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Identification of new quinic acid derivatives as histone deacetylase inhibitors by fluorescence-based cellular assay

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ABSTRACT

A fluorescence-based cellular assay system was established to identify potential epigenetic modulator ligands. This assay method is to detect the de-repression of an EGFP reporter in cancer cells by the treatment of HDAC (histone deacetylase) or DNMT (DNA methyltransferase) inhibitor. Using this system, we conducted a preliminary screening of in-house natural product library containing extracts and pure compounds, and identified several active compounds. Among them, novel quinic acid derivatives were recognized as excellent HDAC inhibitors by both enzymatic and cell-based HDAC assays.

Epigenetic modulation comes from interaction between various enzymes, such as HDAC (histone deacetylase), HAT (histone acetyltransferase), DNMT (DNA methyltransferase), HMT (histone methyltransferase), and etc, and these interactions depend on the balance of the presence of the enzymes.[1] Particularly, the acetylation of histone plays an important role in the epigenetic regulation of the cell. The acetylation of histone will be increased or decreased, depending on the interactivity between histone acetyl-transferases (HATs) and HDACs. Although these enzymes are best of description for regulation of chromatin architecture, in the recent days, general control mechanisms involving various protein complexes are being reported.[2,3] A synthetic hydroxamate HDAC inhibitors (SAHA,[4] belinostat,[5] and panobinostat[6]), and the natural product cyclic depsipeptide romidepsin,[7] have already been approved for the treatment of cancer. Such successful cases lead many scientists to be interested in developing drugs targeting epigenetic pathways.

In our study, to identify potential HDAC or DNMT inhibitors by an efficient fluorescence imaging-based screening method, we selected the cell line in which EGFP expression is repressed by DNMT and/or HDAC (Fig. 1).[8,9] This method is established based on the hypothesis that methylated DNA is specifically recognized by methyl CpG-binding domain (MBD) proteins which can recruit transcriptional co-repressors, including HDAC.[10] When these cells are treated with HDAC or DNMT inhibitors, the EGFP expression is derepressed.[9] This method is useful to preliminarily screen out candidate epigenetic modulators in cells.[12] The hit compounds identified from this assay will be further tested by enzymatic HDAC or DNMT assay to confirm the activity.

In-house 200 natural product compounds and 103 extracts were tested against EGFP-repressed c127LT cells,[13] and among them, 13 compounds and 18 extracts reactivated the EGFP expression. The fluorescence-based assay result for RLE12 (3,5-*O*-*trans*-dicaffeoylquinic acid methyl ester), one of the representative hit compound in Figure 2, is demonstrated in Table 1, in comparison with TSA[14] and MS275.[15] Then, enzymatic[16] and cell-based HDAC assays[17] were conducted to evaluate the HDAC inhibition activities of 13 hit compounds, and only 5 compounds showed micro molar HDAC inhibitory activities. Figure 3a demonstrates the results of enzymatic HDAC assay for the most active hit RLE12,[18–20] and Figure 3b illustrates cell-based HDAC assay. To confirm the binding of RLE12 in the substrate site for HDAC, an enzyme kinetic assay was conducted in comparison with TSA, and the Lineweaver–Burk plot revealed that RLE12 engages in competitive inhibition against acetylated lysine substrate (Fig. S2 in Supplementary material). As a result, RLE12 is identified as HDAC inhibitor with IC_{50} value of 4.99 μM, which is comparable to well-known nonhydroxamate HDAC inhibitor MS275 (IC_{50} = 6.73 μM in enzymatic assay).[15] RLE12 is also nonhydroxamate HDAC inhibitor, a methyl quinate derivative having both 3- and 5-hydroxy group connected with caffeic acid. The structurally related natural product compounds were reported to have mild HDAC inhibition activity, such as caffeic acid (IC_{50} = 2.54 mM) and chlorogenic acid (IC_{50} = 375 μM), and DNMT inhibition activity.[11,21,22] Next, we also tested other quinic acid derivatives (chlorogenic acid, RLE30, and RLE13)[18,20] by

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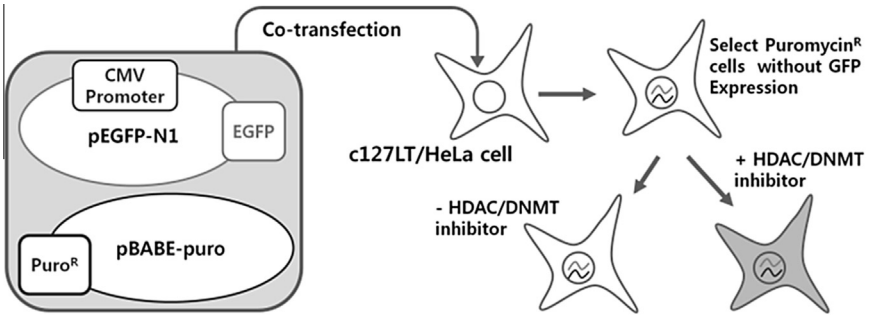


Figure 1. Fluorescence-based cellular screening system.

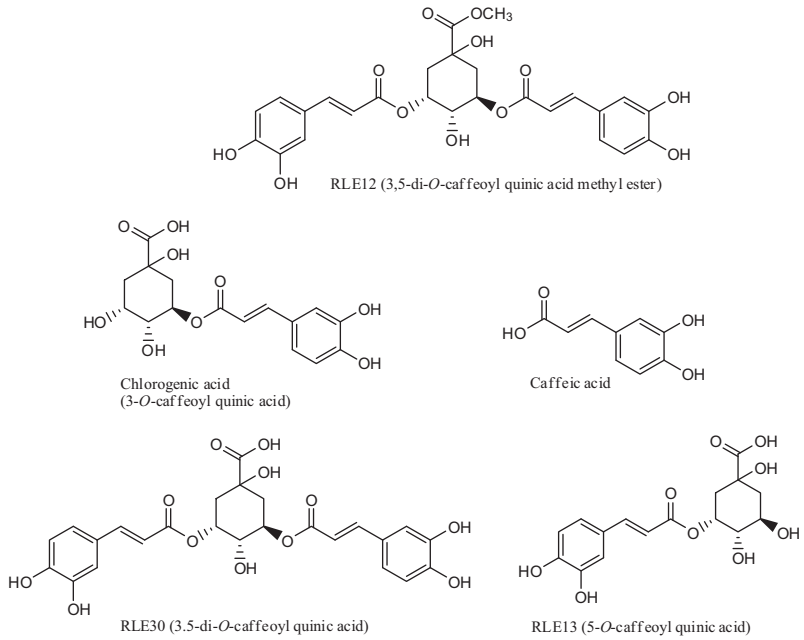


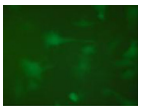
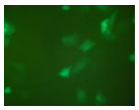
Figure 2. Structures of quinic acid derivatives and caffeic acid.

fluorescence imaging-based assay and HDAC assay. With the exception of RLE12 (Table 2), all other quinic acid derivatives induced EGFP expression very slightly, and in parallel, they showed HDAC inhibition activities noticeably lower than RLE12 (Table 3). These results suggest that intermolecular interactions between drug and HDAC active site might be affected by the methyl quinate group, and the chirality and number of caffeoyl ester group.

Docking study[14,23] was carried out using the Surflex-Dock[24] to examine the differences in binding poses of RLE12 derivatives, in correlation with HDAC inhibitory activities. To compare the Surflex-Dock score output (Table 3), RLE12 obtained the highest binding score ($-\log K_d = 9.03$) out of all other quinic acid derivatives, in agreement with its highest rank of HDAC inhibition activity. The binding score for carboxylate derivative RLE30 is 7.73, indicating that predicted K_d value of RLE30 is about 20 times higher than that of RLE12. Considering that the IC_{50} value of RLE30 is also 29 times higher than that of RLE12, the difference in HDAC inhibition activity of these compounds should be due to the differences in intrinsic interaction energy upon binding to the active site of HDAC.

The binding pose of RLE12 is the active site of human HDAC2[14] is shown in Figure 4a, in comparison with the X-ray pose of SAHA. RLE12 fits into the SAHA-binding pocket, with one caffeoyl group inserted into the zinc-containing narrow pocket and the other capped the entrance of the active site.

Table 1
Activation of EGFP expression by RLE12 and known HDAC inhibitors in EGFP-repressed c127LT cells

Control	TSA(200nM)	MS275(1μM)	RLE12 (10μM)
			

Inside the pocket, the catechol OH groups not only binds to zinc, but also forms a bidentate hydrogen bond with the nitrogen atom of the imidazole His145 and His146. The other catechol laying over the surface of hHDAC2 forms a hydrogen bond network with Gln31 (Fig. 4b). The T-shaped pose of RLE12 makes the drug cover a wide range of area at the capping group binding site of HDAC. The channel of binding site is composed of the hydrophobic side chains, such as Phe155, Phe210, and Tyr308 and interacts with aliphatic linker unit of RLE12 by forming CH–π interaction.[25] Figure 4c demonstrates the hydrophobic interaction formed between hydrophobic region (dark brown) and drug molecules at the entrance of the active site. Particularly, RLE12 only has interaction

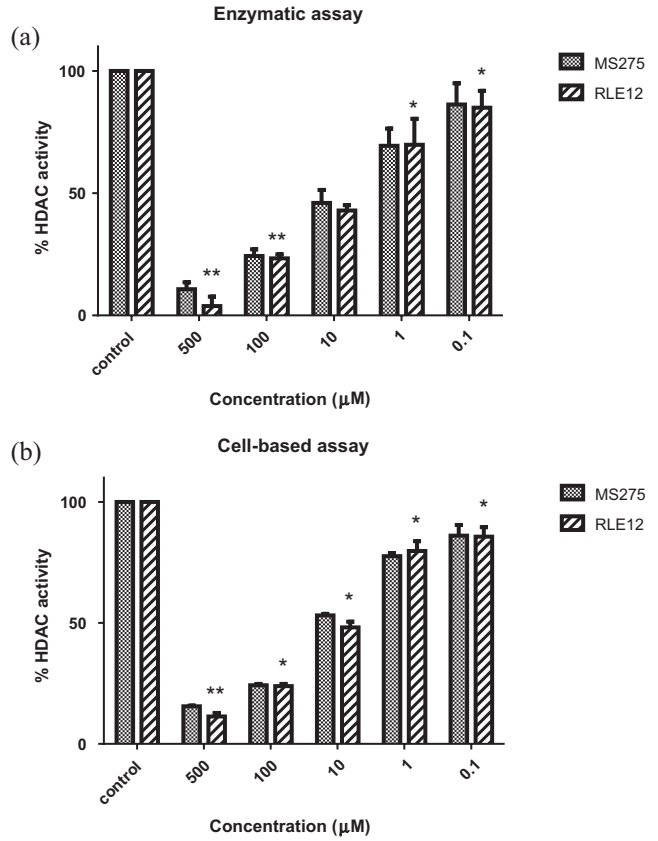
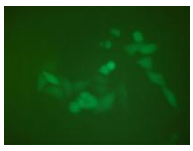


Figure 3. HDAC inhibition enzymatic assay (a), and cell-based assay (b). RLE12 and MS275 were treated onto HeLa nuclear extract (a), and on HeLa cells (b), respectively. HDAC activity was measured using the colorimetric HDAC assay kit. Data are presented as mean ± SD. $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$.

Table 2
Activation of EGFP expression by RLE12 and known HDAC inhibitors in EGFP-repressed c127LT cells

Control	MS275	RLE12
		
Chlorogenic acid	RLE13	RLE30
		

The concentration of all compounds is 10 μM.

with hydrophobic part of capping group, while chlorogenic acid and caffeic acid do not have this interaction. To compare the binding poses of quinic acid derivatives (Fig. 4d), the catechol OH group commonly plays a role of zinc binding group. The distance between zinc and the oxygen atom of catechol OH is 2.09 Å, 2.12 Å, and 2.16 Å, for RLE12, chlorogenic acid, and caffeic acid, respectively, indicating that the strongest chelation with zinc ion is formed by RLE12. These docking results well supports a significantly improved HDAC inhibitory of RLE12 in comparison with its congeners, chlorogenic acid and caffeic acid.

Table 3
Results of HDAC inhibition assays and docking analysis

Compounds	Enzymatic assay (IC_{50}, μM)[b]	Cell-based assay (IC_{50}, μM)[b]	Surflex-Dock score ($-\log K_d$)
MS275	6.73 ± 0.38	11.18 ± 0.44	9.64
RLE12	4.99 ± 1.66	7.59 ± 1.86	9.03
Chlorogenic acid	>200	>200	6.85
RLE30	143.24 ± 6.98	NA[a]	7.73
RLE13	170.4 ± 3.59	>200	6.82

[a] NA is no activity (IC50 > 500 μM).
[b] Data are presented as mean ± standard deviation (SD) of at least independent experiments performed in duplicate.

Inhibition of HDAC generally leads to the accumulation of acetylated histone H3 and H4 in total cellular chromatin. Known HDAC inhibitors, such as TSA and MS275, were reported to cause an increase of acetylated histone H3 and H4 level.[26] To confirm the HDAC inhibition activity of RLE12, western blotting to measure the level of acetylated histone H3 was performed (Fig. 5). First, we examined the change of expression level of acetylated histone H3 depending on concentration of TSA and MS275, after treatment of drugs into HeLa cells at the concentration ranges, 2–2000 nM and 0.1–100 μM, respectively. The amount of acetylated histone H3 was increased by TSA and MS275 in a concentration-dependent manner.

As expected, treatment of RLE12 resulted in the accumulation of acetylated histone H3, and the effect of RLE12 on histone H3 acetylation is quite similar to that of MS275. The results suggest that RLE12 inhibits HDAC and suppresses the elimination of acetyl group of histone H3, eventually will be involved in epigenetic regulation mechanisms in biological system.

The fluorescence-based cellular screening system established in our study aims to discover and identify novel epigenetic modulators. It is based on the cell line in which the expression of fluorescent protein gene is repressed, then utilizing this transgenic cell to identify ligands to de-repress the gene expression. Using this screening method, we were able to find hit compounds very effectively from the library containing only 200 natural product compounds, identifying 13 natural products hit compounds. Among them, 5 compounds showed HDAC inhibitory activity, and of them, RLE12 showed characteristics similar to nonhydroxamte HDAC inhibitor MS275. The remaining 8 compounds that didn't show HDAC inhibitory activity were tested for DNMT1 inhibitory activity, and 1 compound was identified as a novel DNMT1 inhibitor (results not shown). Considering that many natural product compounds are being developed as chemotherapeutic and chemopreventive agents targeting various epigenetic enzymes,[28] our screening system can be useful for natural product library screening to identify potential epigenetic modulator ligands.

Through this study, we identified RLE12, a new caffeolyquinic acid derivative, as a nonhydroxamate HDAC inhibitor. RLE12 was patented as active compounds against many different diseases, such as antiviral agents (HIV1, Hepatitis B),[20,29] anti-diabetic,[30] antiinflammatory,[19,31] and Alzheimer's disease.[32] However, HDAC inhibitory activity of RLE12 has not been reported in the literatures yet. Our study discovered that the molecular target of RLE12 is HDAC for the first time. We suggest that RLE12 should give various biological activities by playing a role of epigenetic modulator. Further studies on the mechanisms of biological action of RLE12 are underway.

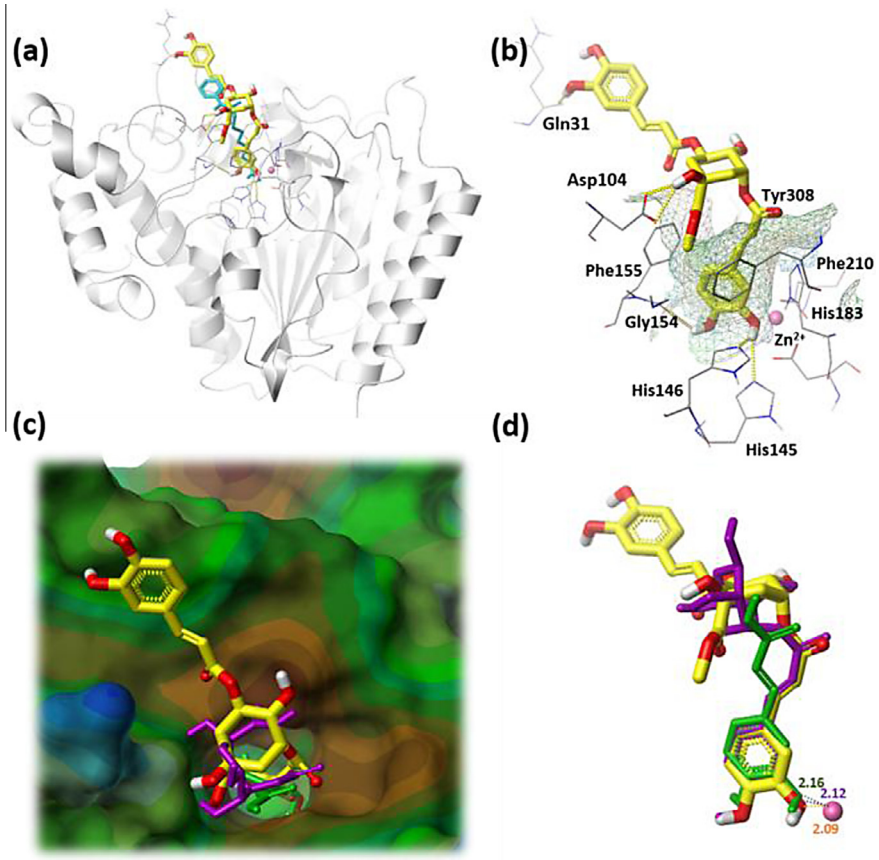


Figure 4. (a) Binding pose of RLE12 (yellow) overlayed over the X-ray pose (PDB id: 4lxz) of SAHA (blue). (b) Docked pose of RLE12 (yellow) in the active site of hHDAC2. (c) Top view of docked poses. The structure of hHDAC2 is rendered in MOLCAD lipophilic potential surface, and colored from blue (hydrophilic) to dark brown (hydrophobic). (d) Superposition of docked poses of caffeic acid (green) and chlorogenic acid (violet), and RLE12 (yellow). The zinc ion positioned in the active site of HDAC2 is rendered as magenta sphere.

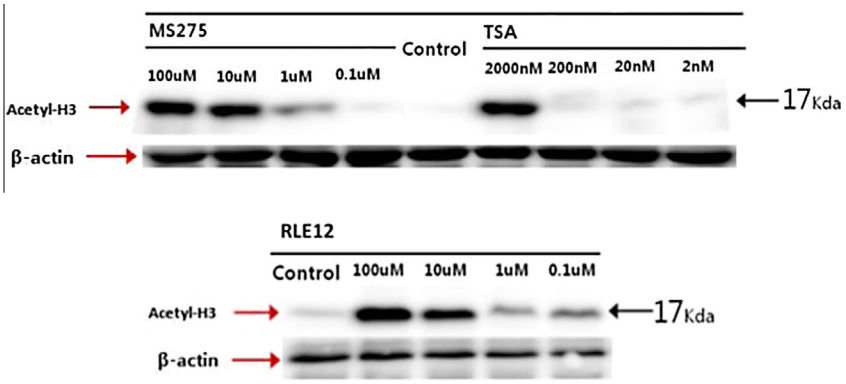


Figure 5. Western blot analysis[27] of acetylated histone H3. TSA, MS275, and RLE12 were treated on a 6-well plate cultured HeLa cells at different concentrations, and 24 h later, nuclear extracts were prepared and the level of acetylated histone H3 was examined by western blot analysis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.03.010.

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12. Our design rationale was obtained to articles, i.e. method in enzymology, Vol. 414, 21–36[13] that involve the reversal of transcriptional repression could be used to interfere with either DNA methylation or histone deacetylation pathways. Several points were considered for making construct, which stably expresses a reporter gene in human cells with selection marker in one. Therefore, CMV promoter is selected to drive a reporter gene (EGFP gene) because the promoter is widely known to be very strongly and constitutive expressed. The HeLa cells and the c127LT cells (ATCC), were respectively used to establish fluorescence screening assay, through co-transfection of pBABE-puro vector and pEGFP-N1 vector. The pEGFP-N1 vector contains the CMV promoter and the EGFP gene, and pBABE-puro vector contains the puromycin (the selection marker) resistant gene. They were both put through the inspection stages and were prepared as cell line. To select the cell line, that should be contemplated available for expression of various HDACs and DNMTs. It should be sensitive to known HDAC inhibitors. It should not take effect drug efflux transporters. It should be adhesion cell line for imaging. It should grow under standard conditions that can facilitate miniaturization. HeLa and c127LT cell were satisfied above all conditions and selected for establishment of fluorescence-based screening system. These cells were seeded in 6-well plate, a density of 1×10^5 cell/well, and incubated overnight to reach 80% confluence. Grown HeLa and c127LT cells are co-transfected with two vectors using Lipofectamine 2000 (invitrogen), the transfected cells incubated using DMEM medium with 10% FBS and 5% penicillin in CO_2 incubator. After incubation for 24hr, the cells are divided 1:5 into separate dishes (100 mm). When these are reattached to dishes, changing medium with puromycin (2 μg/ml), that kill curve of HeLa cell and c127LT cell was measured to determine puromycin concentration. The selection was continued for 3 weeks with selection media being replaced every other day. Observing colonies formation by inspection and marking on bottom of dish. When these reaches 1–3 mm in diameter, there were able to take off the colony using 5 μl of trypsin and then transfer each well of 24-well plate already containing fresh selection media. Continuously, the selected cells were cultured by selection media and last cultured at 75T flask and stored cell stock in LN2 tank. The EGFP-negative phenotype cells were plated into 96-well corning glass-bottom black plate at 2×10^4 cells/well and cultured in DMEM media with 10% FBS, 5% penicillin at 37 °C, 5% CO_2 incubator for 18 h. Treatment of nothing and DMSO (1%) was used to negative control and 25 mM sodium butyrate, 200 nM Trichostatin A (TSA), 10 μM MS275 was used to positive control. At the same time, using compounds (number of 213) of natural product library were treated with final concentration as 10 μM and incubated for 24 h, after fluoresced cells were shown by fluorescence microscope.
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16. HDAC enzymatic assay was conducted using HDAC enzymatic assay kit (Enzo, BML-AK500-0001, Lot No. 03301101A). The experimental procedure was carried out following protocol of manufacturer. The fluorescent activity was determined using Spectra Max Gemini XS micro plate spectrofluorometer, SOFTMAX PRO V. 3.1.2 system, Molecular Devices, with excitation wavelength at 355 nm, emission wavelength at 460 nm, and a cut off filter at 455 nm. The total HDAC assay volume was 50 μl and all the assay components were diluted in HDAC assay buffer (50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$). The reaction was carried out in half-volume white 96-well plates (Costar 3693). The HDAC assay mixture contained HDAC substrate (1×, 12.5 μl/well), Nuclear Extract from HeLa Cells (diluted to 7.5 μl), and inhibitor (diluted to 5 μl final volume). Positive controls contained all the above components except the inhibitor. The negative controls contained neither enzyme nor inhibitor. And then, each sample was replaced with an HDAC assay buffer.
17. HeLa cell was seeded at a density of 3×10^4 cells/well into black clear bottom 96-well plate (Costar 3603) and incubated overnight to reach 70% confluence. Medium was used as a blank. The assay was performed using HDAC Cell-based Activity Assay kit (Cayman Chemical Company, catalog No. 600150) according to protocol of manufacturer. The Spectra Max Gemini XS micro plate spectrofluorometer was used to determine the fluorescent activity with the SOFTMAX PRO V. 3.1.2 system (Molecular Devices) with excitation at 355 nm and emission at 460 nm with a cut off filter of 455 nm.
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23. This work was carried out with Sybyl-x2.0 (Tripos International, St. Louis, MO), operating under Linux OS (CentOS release 5.9). We conducted docking analysis using the Surflex-Dock algorithm, combined with CScore, which are not only used to examine the binding mode of the compounds at the active site of HDAC, but also to obtain a basis for further synthesis compounds. Natural products were energy-minimized using the conjugated gradient method with Gasteiger-Hückel charge until a convergence value of 0.001 kcal/(Å mol), using the Tripos force field. The crystal structure of HDAC2 complexed with SAHA was obtained from the Protein Data Bank (PDB entry code: 4lxz). The SFXC file was built using the mol2 prepared protein structure. The protomol was generated using each reference ligand with a threshold of 0.50 and bloat set to 0. Each atom in the protein and ligand is labeled as nonpolar (e.g., the H of a C—H) or polar (e.g., the H of an N—H or the O of a C═O), and generated protomol mimics the interactions. The main setting was 50 solutions per each compound and other parameters accepted the Surflex-Dock Geom settings. Surflex-Dock's scoring function was trained to estimate the dissociation constant (Kd) expressed in $-\log K_d$ unit. Then, the best poses from each run were combined and re-ranked using a consensus scoring (CScore).
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27. Cultured HeLa cell at 6-well plate (a number of $1x10^4$ cells), until 70–80% confluence. Treatment of negative control was 1% DMSO, while positive controls were used to TSA, MS275 for dependent on concentration (respectively, 2 nM ~ 2 μM and 0.1–10 μM). Nuclear protein extract was obtained using Nuclear Extract Kit (Active Motif, Bio-Medical Science Co., Ltd) according to provided protocol. After concentration determination by Bradford assay, 30 μg of protein was separated on 15% SDS–PAGE for 1 h in 120 V and transferred into PDVF membrane (0.45 μm) for 1 h in 100 V. Membrane was blocked with 8% skim milk with TBST for 18hr and incubated with 1:400 anti-acetyl-Histone H3 (Millipore/Upstate 06-599), and 1:5000 β-actin (ab8229) in TBST for 2 h at rt. Following secondary antibodies (HRP mouse; Santa Cruz sc-2005 and HRP rabbit; Santa Cruz sc-2030) incubation for 1hr at room temperature, the blotted band were detected with enhanced chemiluminescence reagent.
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