Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 4208–4212

# Design, synthesis, and biological activity of folate receptor-targeted prodrugs of thiolate histone deacetylase inhibitors

Takayoshi Suzuki,[a,*] Shinya Hisakawa,[a] Yukihiro Itoh,[a] Nobuaki Suzuki,[a] Katsumasa Takahashi,[a] Masatoshi Kawahata,[b] Kentaro Yamaguchi,[b] Hidehiko Nakagawa[a] and Naoki Miyata[a,*]

[a]*Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan*
[b]*Evaluation Group, Drug Research Department, R&D Division, Pharmaceuticals Group, Nippon Kayaku Co., Ltd, 31-12, Shimo 3-chome, Kita-ku, Tokyo 115-8588, Japan*

Received 22 April 2007; revised 10 May 2007; accepted 11 May 2007
Available online 17 May 2007

**Abstract**—Aiming to develop selective anticancer drugs, we designed and synthesized three disulfides bearing a folic acid moiety as candidate folate receptor (FR)-targeted prodrugs of thiolate histone deacetylase inhibitors. Among them, compound **1** displayed growth-inhibitory activity toward folate receptor-positive MCF-7 breast cancer cells. The activity of **1** was significantly reduced by free folic acid, suggesting that cellular uptake of **1** is mediated by FR.


Histone deacetylases (HDACs) have recently emerged as a new target for the development of anticancer drugs, and some small-molecular HDAC inhibitors, including suberoylanilide hydroxamic acid (SAHA) (also known as vorinostat) and MS-275, have been developed as anticancer drugs (Fig. 1).[1–6] Inhibition of HDACs causes histone hyperacetylation which leads to the disruption of the chromatin structure and the transcriptional activation of genes associated with cancer. Indeed, HDAC inhibitors have shown anticancer activity in vitro, in animal models and in patients with solid tumors and hematological malignancies.[7] Nevertheless, they have been reported to cause adverse events, such as nausea, vomiting, anorexia, anemia, thrombocytopenia, and fatigue, in the course of clinical trials.[7–9] Therefore, it is necessary to find HDAC inhibitors that show selective anticancer activity.

We have focused on folate receptor (FR)-targeted prodrugs for selectively targeting cancer cells. The vitamin folic acid and its analogues display extremely high affinity for the folate receptor on the cell surface, and are internalized via receptor-mediated endocytosis.[10] Since the FR is overexpressed on certain malignant cell types and is undetectable or present only at low levels in most normal tissues,[11–13] targeting of the FR has been proposed as a potential mechanism for delivery of drugs to treat cancer.[14] In addition, small molecules including folate-drug conjugates may avoid the limitations associated with antibody-mediated targeting.[15,16] Here, we report on the design, synthesis, and biological activity of FR-targeted prodrugs of HDAC inhibitors.

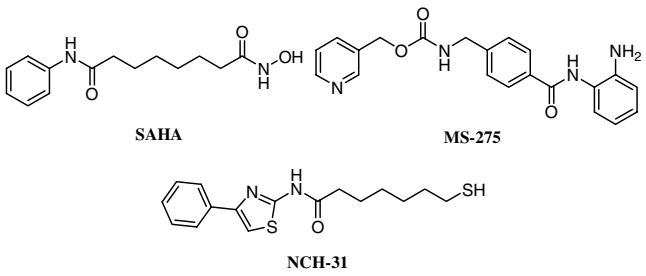



**Figure 1.** Structures of SAHA, MS-275, and the thiolate HDAC inhibitor NCH-31.

We previously reported that thiol-based analogues, including NCH-31 (Fig. 1), are potent HDAC inhibitors.[17–19] Thiols are thought to inhibit HDACs by coordinating the zinc ion, which is required for deacetylation

*Keywords*: Histone deacetylase inhibitor; Selective anticancer activity; Folate conjugate; Prodrug.
* Corresponding authors. Tel./fax: +81 52 836 3407; e-mail addresses: suzuki@phar.nagoya-cu.ac.jp; miyata-n@phar.nagoya-cu.ac.jp

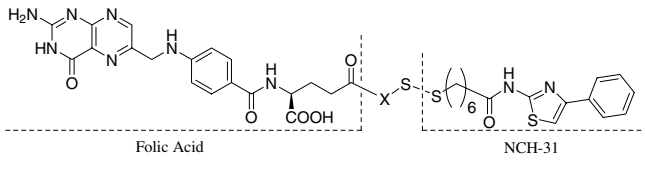
0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.05.040

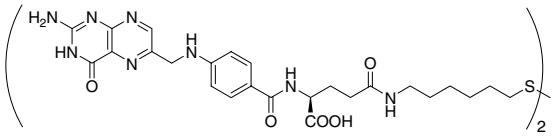
of the acetylated lysine substrate. Further, thiolate analogues showed potent cancer cell growth-inhibitory activities.[20,21] Based on these findings, we designed FR-targeted prodrugs of HDAC inhibitors. Unlike hydroxamates and *o*-aminoanilides, such as SAHA and MS-275, thiolate HDAC inhibitors can be conjugated with a folic acid moiety via a disulfide bond, which would be reduced under reductive conditions, releasing the free thiol as an active species. We designed the folic acid-NCH-31 conjugates **1** and **2** (Fig. 2), which are expected to be recognized by the FR located on the cell surface, to enter cells via receptor-mediated endocytosis, and then to release the HDAC inhibitor NCH-31 upon cleavage of the disulfide bond in the cellular environment. We also designed the symmetrical disulfide **3** bearing a folic acid moiety. The reduced form of compound **3** itself could behave as an HDAC inhibitor.

The synthesis of the folic acid-NCH-31 conjugate **1** is outlined in Scheme 1. Mercaptoethylamine **4** was converted into 2-(2-(2-pyridinyl)disulfanyl)ethylamine **5** by the Boc protection of **4**, followed by treatment with 2,2′-dithiopyridine and Boc deprotection. Compound **5** was then coupled with *N*-Boc-L-glutamic acid α-*tert*-butyl ester to give the amide **6**. Treatment of compound **6** with NCH-31[17,20] in DMF afforded the sulfur-exchanged product **7**. Universal deprotection of **7** using hydrochloric acid yielded the NCH-31-glutamic acid linked compound **8**. The folic acid-NCH-31 conjugate **1** was obtained in 92% yield by the reaction of **8** with pteroyl azide **9**[22] in DMSO in the presence of tetramethylguanidine.

Scheme 2 shows the preparation of the other folic acid-NCH-31 conjugate **2**. Compound **2** was synthesized from 2,2′-(ethylenedioxy)diethylamine **10**. Reaction of the diamine **10** with an equivalent amount of $(Boc)_2O$ gave the mono-Boc-protected compound **11**. Coupling between amine **11** and 3-mercaptopropanoic acid in the presence of EDCI and HOBt afforded **12**. The folic acid-NCH-31 conjugate **2** was prepared from the thiol **12** in the same manner as described for the synthesis of **1**.

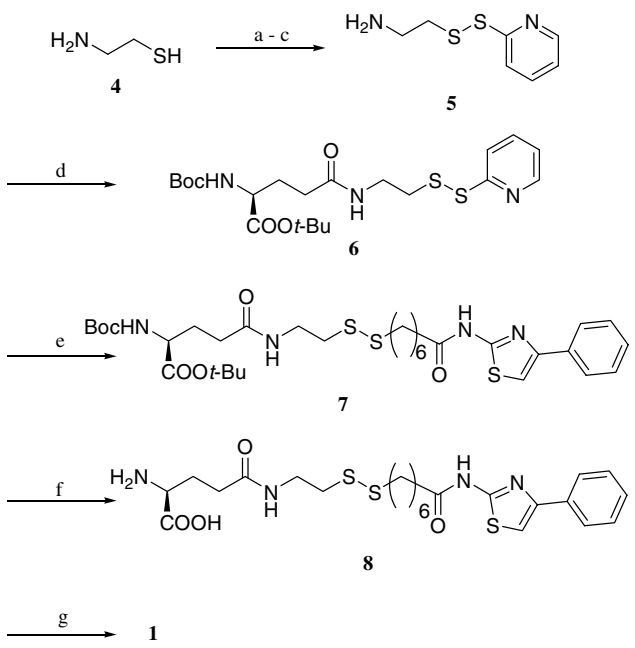
The attempted route to **3** is shown in Scheme 3. In this scheme, we anticipated that disulfide dimer **3** would be



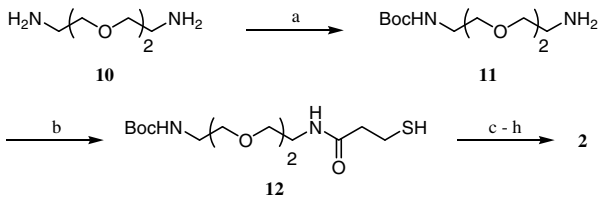



**3**

**Figure 2.** Candidate FR-targeted prodrugs of thiolate HDAC inhibitors.

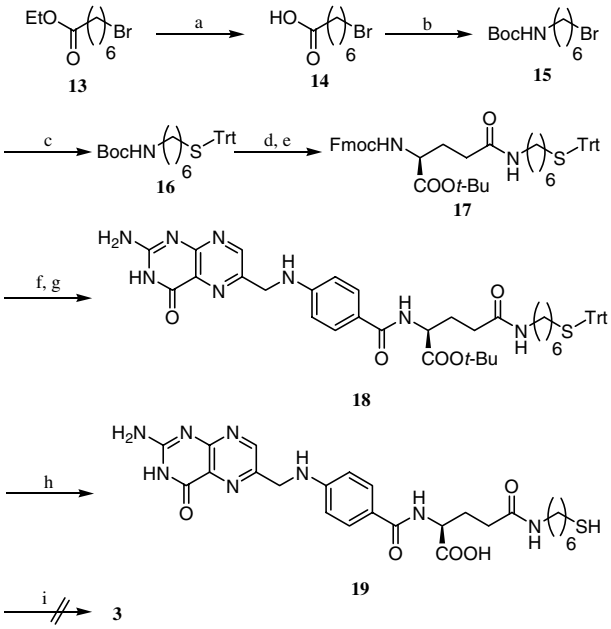


**Scheme 1.** Reagents and conditions: (a) $(Boc)_2O$, $Et_3N$, $CH_2Cl_2$, rt; (b) 2,2′-dithiopyridine MeOH, rt; (c) HCl, AcOEt, rt, 39% (three steps); (d) Boc-Glu-O-*t*-Bu, EDCI, HOBt, $Et_3N$, $CH_2Cl_2$, rt, 76%; (e) NCH-31, DMF, rt, 86%; (f) HCl, AcOEt, rt, quant; (g) pteroyl azide (**9**), tetramethylguanidine, DMSO, rt, 92%.



**Scheme 2.** Reagents and conditions: (a) $(Boc)_2O$, $CH_2Cl_2$, 0 °C to rt, quant; (b) 3-mercaptopropanoic acid, EDCI, HOBt, $CH_2Cl_2$, rt, 53%; (c) 2,2′-dithiopyridine MeOH, rt; (d) HCl, AcOEt, rt; (e) Boc-Glu-O-*t*-Bu, EDCI, HOBt, $Et_3N$, $CH_2Cl_2$, rt; (f) NCH-31, DMF, rt; (g) HCl, AcOEt, rt; (h) **9**, tetramethylguanidine, DMSO, rt, 59% (six steps).

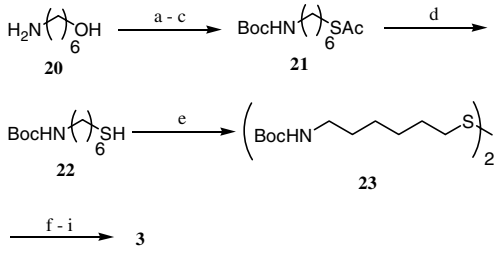
obtained from the corresponding thiol monomer by reaction with $I_2$. The 7-bromoheptanoic acid ethyl ester **13** was hydrolyzed to give the carboxylic acid **14**, after which Curtius rearrangement of the acyl azide prepared from **14** using diphenylphosphoryl azide (DPPA) provided the isocyanate. This, on treatment with *tert*-butanol, gave the *N*-Boc compound **15**. Treatment of **15** with triphenylmethanethiol in the presence of NaOMe afforded compound **16**. Deprotection of the Boc group of **16** and coupling with *N*-Fmoc-L-glutamic acid α-*tert*-butyl ester gave the amide **17**. The Fmoc group of **17** was removed using piperidine and coupling with pteroyl azide **9** afforded compound **18**. Removal of the *tert*-butyl group and the triphenylmethyl group of **18** under acidic conditions gave the thiol **19**. Although **19** was successfully obtained from **13** in eight steps, it was poorly soluble. We examined a variety of solvents for the dimerization of **19** using $I_2$, but a suspension of **19** with $I_2$ failed to provide the disulfide dimer **3**.



**Scheme 3.** Reagents and conditions: (a) LiOH, THF, EtOH, $H_2O$, rt, quant; (b) 1—DPPA, $Et_3N$, reflux, 2—*t*-BuOH, toluene, reflux, 44%; (c) NaOMe, HS-Trt, toluene, EtOH, 60 °C, 94%; (d) TFA, $CH_2Cl_2$, rt; (e) Fmoc-Glu-O-*t*-Bu, EDCI, HOBt, $CH_2Cl_2$, rt, 76% (two steps); (f) piperidine, DMF, rt, 87%; (g) **9**, tetramethylguanidine, DMSO, rt, 21%; (h) TFA, $CH_2Cl_2$, rt, 94%; (i) $I_2$.

We succeeded in obtaining **3** through the route outlined in Scheme 4. In this route, a disulfide bond was formed in the early stage. Initially, 6-aminohexanol **20** was converted to compound **21** by *N*-Boc protection, O-tosylation, and treatment with potassium thioacetate. The acetyl group of **21** was then removed to give the thiol **22**, and the disulfide dimer **23** was obtained by the reaction of the thiol monomer **22** with $I_2$. The desired disulfide **3** was successfully obtained from **23** in 74% yield using the same procedure as described for the synthesis of **1**.

Compounds **1**–**3** were initially tested in an in vitro HDAC inhibition assay under reductive conditions (Table 1).[23] Among these compounds, compound **1** showed the most potent activity inhibiting HDACs with an $IC_{50}$ of 0.27 μM, and the activity was comparable with that of NCH-31. This result suggested that the



**Scheme 4.** Reagents and conditions: (a) $(Boc)_2O$, THF, rt; (b) TsCl, $Et_3N$, THF, rt; (c) KSAc, acetone, rt, 75% (three steps); (d) aq NaOH, MeOH, THF, rt, 71%; (e) $I_2$, MeOH, rt, 95%; (f) HCl, AcOEt, rt; (g) Boc-Glu-O-*t*-Bu, EDCI, HOBt, $Et_3N$, $CH_2Cl_2$, rt; (h) HCl, AcOEt, rt; (i) **9**, tetramethylguanidine, DMSO, rt, 74% (four steps).

**Table 1.** HDAC inhibition data for NCH-31, **1**–**3**, and **19**[a]

| Entry | Compound | $IC_{50}$ (μM) |
|---|---|---|
| 1 | NCH-31 | 0.17[b] |
| 2 | **1**[c] | 0.27 |
| 3 | **2**[c] | 3.0 |
| 4 | **3**[c] | 9.0 |
| 5 | **19** | 21 |

[a] Values are means of at least three experiments.
[b] Data taken from the literature (Ref. 20).
[c] Incubated with DTT (250 μM).

disulfide bond of compound **1** was reduced to release NCH-31 under the reductive conditions. On the other hand, the HDAC-inhibitory activities of compounds **2** and **3** were weaker than that of **1**. The reason for the weaker activity of **2** is unclear, but it may be because compound **2** is resistant to reduction as compared with compound **1**.

To confirm the effectiveness of the folic acid-based prodrugs of HDAC inhibitors, compounds **1** and **2** were tested in a cancer cell growth inhibition assay[24] using FR-positive human breast cancer MCF-7 cells.[25] Consistent with the results in the enzyme assay, compound **1** displayed dose-dependent cell growth-inhibitory activity (Fig. 3). Further, a competition experiment with 100 μM free folic acid significantly reduced the cell growth-inhibitory activity of **1**, demonstrating that the FR is responsible for the cellular entry of **1** (Fig. 4). In addition, treatment of MCF-7 cells with compound **1** produced an increase in the accumulation of acetylated histone H4 (Fig. 5),[26] which indicated that the cell growth-inhibitory activity of compound **1** significantly correlates with the inhibition of HDACs. Furthermore, the activity of **1** to cause histone hyperacetylation was significantly reduced by 100 μM free folic acid (Fig. 5). These results also suggested that the uptake of compound **1** is FR mediated.

In summary, we have designed and synthesized FR-targeted prodrugs of thiolate HDAC inhibitors that possess a cleavable disulfide bond. The folic acid-NCH-31 conjugate **1** showed potent HDAC-inhibitory activity under reductive conditions. Furthermore, compound **1** exerted growth-inhibitory activity against FR-positive breast cancer MCF-7 cells, and the cellular uptake of **1** was considered to be FR mediated, based on a competition experiment with free folic acid. Our strategy of utilizing

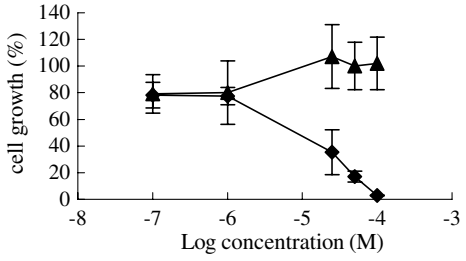



**Figure 3.** Growth inhibition of FR-positive MCF-7 cells by compounds **1** (●) and **2** (▲).

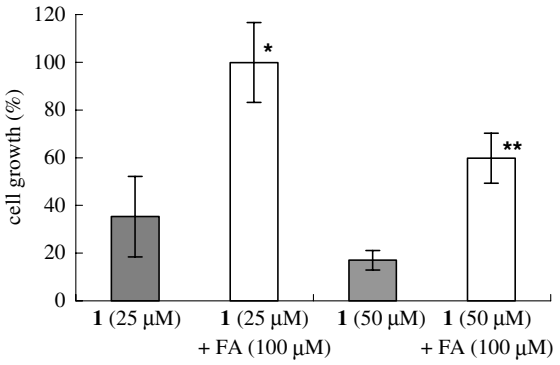



**Figure 4.** Growth inhibition of FR-positive MCF-7 cells by **1** and **1** plus 100 μM free folic acid (FA). $^{*}p < 0.05$; $^{**}p < 0.01$ by Student's *t* test.

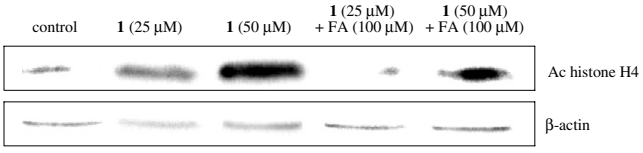



**Figure 5.** Western blot analysis of histone hyperacetylation in MCF-7 cells produced by **1** and **1** plus 100 μM free folic acid (FA).

a disulfide bond to connect a thiolate HDAC inhibitor with folic acid should be applicable to other anticancer agents bearing a thiol group, such as thiolate matrix metalloproteinase inhibitors[27] and thiolate farnesyltransferase inhibitors.[28] Our findings in this study provide the basis for a new approach to developing candidate antitumor agents with potentially fewer side effects.

## Acknowledgments

This work was supported in part by Grants-in-Aid for Young Scientists (B) from the Ministry of Education, Science, Culture, Sports, Science, and Technology, Japan, and a grant from Takeda Science Foundation.

## References and notes

1. Miller, T. A.; Witter, D. J.; Belvedere, S. *J. Med. Chem.* **2003**, *46*, 5097.
2. Miller, T. A. *Expert Opin. Ther. Pat.* **2004**, *14*, 791.
3. Biel, M.; Wascholowski, V.; Giannis, A. *Angew. Chem. Int. Ed. Engl.* **2005**, *44*, 3186.
4. Suzuki, T.; Miyata, N. *Curr. Med. Chem.* **2006**, *13*, 935.
5. Richon, V. M.; Emiliani, S.; Verdin, E.; Webb, Y.; Breslow, R.; Rifkind, R. A.; Marks, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3003.
6. Saito, A.; Yamashita, T.; Mariko, Y.; Nosaka, Y.; Tsuchiya, K.; Ando, T.; Suzuki, T.; Tsuruno, T.; Nakanishi, O. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4592.
7. Kelly, W. K.; Richon, V. M.; O'Connor, O.; Curley, T.; MacGregor-Curtelli, B.; Tong, W.; Klang, M.; Schwartz, L.; Richardson, S.; Rosa, E.; Drobnjak, M.; Cordon-Cordo, C.; Chiao, J. H.; Rifkind, R.; Marks, P. A.; Scher, H. *Clin. Cancer Res.* **2003**, *9*, 3578.
8. Kelly, W. K.; O'Connor, O. A.; Krug, L. M.; Chiao, J. H.; Heaney, M.; Curley, T.; MacGregore-Cortelli, B.; Tong, W.; Secrist, J. P.; Schwartz, L.; Richardson, S.; Chu, E.; Olgac, S.; Marks, P. A.; Scher, H.; Richon, V. M. *J. Clin. Oncol.* **2005**, *23*, 3923.
9. Ryan, Q. C.; Headlee, D.; Acharya, M.; Sparreboom, A.; Trepel, J. B.; Ye, J.; Figg, W. D.; Hwang, K.; Chung, E. J.; Murgo, A.; Melillo, G.; Elsayed, Y.; Monga, M.; Kalnitskiy, M.; Zwiebel, J.; Sausville, E. A. *J. Clin. Oncol.* **2005**, *23*, 3912.
10. Antony, A. C. *Blood* **1992**, *79*, 2807.
11. Ross, J. F.; Chaudhuri, P. K.; Ratnam, M. *Cancer* **1994**, *73*, 2432.
12. Weitman, S. D.; Lark, R. H.; Coney, L. R.; Fort, D. W.; Frasca, V.; Zurawski, V. R.; Kamen, B. A. *Cancer Res.* **1992**, *52*, 3396.
13. Weitman, S. D.; Weinberg, A. G.; Coney, L. R.; Zurawski, V. R.; Jennings, D. S.; Kamen, B. A. *Cancer Res.* **1992**, *52*, 6708.
14. Leamon, C. P.; Reddy, J. A. *Adv. Drug Deliv. Rev.* **2004**, *56*, 1143.
15. Canevari, S.; Mezzanzanica, D.; Menard, S.; Ferrini, S.; Moretta, L.; Colnaghi, M. I. *Int. J. Cancer* **1992**, *7*, 42.
16. Yamaguchi, T.; Tsurumi, H.; Kotani, T.; Yamaoka, N.; Otsuji, E.; Kitamura, K.; Takahashi, T. *Jpn. J. Cancer Res.* **1994**, *85*, 167.
17. Suzuki, T.; Kouketsu, A.; Matsuura, A.; Kohara, A.; Ninomiya, S.; Kohda, K.; Miyata, N. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3313.
18. Suzuki, T.; Matsuura, A.; Kouketsu, A.; Nakagawa, H.; Miyata, N. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 331.
19. Suzuki, T.; Kouketsu, A.; Itoh, Y.; Hisakawa, S.; Maeda, S.; Yoshida, M.; Nakagawa, H.; Miyata, N. *J. Med. Chem.* **2006**, *49*, 4809.
20. Suzuki, T.; Nagano, Y.; Kouketsu, A.; Matsuura, A.; Maruyama, S.; Kurotaki, M.; Nakagawa, H.; Miyata, N. *J. Med. Chem.* **2005**, *48*, 1019.
21. Suzuki, T.; Hisakawa, S.; Itoh, Y.; Maruyama, S.; Kurotaki, M.; Nakagawa, H.; Miyata, N. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 1558.
22. Luo, J.; Smith, M. D.; Lantrip, D. A.; Wang, S.; Fuchs, P. L. *J. Am. Chem. Soc.* **1997**, *119*, 10004.
23. The HDAC activity assay was performed using an HDAC fluorescent activity assay/drug discovery kit (AK-500, BIOMOL Research Laboratories). HeLa Nuclear Extracts (0.5 μL/well) were incubated at 37 °C with 25 μM of Fluor de Lys™ substrate and various concentrations of samples. Reactions were stopped after 30 min by adding Fluor de Lys™ Developer with trichostatin A which stops further deacetylation. Then, 15 min after addition of this developer, the fluorescence of the wells was measured on a fluorometric reader with excitation set at 360 nm and emission detection set at 460 nm, and the % inhibition was calculated from the fluorescence readings of inhibited wells relative to those of control wells. The concentration of compound which results in 50% inhibition was determined by plotting the log[Inh] versus the logit function of the % inhibition. $IC_{50}$ values are determined using a regression analysis of the concentration/inhibition data.
24. MCF-7 human breast cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing penicillin and streptomycin, which was supplemented with fetal bovine serum as described in the ATCC instructions. MCF-7 cells were plated in 96-well plates at initial densities of 5000 cells/well (50 μL/well) and incubated at 37 °C. After 24 h, cells were exposed to a solution of test compounds in DMEM (50 μL) at various concentrations in DMEM at 37 °C in

5% $CO_2$ for 72 h. Then, 10 μL of alamarBlue™ was added, and cells were incubated at 37 °C for 3 h. The fluorescence of the wells was measured on a fluorometric reader with excitation set at 530 nm and emission detection set at 590 nm, and the percentage of cell growth was calculated from the fluorescence readings.

25. Lee, E. S.; Na, K.; Bae, Y. H. *J. Control. Release* **2003**, *91*, 103.
26. MCF-7 cells were cultured in DME culture medium containing penicillin and streptomycin, which was supplemented with fetal bovine serum as described in the ATCC instructions. MCF-7 cells ($5 \times 10^5$) were treated for 14 h with samples at the indicated concentrations in 10% FBS-supplemented DMEM then collected and extracted with SDS buffer. Protein concentrations of the lysates were determined using a Bradford protein assay kit (Bio-Rad Laboratories); equivalent amounts of proteins from each lysate were resolved in 15% SDS–polyacrylamide gel and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). After blocking for 30 min with Tris-buffered saline (TBS) containing 3% skim milk, the transblotted membrane was incubated overnight at 4 °C with hyperacetylated histone H4 antibody (Upstate Biotechnology) (1:2000 dilution) or β-actin antibody (Abcam) (1:1000 dilution) in TBS containing 3% skim milk. After probing with the primary antibody, the membrane was washed twice with water, then incubated with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (diluted 1:5000) for 2 h at room temperature, and further washed twice with water. The immunoblots were visualized by enhanced chemiluminescence.
27. Whittaker, M.; Floyd, C. D.; Brown, P.; Gearing, A. J. H. *Chem. Rev.* **1999**, *99*, 2735.
28. Bell, I. M. *J. Med. Chem.* **2004**, *47*, 1869.