:1) to give **19** (0.009 g, 0.021 mmol, 19%). Mp 142–144 °C. IR (KBr) 3368, 3233, 1642, 1614, 1454, 1304 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.22 (3H, s, NCH_3), 3.56 (2H, s, NCH_2Ph), 3.60 (2H, s, PhCH_2N), 6.83–6.86 (2H, m, Ph*H*), 7.06–7.10 (2H, m, Ar*H*), 7.27–7.34 (2H, m, thienyl*H*), 7.38 (2H, d, *J* = 8.0 Hz, Ph*H*), 7.50 (2H, d, *J* = 8.0 Hz, Ph*H*), 7.59 (2H, d, *J* = 8.0 Hz, Ph*H*), 7.84–7.88 (3H, m, Ar*H*). FAB-MS *m/z*: 428 $(M+H)^+$. HR-FAB-MS *m/z*: $(M+H)^+$ calcd for $C_{26}H_{26}N_3OS$, 428.1797; found, 428.1793.

5.2.23. *N*-(2-Aminophenyl)-4-((N-(4-(thiophen-2-yl)benzyl) methylsulfonamido) methyl)benzamide (20)

A suspension of **18** (0.050 g, 0.11 mmol) in CH_2Cl_2 (0.5 mL) was treated with methanesulfonyl chloride (8.5 μM, 0.35 mmol) and TEA (0.03 mL, 0.22 mmol) at room temperature overnight. The mixture, after purification by PLC ($CHCl_3$) to an oily product (0.045 g, 0.086 mmol, 78%), was reduced in a manner similar to **19**, yielding **20** (0.010 g, 0.020 mmol, 24%). IR (KBr) 3335, 1649, 1450, 1315, 1146 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.88 (3H, s, SO_2CH_3), 4.37 (2H, s, NCH_2Ph), 4.44 (2H, s, PhCH_2N), 6.88 (1H, d, *J* = 4.0 Hz, Ar*H*), 7.08–7.34 (9H, m, Ar*H*), 7.44 (2H, d, *J* = 8.0 Hz, Ar*H*), 7.60

(2H, d, J = 8.0 Hz, ArH), 7.93 (2H, d, J = 8.0 Hz, ArH). FAB-MS m/z: 492 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{26}H_{26}N_3O_3S_2$, 492.1416; found, 492.1424.

5.2.24. *N*-(2-Aminophenyl)-4-((3,3-dimethyl-1-(4-(thiophen-2-yl)benzyl)ureido) methyl)benzamide (21)

A suspension of **18** (0.050 g, 0.11 mmol) in CH_2Cl_2 (0.5 mL) was treated with dimethylcarbamyl chloride (0.010 mL, 0.11 mmol) and TEA (0.03 mL, 0.22 mmol) at room temperature overnight. The mixture, after purification by PLC ($CHCl_3$) to an oily product (0.053 g, 0.10 mmol, 94%), was reduced in a manner similar to **19**, giving **21** (0.019 g, 0.039 mmol, 38%). IR (KBr) 3256, 1620, 1454, 1393, 1315 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.92 (6H, d, J = 2.4 Hz, NCH_3), 4.30 (2H, s, NCH_2Ph), 4.35 (2H, s, PhCH_2N) 7.09–7.32 (9H, m, ArH), 7.58 (3H, m, ArH), 7.86 (2H, d, J = 7.6 Hz, ArH), 8.07 (1H, s, ArH). FAB-MS m/z: 485 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{28}H_{29}N_4O_2S$, 485.2011; found, 485.2016.

5.2.25. *N*-(2-Aminophenyl)-4-(((2-hydroxyethyl)(4-(thiophen-2-yl)benzyl)amino) methyl)benzamide (22)

A solution of **18** (0.10 g, 0.23 mmol) in MeCN (1 mL) was reacted with 2-bromoethanol (0.033 mL, 0.46 mmol) and TEA (0.064 mL, 0.46 mmol) at 60 °C for 12 h. The product was, after purification with PLC (0.061 g, 0.13 mmol, 54%), was reduced in a manner similar to **19**, affording **22** (0.11 g, 0.24 mmol, 45%). Mp 146–148 °C. IR (KBr) 3240, 1651, 1612, 1454, 1292 cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.71 (2H, t, J = 5.2 Hz, CH_2CH$_2$OH), 3.63 (2H, t, J = 5.2 Hz, CH$_2$CH_2OH), 3.66 (2H, s, NCH_2Ph), 3.71 (2H, s, PhCH_2N), 6.85–6.87 (2H, m, PhH), 7.07–7.12 (2H, m, ArH), 7.28–7.33 (4H, m, ArH), 7.45 (2H, d, J = 8.0 Hz, PhH), 7.59 (2H, d, J = 8.0 Hz, PhH), 7.84–7.89 (3H, m, ArH). FAB-MS m/z: 458 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{27}H_{28}N_3O_2S$, 458.1902; found, 458.1903.

5.2.26. *N*-(2-Aminophenyl)-4-(((biphenyl-4-ylmethyl)(2-hydroxyethyl)amino) methyl)benzamide (23)

Compound **23** was prepared from **15b** (0.21 g, 0.48 mmol) according to the procedure used for **22** (0.11 g, 0.23 mmol, 20%). Mp 152–154 °C. IR (KBr) 3321, 3211, 1643, 1612, 1454, 1312 cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.53 (2H, m, CH_2CH$_2$OH), 3.51–3.57 (2H, m, CH$_2$CH_2OH), 3.65 (2H, s, NCH_2Ph), 3.71 (2H, s, PhCH_2N), 4.47 (1H, t, J = 5.2 Hz, CH$_2$CH$_2$OH), 4.89 (2H, s, PhNH_2), 6.59 (1H, t, J = 7.6 Hz, PhH), 6.78 (1H, d, J = 7.6 Hz, PhH), 6.97 (1H, t, J = 7.6 Hz, PhH), 7.16 (1H, d, J = 7.6 Hz, PhH), 7.35 (1H, t, J = 7.6 Hz, PhH), 7.44–7.54 (6H, m, PhH), 7.65 (4H, dd, J = 9.2, 7.6 Hz, PhH), 7.95 (2H, d, J = 8.0 Hz, PhH), 9.63 (1H, s. CONH). FAB-MS m/z: 452 $(M+H)^+$. HR-FAB-MS m/z: $(M+H)^+$ calcd for $C_{29}H_{30}N_3O_2$, 452.2338; found, 452.2334.

5.3. Solubility tests of HDAC inhibitors in 10% DMSO

A solution (100 mg/mL) of each HDAC inhibitor in DMSO was diluted with H_2O to prepare a 10% DMSO suspension (10 mg/mL), which was subsequently sonicated for 15 min, stirred with a Vortex for 0.5 min and allowed to stand for 5 min. Dilution with 10% DMSO followed by the same procedures was repeated until residual fine particles which were suspended but not precipitated, started to appear on observation with a magnifying glass (×10).

5.4. Cells, animals and antibodies

HCT116 cells were cultured in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 50 μg/mL penicillin G, and 50 μg/mL streptomycin sulfate (Invitrogen) in a 5% CO_2 and 95% air atmosphere at 37 °C. Female BALB/c-nu/nu mice (4 weeks of age) were purchased from Japan SLC Inc. The mice were used at 6 weeks of age. Antibodies specific to p21/WAF1 and β-actin were purchased from Sigma–Aldrich, and anti-acetyl-histone H3 was from Millipore.

5.5. Evaluation of HDAC1 inhibition

The IC_{50} values of **3**, **6**, **9a** and **9b** were determined using a SCADS inhibitor kit by the Screening Committee of New Anticancer Agents.[23,24] Those of other compounds were measured utilizing HDAC1 Fluorimetric Drug Discovery Kit-AK-511 (BIOMOL).

5.6. Evaluation of growth inhibition in HCT116 cells

HCT116 cells were cultured in McCoy's 5a medium with 10% FBS. The cells (1×10^4 /mL) were inoculated onto standard 96-well microtiter plates. Following 24 h of culture, serially diluted samples were added to the wells. After a 3-day culture, cell growth was evaluated with the assay of WST-1 (Dojindo), and IC_{50} values were calculated.

5.7. Western blot analysis

HCT116 cells were plated onto 60-mm diameter dishes (1.0×10^6/dish). After incubating for 24 h, the cells were washed twice with the serum-free medium (1 mL) and suspended with the serum-free medium (5 mL) for 24 h. After removal of the medium, the cells were washed twice with the serum-free medium (1 mL) and incubated in the serum-free medium (5 mL) with a test compound (10 μM) for another 24 h. The medium was discarded, and floating and adherent cells were washed twice with PBS (each 1 mL), spraped, and resuspended in 200 μL of modified Covance Laboratoes lysis buffer[25] (pH 8.0 and 1% Nonidet P-40 were adopted in place of pH 7.4 and 1% Triton-X 100, respectively). The lysates were centrifuged at 14,000g for 15 min at 4 °C. The protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific). An equal amount of protein was then resolved by SDS–PAGE and transferred to PVDF membrane. The blots (1 × Tris–HCl buffer saline, 5% non-fat milk and 0.05% Tween 20) were probed with an antibody specific to each protein and detected using ECL chemiluminescence.

5.8. Examination of cell cycle regulation by flow cytometry

HCT116 cells were plated onto 60-mm diameter dishes (1.0×10^6/dish). After incubation for 24 h, the cells were treated as for the Western blot analysis. The adherent cells were treated with 0.25% trypsin (Invitrogen) and combined with the floating cells. All the cells were treated with a Cycle Test Plus DNA reagent Kit (Catalog No. 340242, Becton Dickinson). DNA content was measured with a FACS™Cant II.

5.9. TUNEL assay by flow cytometry

HCT 116 cells (1.0×10^6/dish) were treated as above. The collected floating and adherent cells were washed twice with PBS (each 1 mL). The cells were fixed with PBS (0.5 mL) (containing BSA (2.5 mg) and 0.1% TRITON X-100) and 1% paraformaldehyde (5 mL) for 20 min at 0 °C, washed twice with the above PBS (each 5 mL) and permeabilized by PBS (0.5 mL) (containing BSA (2.5 mg) and 0.1% TRITON X-100) and 70% EtOH. The TUNEL reaction was carried out by incubating cells (60 min; 37 °C) with 50 μM of TUNEL mixture (DeadEnd™ Fluorometirc TUNEL System) and treated with PI solution (Dojindo) and RNase (Wako) according to the procedure (Catalog No. G3250, Promega). Rates of cell fragmentation were measured with FACS™Cant II.

5.10. In vivo antitumor activity

HCT116 cells (1.3×10^6/100 μL medium) were injected subcutaneously into the back of nude mice (6 weeks of age). Compounds **22** and MS-275 (each 0.9 mg/200 μL) as well as vehicle (0.1% Tween 80, 200 μL) were administered orally to the nude mice with tumors (tumor size: 94–110 mm^3) (n = 10, 11 and 10, respectively) on days 0, 2, 4, 7, 9, 11, 14 and 16. Tumor length and width as well as body weights were monitored. Tumor volume (mm^3) was calculated by measuring tumor length and width (in mm) as described.[26] T/C was calculated by dividing tumor volume for treated mice by tumor volume for control mice. At day18, the cervical spine was dislocated. Tumor masses were removed and weighed as they were.

5.11. Statistical analysis

The statistical significance of the effects of drugs virus control was analyzed with Student's t-test.

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