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Lactam-Based HDAC Inhibitors for Anticancer Chemotherapy: Restoration of RUNX3 by Posttranslational Modification and Epigenetic Control

Misun Cho,[a] Eunhyun Choi,[c] Jae Hyun Kim,[a] Hwan Kim,[a] Hwan Mook Kim,[d] Jang Ik Lee,[e] Ki-Chul Hwang,[f] Hyun-Jung Kim,*[b] and Gyoonhee Han*[a]

Expression and stability of the tumor suppressor runt-related transcription factor 3 (RUNX3) are regulated by histone deacetylase (HDAC). HDAC inhibition alters epigenetic and posttranslational stability of RUNX3, leading to tumor suppression. However, HDAC inhibitors can nonselectively alter global gene expression through chromatin remodeling. Thus, lactam-based HDAC inhibitors were screened to identify potent protein stabilizers that maintain RUNX3 stability by acetylation. RUNX activity and HDAC inhibition were determined for 111 lactam-based analogues through a cell-based RUNX activation and HDAC inhibition assay. 3-[1-(4-Bromobenzyl)-2-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-*N*-hydroxypropanamide (**11-8**) significantly increased RUNX3 acetylation and stability with relatively low RUNX3 mRNA expression and HDAC inhibitory activity. This compound showed significant antitumor effects, which were stronger than SAHA, in an MKN28 xenograft model. Thus, we propose a novel strategy, in which HDAC inhibitors serve as antitumor chemotherapeutic agents that selectively target epigenetic regulation and protein stability of RUNX3.

Introduction

Histone deacetylases (HDACs) are a family of enzymes that catalyze histone deacetylation and subsequent epigenetic regulation of gene transcription. HDACs are associated with the pathogenesis of several diseases, including cancers.[1] Recent research indicates that HDACs also regulate deacetylation of nonhistone proteins, such as, transcription factors, chaperones, structural proteins, and tumor suppressor proteins.[2] Therefore, pharmacological inhibitors of HDACs are considered to be potential therapeutic agents for treating cancers.[3] Two HDAC inhibitors, vorinostat (SAHA, **1**) and romidepsin (FK228), have been approved by the US Food and Drug Administration (FDA) for treatment of cutaneous T-cell lymphoma (CTCL).[4]

In gastric cancer, the functional inactivation of runt-related transcription factor 3 (RUNX3) is related to tumor development. Thus, RUNX3 can function as a tumor suppressor.[5] RUNX3 activity is regulated by epigenetic silencing and posttranslational modification.[5b,6] Hypermethylation of the CpG island, histone H3K9 methylation, H3 deacetylation, and H3K27 methylation in the *RUNX3* promoter repress transcription of *RUNX3*.[5b] Nuclear translocation and stability of RUNX3 are regulated by transforming growth factor (TGF)-β signaling. Activation of TGF-β stimulates nuclear translocation of endogenous RUNX3, which inhibits cancer cell growth. In addition, TGF-β activation enhances acetylation of RUNX3 by p300 histone acetyltransferase (HAT) and stabilizes RUNX3 due to inhibition of ubiquitin-mediated degradation.[6c] Recent studies demonstrate that HDAC inhibitors, such as trichostatin A (TSA, **2**), contribute to reactivation and stabilization of RUNX3 and subsequent inhibition of cancer cell growth.[6b,c,7]

HDAC inhibitors have emerged as putative epigenetic drugs for anticancer treatment. However, these agents have substantial limitations, including epigenetic non-specificity (pleiotropic effects) and drug resistance.[8] The traditional rationale of epigenetic cancer therapy, which focuses on histone proteins, needs to be refined to selectively modify tumor-specific nonhistone proteins.[9] Restoration and stabilization of RUNX3 by HDAC inhibition is a novel approach for anticancer chemother-

[a] *Dr. M. Cho, J. H. Kim, Dr. H. Kim, Prof. G. Han*
Translational Research Center for Protein Function Control (TRCP)
Department of Biotechnology and
Department of Biomedical Sciences (WCU Program)
Yonsei University, Seodaemun-gu, Seoul 120-752 (Republic of Korea)
E-mail: gyoonhee@yonsei.ac.kr

[b] *Dr. H.-J. Kim*
BioRunx Co. Ltd., 12 Gaesin-dong, Huengduk-gu
Cheongju, Chungbuk 361-763 (Republic of Korea)
E-mail: hjkim7@yuhs.ac

[c] *Dr. E. Choi*
Severance Integrative Research Institute for
Cerebral and Cardiovascular Disease
Yonsei University Health System
Seodaemun-Gu, Seoul 120-752 (Republic of Korea)

[d] *Prof. H. M. Kim*
College of Pharmacy, Gachon University of Medicine and Science
Incheon 406-799 (Republic of Korea)

[e] *Prof. J. I. Lee*
College of Pharmacy, Yonsei University
85 Songdogwahak-ro, Yeonsu-gu, Incheon 406-840 (Republic of Korea)

[f] *Prof. K.-C. Hwang*
Severance Biomedical Science Institute
Cardiovascular Research Institute
Yonsei University College of Medicine
Seodaemun-gu, Seoul 120-752 (Republic of Korea)

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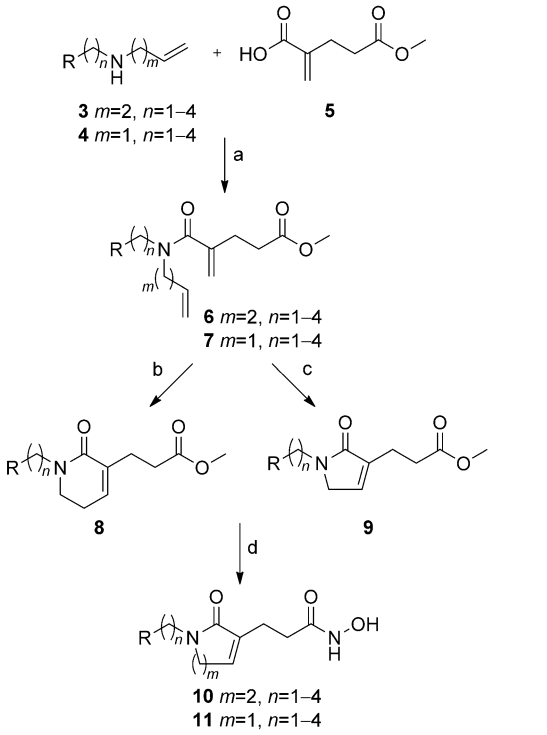
apy. We developed lactam-based HDAC inhibitors and selected those inhibitors that restored RUNX3 transcriptional expression by histone acetylation and stabilization of RUNX3 protein by acetylation at the posttranslational level.

In the present study, we investigated the effects of our in-house chemical library of lactam-based HDAC inhibitors on the restoration of *RUNX3* gene expression and RUNX3 acetylation and stabilization. Cell-based mechanistic studies provided a rationale for in vivo analysis. Our results suggest that epigenetic and posttranslational regulation of RUNX3 by lactam-based HDAC inhibitors might contribute to attenuation of gastric cancer pathogenesis. These novel data suggest that HDAC inhibitors can be developed as target-specific anticancer agents.

Results and Discussion

Chemical library of lactam-based HDAC inhibitors

In our previous studies, we reported novel lactam-based HDAC inhibitors (**10**, **11**) that consist of three parts. The inhibitors have diverse cap groups with substituted aromatic rings, δ- or γ-lactam cores, and hydroxamate moieties that function as zinc binders (Figure 1).[10] Several compounds potently inhibited HDAC activities and reduced cancer cell growth.[10,11] Through docking simulation study and quantitative structure–activity relationship (QSAR), we indicated that our active lactam compounds bound to the active pocket of HDAC; hydroxamate moieties were chelated with a zinc ion in the active site similar to TSA and SAHA, and the aromatic cap groups had hydrophobic interactions with the enzyme surface.[12] The lactam core interacted with the hydrophobic aromatic side chain, and γ-lactam especially gave stabilization by π–π interaction with phenylalanine residues in the narrow active pocket.[10b,11b,c,12]



Scheme 1. General synthetic procedures of lactam-based HDAC inhibitors (**10**, $m=2$; **11**, $m=1$; $n=1$–4). *Reagents and conditions*: a) EDC, DMAP, CH_2Cl_2; b) Grubbs catalyst I, CH_2Cl_2; c) Grubbs catalyst II, CH_2Cl_2; d) $KONH_2$ (1.7 M in MeOH), MeOH, 0 °C.

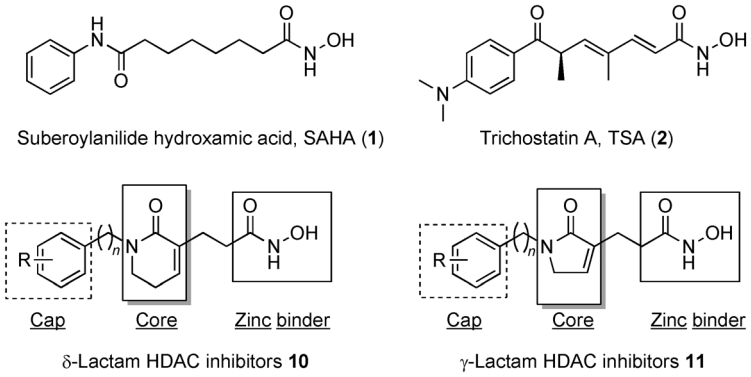


Figure 1. HDAC inhibitors.

The synthetic procedures of δ- and γ-lactam based HDAC inhibitors (**10**, **11**) are presented in Scheme 1. The secondary amines (**3**, **4**), which were obtained by *N*-alkylation or reductive amination, were coupled with monoacid (**5**) using a 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-mediated coupling reaction to afford amines **6** and **7**. Metathesis reaction with Grubbs' catalyst II (2–3 mol%) produced the δ-lactam rings (**8**) and Grubbs' catalyst I generated the γ-lactam rings (**9**) in good yields. The final compounds (**10**, **11**), which were transformed from methyl esters into hydroxamic acids, were obtained by subsequent reaction with potassium hydroxylamine in methanol at 0 °C.[11a,b]

A total of 111 lactam-based HDAC inhibitors were prepared to study their abilities to restore and stabilize RUNX3. There were 50 δ-lactam- and 62 γ-lactam-based analogues with a wide spectrum of HDAC inhibitory activities ranging from IC_{50} values of 0.01 μM to more than 10 μM (see table S1).

Screening: RUNX activity and HDAC inhibitory activity

We needed an assay system that could evaluate whether our lactam-based HDAC inhibitors affected RUNX proteins. We used an effective luciferase assay system in 6xOSE2-C2C12 cells to monitor RUNX transcriptional activity (Figure 2 and table S1).[13] RUNX activity is presented as percentage of activation compared to a positive control, fibroblast growth factor (FGF)-2, which is known to simulate RUNX2 expression and function and highly stimulates 6xOSE2-luciferase activity.[14] All three RUNX proteins share structural similarity and recognize the same consensus sequences in the promoter regions of target genes.[14] Overexpression of RUNX3 also stimulated RUNX activity (data not shown). In addition, RUNX3 is ex-

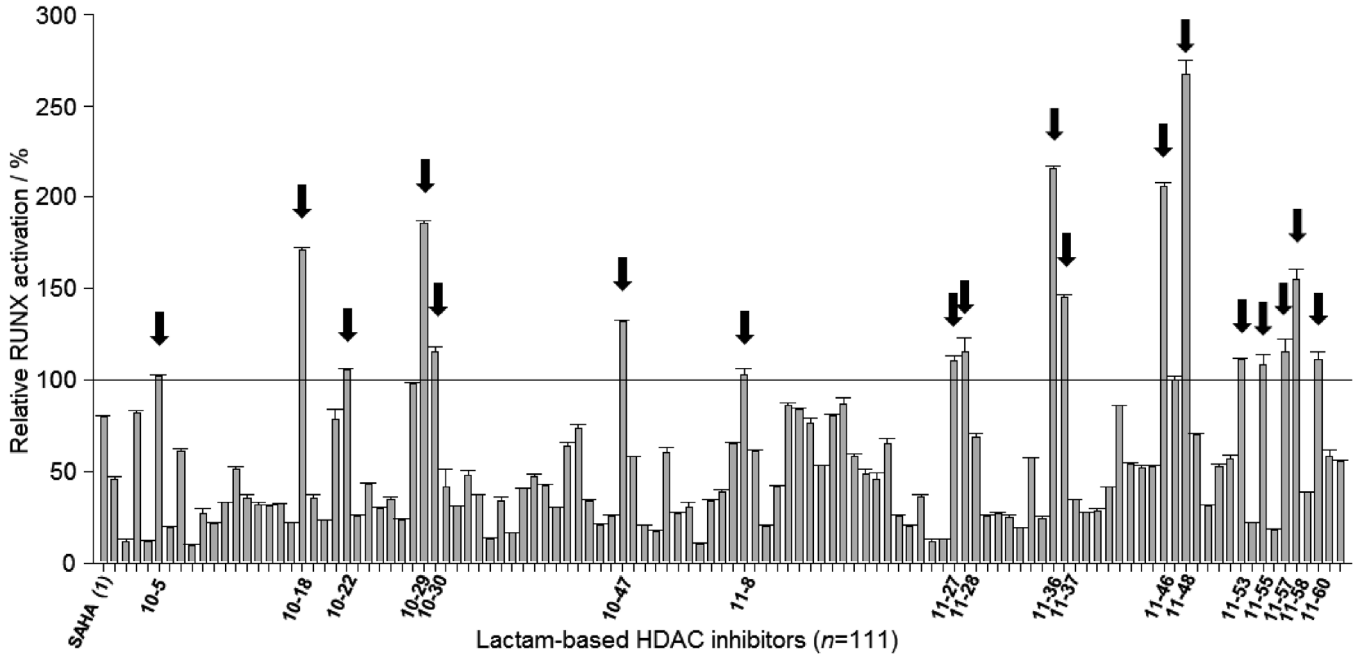


Figure 2. Percentage of RUNX transcriptional activation relative to FGF-2. Data represent the mean ± standard deviation (SD) of at least two independent experiments, and the order of compound names are provided in table S1. Compounds that show > 100% RUNX activity are highlighted with black arrows.

pressed in this cell line.[13] Based on these studies, the 6xOSE2-C2C12 cell-based assay is useful for screening compounds that regulate the activity of RUNX3 and other RUNX family members. Lactam analogues were compared to SAHA, which was the first epigenetic anticancer drug approved by the US FDA.[15]

We screened the effects of 111 compounds on RUNX transcriptional activity in 6xOSE2-C2C12 cells (Figure 2). Eighteen lactam analogues induced RUNX activity to greater than 100% and were selected for further analysis (Table 1). Three activators, **11-36**, **11-48** and **11-46**, which induced RUNX activation by > 200%, were categorized as group I. Three good activators, **10-18**, **11-58** and **10-29**, which induced RUNX activation from 150% to 200%, were classified as group II, and twelve activators, **11-28**, **11-37**, **11-27**, **10-47**, **11-57**, **11-53**, **11-60**, **11-55**, **11-8**, **10-5**, **10-22** and **10-30**, induced RUNX activation from 100% to 150% (Table 1).

Compounds of group I showed HDAC inhibitory activities from 0.05 μM to 0.1 μM (**11-36**: 0.05 μM, **11-48**: 0.07 μM, **11-46**: 0.1 μM) and group II compounds had IC_{50} values in the range of 0.03–0.1 μM (**10-18**: 0.05 μM, **11-58**: 0.03 μM, **10-29**: 0.1 μM). Group III compounds displayed a wide spectrum of HDAC inhibitory activities from 0.3 μM to 0.01 μM (see table S1). SAHA (**1**) showed moderate RUNX activity [(80.2 ± 0.41)%] and HDAC inhibitory activity [IC_{50} = (0.11 ± 0.021) μM] as much as group III compounds (see table S1). Based on these results and classification, we confirmed that all compounds in Table 1 show potent HDAC inhibition with IC_{50} values < 0.3 μM (see table S1). Although potency of HDAC inhibition does not always correlate with potency of RUNX activation, it is definitely involved in regulation of RUNX transcriptional activity.

Table 1. Grouping of lactam-based HDAC inhibitors by RUNX transcriptional activation.

Relative RUNX transcriptional activation to FGF-2 [%][a]									
Group I: > 200%		Group II: 150–200%				Group III: 100–150%			
Compd	RUNX [%]	Compd	RUNX [%]	Compd	RUNX [%]	Compd	RUNX [%]	Compd	RUNX [%]
11-36	215.4 ± 1.56	**10-18**	170.8 ± 1.52	**11-28**	115.2 ± 7.67	**11-57**	115.3 ± 7.12	**11-8**	102.6 ± 3.94
11-48	266.9 ± 7.95	**11-58**	155.4 ± 4.95	**11-37**	145.5 ± 0.87	**11-53**	111.4 ± 0.84	**10-5**	102.5 ± 0.59
11-46	206.1 ± 2.15	**10-29**	185.5 ± 1.70	**11-27**	110.3 ± 2.99	**11-60**	111.0 ± 4.60	**10-22**	105.4 ± 0.80
				10-47	132.1 ± 0.53	**11-55**	108.2 ± 5.87	**10-30**	115.4 ± 2.74

[a] Data represent the mean ± SD of at least two independent experiments. Compounds are listed in the order of HDAC inhibitory activity (see table S1).

Regulation of RUNX3 acetylation

RUNX3 acetylation by p300 plays a key role in the maintenance of RUNX3 stability and transcriptional activity. Deacetylation of acetylated RUNX3 by HDACs promotes proteasome-mediated degradation of RUNX3.[6c] We examined RUNX3 acetylation and protein levels after treating cells with the HDAC inhibitors. Myc-tagged RUNX3 expression vectors were transfected into HEK293 cells followed by treatment with selected HDAC inhibitors.[6c] All of the group I and II compounds (**11-36**, **11-48**, **11-46**, **10-18**, **11-58**, and **10-29**) and three compounds (**10-47**, **11-37**, and **11-8**) in group III were examined in this experiment (Figure 3 A). TSA (**2**) is a positive control, because it is known to increase expression and acetylation of RUNX3.[6c] The ratio of acetylated

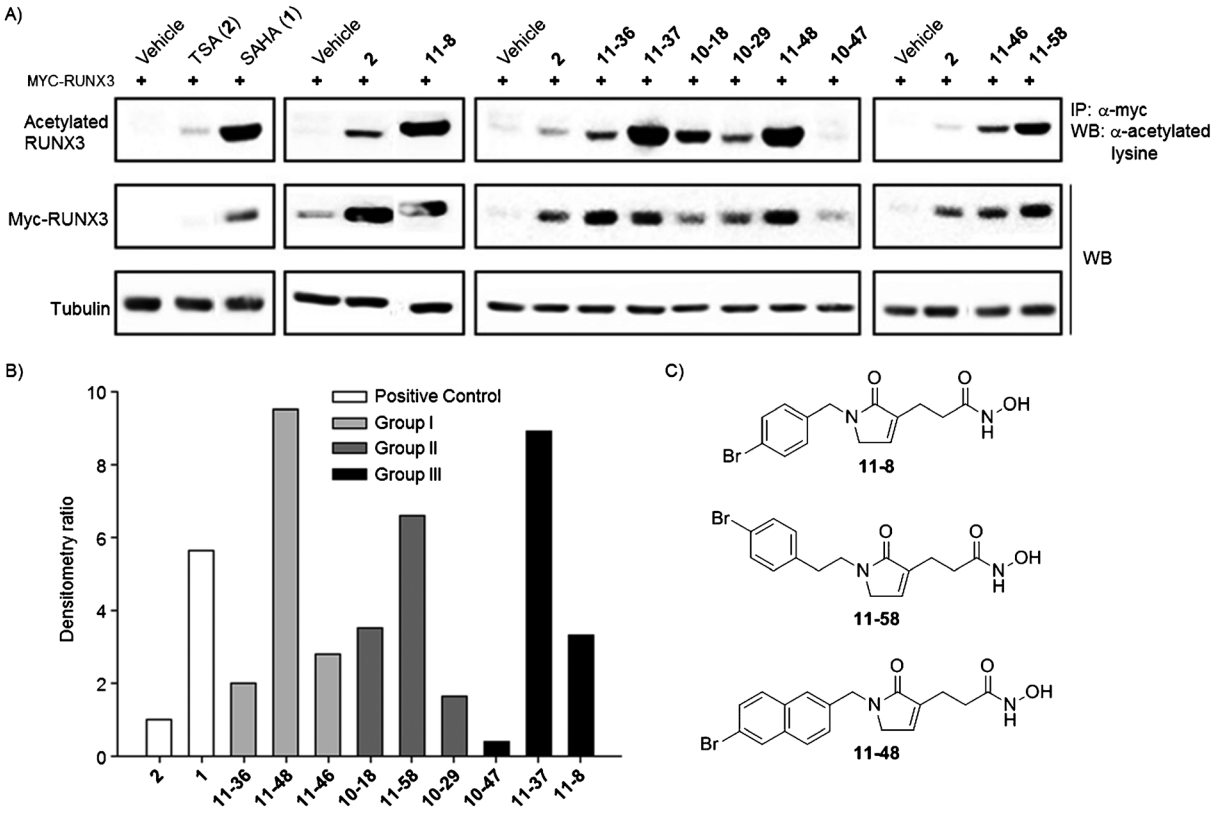


Figure 3. RUNX3 acetylation of selected lactam-based HDAC inhibitors. A) Acetylated RUNX3 levels were analyzed by immunoprecipitation in HEK293 cells at 1 μM test compound; B) Relative densitometry ratio of RUNX3 acetylation based on the results of the western blot shown in Figure 3A (at 1 μM test compound); C) Selected lactam-based HDAC inhibitors based on RUNX3 acetylation assay.

RUNX3 following treatment with each of the above compounds relative to levels following treatment with TSA is shown in Figure 3B.

Most of the compounds, including SAHA (**1**), **11-8**, **11-36**, **11-37**, **10-18**, **10-29**, **11-48**, **11-46**, and **11-58**, significantly increased the levels of acetylated RUNX3, resulting in RUNX3 stabilization compared to control (Figure 3A). In particular, **11-8**, **11-37**, **10-18**, **11-48**, **11-58**, and SAHA (**1**) dramatically increased acetylation of RUNX3 compared to TSA (Figure 3). The highest levels of acetylated RUNX3 and protein stabilization were achieved by **11-27**, **10-18**, **11-48**, **11-58**, and **11-8** in groups I, II, and III, respectively (Figure 3A,B). Among these, the HDAC inhibitory activities of **11-37** and **10-18** [$IC_{50} = (0.02 \pm 0.0134)$ μM and (0.03 ± 0.0234) μM, respectively] were significantly more potent. Thus, **11-37** and **10-18** might promote nonselective chromatin remodeling and associated changes in global gene expression. In comparison to TSA (**2**), **10-18** achieved weaker stabilization of RUNX3 protein, while it significantly promoted RUNX expression (Table 1). Therefore, we excluded these compounds from the subsequent studies. Thus, **11-8**, **11-48**, and **11-58**, which had γ-lactam cores and bromo-substituents on the aromatic rings, were selected as the lead compounds for further experiments (Figure 3C).

RUNX3 mRNA expression and RUNX3 stability

RUNX3 has been reported to be reactivated by an HDAC inhibitor in the MKN28 human gastric cancer cell line, in which the *RUNX3* gene has a hemizygous deletion.[5c] Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to determine if **11-8**, **11-58**, and **11-48** regulate *RUNX3* at the transcriptional level. SNU16 cells were used as a positive control for detecting RUNX3 mRNA, because RUNX3 is highly expressed in this cell line despite the presence of a mutation in *RUNX3* (Figure 4).[5c] Compound **11-48** strongly induced RUNX3 mRNA expression. Induction of *RUNX3* gene expression by 1 μM **11-48** was much higher than that achieved by TSA (**2**, Figure 4A). Compound **11-8** weakly induced RUNX3 mRNA expression. At the same concentration (1 μM), **11-58** and SAHA (**1**) poorly induced RUNX3 mRNA expression.

Compounds **11-8** and **11-58** were tested in concentration dependent manner to confirm this result (Figure 4B). Compound **11-8** showed very low levels of RUNX3 mRNA expression, whereas **11-58** moderately expressed RUNX3 mRNA at 0.1 μM and 1 μM. At 10 μM, both compounds remarkably increased RUNX3 mRNA expression, comparable to the positive control SNU16 (Figure 4B). Despite slight discrepancies in the levels of induction of RUNX3 mRNA in Figure 4A and 4B, **11-8** and **11-58** showed weak effects on RUNX3 mRNA expression at less than 1 μM in comparison with **11-48**.

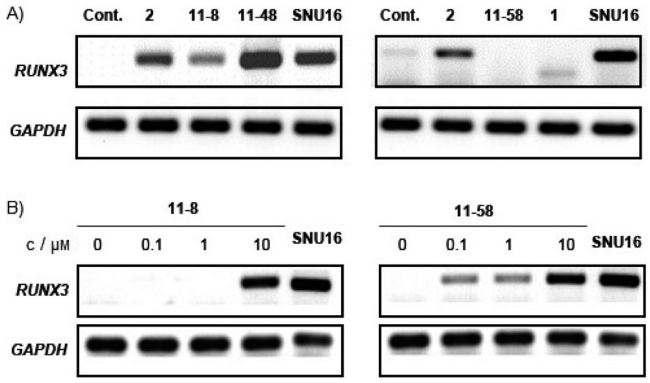


Figure 4. RUNX3 mRNA expression in MKN28 cells after treatment with **11**–**8**, **11**–**48**, and **11**–**58** (1 μM each). A) The effects of the three compounds and SAHA on RUNX mRNA expression; B) Dose-dependent effects of **11**–**8** and **11**–**58** on RUNX3 mRNA expression. All of the compounds were treated at micromolar concentration (μM); **2**, TSA; **1**, SAHA, Cont., control.

The effects of compounds **11-8**, **11-58**, and **11-48** on RUNX3 stability were evaluated in MKN28-RUNX3-Lac cells (Figure 5). Because this stable cell line expresses RUNX3 only after treatment with isopropyl-β-D-thiogalactopyranoside (IPTG),[15] it is a useful cell-based system to monitor RUNX3 stability together

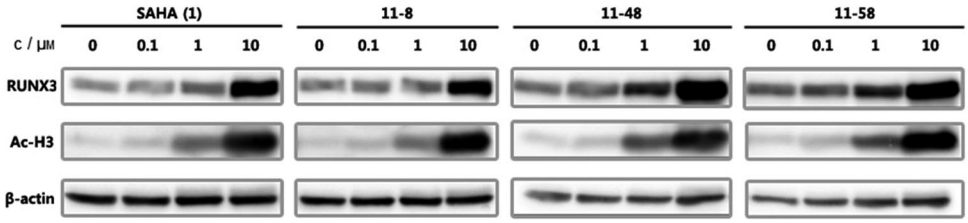


Figure 5. Stability of RUNX3 and acetylated histone H3 (Ac-H3) in MKN28-Lac-RUNX3.

with a RUNX3 immunoprecipitation assay. Cells were pretreated with IPTG for 24 h to induce maximum RUNX3 expression, at which time the RUNX3 expression level was the same in all groups. Cells were then treated with test compounds for an additional 24 h without IPTG treatment to monitor RUNX3 stability. Acetylation of histone H3 was also examined to monitor the effects of compounds on histone proteins. All compounds, **11-8**, **11-58**, **11-48** and SAHA, showed similar levels of RUNX3 stability and histone H3 acetylation at each concentration; their RUNX3 stability and histone acetylation were slightly increased at 1 μM, and they showed excessive increases of RUNX3 stability and histone H3 acetylation at 10 μM (Figure 5). RUNX3 stability at 10 μM was caused by effects of both transcriptional expression of RUNX3 and posttranslational acetylation of RUNX3.

Taken together, although RUNX3 stability in the groups treated with **11-8** or **11-58** was similar to those of the group treated with **11-48** at 1 μM, they induced relatively low levels of RUNX3 mRNA expression as compared with **11-48**, which showed the strongest induction of RUNX3 mRNA expression. Because HDAC inhibitors cause strong induction of mRNA levels and altered gene expression profiles in cells, indicating global chromatin remodeling, **11-48** might cause nonspecific gene expression at the transcription level. Based on these result, RUNX3 restoration by **11-8** and **11-58** was relatively more regulated by RUNX3 acetylation by posttranslational modification rather than controlling *RUNX3* gene expression at the transcriptional level. Therefore, **11-8** and **11-58** might be more suitable HDAC inhibitors for our strategy on RUNX3-targeted drug discovery.

Reduced tumor growth in xenograft models

In vivo xenograft experiments were performed to evaluate antitumor activities of **11-8**, **11-58**, and **11-48**. MKN28 cells were subcutaneously implanted into nude mice. Compounds were administered intravenously at 20 mg kg^{-1} daily for two weeks when tumor volumes reached 50–80 mm^3. Intravenous administration was performed to minimize the effects of ADME (absorption, distribution, metabolism and elimination). Tumor volume was measured every two or three days for 14 days.

Compounds **11-8**, **11-48**, and **11-58** significantly inhibited tumor growth compared to vehicle control (Figure 6 A,B). The positive control, SAHA, also showed significant tumor growth inhibition. There were no significant reductions in body weight in the treatment groups (Figure 6 A,B). The tumor growth inhibitory activities of compounds **11-8**, **11-48**, and **11-58** were similar to SAHA at tumor volume and tumor weight (Table 2). Compound **11-8** and **11-48** inhibited tumor growth at tumor volume (57.5% and 35.1%, respectively) and weight (58.1% and 40.6%, respectively) similar to SAHA (44.6% for tumor volume and 45.6% for weight), and **11-58** (45.2% for tumor volume and 46.7 for weight) also showed tumor growth inhibition like SAHA (56.3% for tumor volume and 51.0% for weight).

Table 2. Tumor growth inhibition in xenograft models.[a]

	Compd	Volume [%]	Weight [%]
Expt I	**SAHA**	44.6	45.6
	11-48	35.1	40.6
	11-8	57.5	58.1
Expt II	**SAHA**	56.3	51.0
	11-58	45.2	46.7

[a] Tumor inhibition compared to vehicle. For details, see Figure 6.

All three compounds showed similar HDAC inhibitory potency and RUNX3 stability as well as in vivo tumor growth inhibitory activity. Among them, **11-48** showed a remarkably high level of RUNX3 mRNA expression, which could be an indicator for a nonselective epigenetic activator. Therefore, **11-58** and

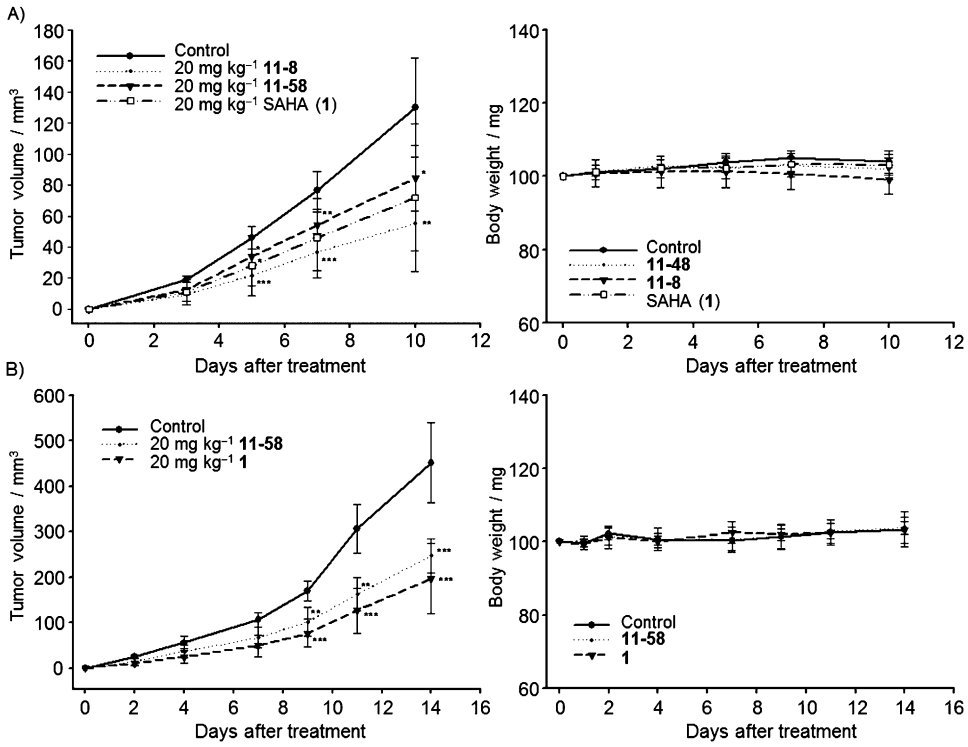


Figure 6. In vivo antitumor activity of **11**–**8**, **11**–**48**, and **11**–**58** in a xenograft model. A) Experiment I: average tumor volume and body weight changes of vehicle (control), SAHA, **11**–**8**, and **11**–**48**. B) Experiment II: average tumor volume and body weight changes of vehicle (control), SAHA, and **11**–**58**. Experiment I and II were performed with xenografts of the human stomach cancer cell line MKN28 in nude mice; experiment I, $n=5$; experiment II, $n=6$; standard errors are provided in the Supporting Information (table S2 and S3); $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, Student *t*-test; ΔV_t (tumor volume) $= V_t$ (measurement of the tumor volume) $- V_0$ (initial tumor volume).

11-8 were selected as potent RUNX3 stabilizers for gastric cancer therapy.

Conclusions

Epigenetic activation often leads to nonselective gene expression and side effects, which are considered to be major limitations of HDAC inhibitors.[15] The approach of stabilizing proteins with HDAC inhibitors is an alternative strategy for restoring expression of tumor suppressors through target-selective acetylation. Specifically, RUNX3 can be stabilized by HDAC inhibitors, resulting in restoration of its tumor suppressor functions. In this study, **11-8**, **11-58**, and **11-48** were selected by measuring acetylation and stabilization of RUNX3. These inhibitors also showed good in vivo anticancer efficacy. However, **11-48** was not selected as a candidate because of very strong epigenetic effects at the transcription level. In previous reports, we already demonstrated that compound **11-8** is orally available with good pharmacokinetic properties,[11c] whereas **11-58** is not (unpublished data). Therefore, **11-8** should be the better candidate to regulate RUNX3 stabilization through epigenetic and target-specific posttranslational modification. Further studies towards the identification of the underlying mechanisms by which these novel HDAC inhibitors stabilize RUNX3 are in progress. The current study provides important insights into a novel anticancer epigenetic approach with HDAC inhibitors for anticancer drug development.

Experimental Section

Chemistry

Chemical data for 18 hit and lead compounds were published in previous papers: **10-5** and **10-22** are presented in ref. [16] and [10a], respectively; **10-18**, **10-29**, **10-30** and **10-47** are published in ref. [11a]; **11-27**, **11-28**, **11-36**, **11-37** and **11-48** are shown in ref. [10b]; **11-8**, **11-46**, **11-53**, **11-55**, **11-57**, **11-58**, **11-60** are reported in ref. [11c]. For convenience, synthetic procedures and characterization data of these compounds are presented in the Supporting Information together with data for intermediate compounds **3-47**, **4-48**, **5**, **6-47**, **7-48,** **8-47** and **9-48**, which are not reported elsewhere.

Biology

HDAC inhibition assay: HDAC inhibition assays were performed as described in previous papers.[17]

Cell cultures: All tissue culture media and antibiotics were purchased from Hyclone (Logan, UT, USA). HEK293 and C2C12-6xOSE cells were maintained in DMEM with 10% fetal bovine serum (FBS) and antibiotics [penicillin (100 IU mL^{-1}), streptomycin (100 μg mL^{-1})] (Invitrogen, Grand Island, NY, USA) at 37 °C in a 5% CO_2 atmosphere. MKN28, MKN28-Lac-RX3, and SNU16 cells were maintained in RPMI1640 media with 10% FBS and antibiotics. The MKN28-Lac-RX3 stable cell line was kindly provided by Prof. Sul-Chul Bae (Chungbuk National University, Cheongju, South Korea).[15] Treatment with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma–Aldrich, St. Louis, MO, USA) was performed for 24 h to induce RUNX3. Drugs were then added to cell cultures for an additional 24 h to assess effects on RUNX3 stability.

Screening for RUNX activity: The C2C12-6xOSE cell line was kindly provided by Prof. Hyun-Mo Ryoo (Seoul National University, Seoul, South Korea). Cells were plated at 1×10^4 cells per well in 96-well plates. FGF-2 and drugs (1 μM each) were added to cells the next day. After 24 h, cells were harvested and analyzed by luciferase assay with the Bright-Glo Luciferase Assay System according to the manufacturer's protocol (Promega, Madison, WI, USA). Lysates were analyzed with the GloMax-multi Detection System (Promega). Fibroblast growth factor (FGF)-2 was used as a positive control for RUNX activation.

Western blot: After cells were lysed with radio-immunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, SDS), protein concentrations were determined with a bicinchoninic acid

assay (BCA) protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were fractionated by SDS-PAGE and transferred onto a nitrocellulose membrane (BioRad, Richmond, CA, USA). After blocking with 5% skim milk in phosphate buffered saline (PBS) containing 0.1% Tween 20, membranes were incubated with the appropriate primary antibodies at 4 °C overnight. Proteins were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Antibodies against myc (9E10), β-actin, and tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-acetylated lysine and anti-acetylated histone 3 antibodies were purchased from Cell Signaling (Danvers, MA, USA).

Immunoprecipitation: The myc-RUNX3 expression vector was kindly provided by Prof. Sul-Chul Bae (Chungbuk National University, Cheongju, South Korea). The myc-RUNX3 expression vector was transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen). Transfected cells were treated with HDAC inhibitors at 1 μM the next day for an additional 24 h. Cells were lysed in ice-cold lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4) and cleared by centrifugation. For immunoprecipitation experiments, 500 μg of protein was incubated with anti-myc antibody and precipitated with protein G beads (Pierce, Rockford, IL, USA) at 4 °C. The beads were washed three times with cold lysis buffer, and the immunoprecipitates were analyzed by western blot with an antibody against acetylated lysine.

RT-PCR: MKN28 cells were plated and treated with drugs the next day. After 24 h, cells were harvested. Total RNA was extracted with an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was performed on 1 μg of total RNA with Superscript III reverse transcriptase according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA). The resulting first-strand cDNA was amplified to measure mRNA levels of RUNX3 with specific primers. Equal quantities of each sample were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining and UV illumination. The mRNA level of GAPDH served as an internal control for RT-PCR analysis. The following primers were used: 5′-GCA GGC AAT GAC GAG AAC TA-3′ (RUNX3 forward primer), 5′-GTC TGG TCC TCC AGC TTC TG-3′ (RUNX3 reverse primer), 5′-CAA AGT TCT CAT GGA TGA CC-3′ (GAPDH forward primer), 5′-CCA TGG AGA AGG CTG GGG-3′ (GAPDH reverse primer).

In vivo xenografts: Athymic 5–6 week old female mice were purchased from Charles River Laboratories (Yokohama, Japan) and maintained in accordance with the Animal Research Committee's Guidelines at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). MKN28 cells (5×10^6/animal) were injected subcutaneously (s.c.) into the flank area. When tumors reached a volume of 50–80 mm^3, which was measured with a digital caliper, mice were randomized into groups of five animals each to receive either vehicle control, SAHA, **11-8**, **11-48**, or **11-58**. The drugs were administered intravenously at 20 mg kg^{-1} in 0.5% Tween 80 (Sigma) in sterile Milli-Q H_2O daily for two weeks. Tumor growth was measured three times a week until the end of the study. The length and width of the tumor were measured with calipers. Tumor volume was calculated according to the formula for an ellipse, volume = 0.523 × (long dimension) × (short dimension)^2.

Statistics software: The level of protein expression was quantified with Scion image densitometry software (Scion Corporation, Frederick, MD, USA). Data were analyzed with a commercial statistics software package (SigmaStat, SPSS Science, Chicago, IL, USA). Student's *t*-test was performed for individual comparisons. Multiple comparisons were assessed by one-way ANOVA or regression analysis.

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