Brief Report

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Novel HDAC6 selective inhibitors with 4-aminopiperidine-1-carboxamide as the core structure enhanced growth inhibitory activity of bortezomib in MCF-7 cells

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Summary **In epigenetics, histone deacetylases (HDACs) are well validated targets for the development of anticancer drugs. In this work, we reported the design and synthesis of a series of twenty-two novel (*E*)-N-hydroxycinnamamide-based HDAC inhibitors with 4-aminopiperidine-1-carboxamide as the core structure. Most newly synthesized compounds displayed high inhibition rates toward HDAC at the concentration of 1 μM. Among them, the inhibition rates of compounds LYP-2, LYP-3, LYP-6, and LYP-15 were more than 75%. Furthermore, compounds LYP-2, LYP-3, and LYP-6 potently inhibited the activity of HDAC6 with selectivity over HDAC1. We chose LYP-2 and LYP-6 to test its antiproliferative effect on breast cancer cells MCF-7. Either LYP-2 or LYP-6 alone moderately suppressed the cell growth, but could synergistically enhance the inhibitory effect of bortezomib. These results suggested that combined HDAC6 inhibitor and bortezomib regimen might be an option for breast cancer treatment.**

Keywords: HDAC, HDAC6, selective, inhibitors, cancer

1. Introduction

In the field of epigenetics, acetylation is the most common covalent modification, playing important roles in the regulation of normal cellular processes such as cell differentiation, proliferation, angiogenesis, and apoptosis (*1*). Dysregulation of acetylation has been associated with diverse human diseases including cancers (*2*). The level of acetylation of histones and non-histone proteins is governed by two antagonistic families of enzymes: histone deacetylases (HDACs) and histone acetyl transferases (HATs) (*3*). HDACs are a family of eraser enzymes that are responsible for removing an acetyl group from the ε-amino groups of lysine residues present within core histones and many non-histone proteins. Silencing or inhibiting HDACs can impair cell cycle, cell growth, chromatin decondensation, cell differentiation, apoptosis, and angiogenesis in several cancer cell types. Therefore, HDACs have emerged as important therapeutic targets for cancers (*4*).

The known HDACs are divided into four classes based on their sequence homology: Class I HDACs including HDAC1, 2, 3, and 8; Class II HDACs including Classes IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10); Class III HDACs, known as sirtuins (sirt1-7); and Class IV HDAC (HDAC11). Class I, II, and IV are all Zn^{2+} dependent while Class III is NAD^{+}-dependent (*5*). HDAC6 belonging to class IIb has recently emerged as an attractive drug target for the treatment of several human diseases including Alzheimer's disease, autoimmune disorders, and cancers (*6*). As a cytoplasmic enzyme, HDAC6 uniquely features two deacetylase domains, a dynein motor binding domain to enable HDAC6 to shuttle cargo along the microtubule and a zinc finger ubiquitin-binding domain at the C-terminus. Functionally, HDAC6 is able to remove the acetyl group from lysine residues

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mainly in non-histone substrates, including α-tubulin (*7*), Hsp90 (*8*), cortactin (*9*), and peroxiredoxin (*10*), and plays important roles in microtubule dynamics and chaperone activities. In contrast to the lethal effect of HDAC1-3 suppression, it has been reported that mice with HDAC6 knocked out are effectively normal (*11*). Indeed, HDAC6 inhibitors do not seem to be cytotoxic toward normal cells and have fewer side effects than pan-HDACis.

Considering the value to develop HDAC6 selective inhibitors, here, we report the design, synthesis and activity studies of 22 novel HDAC inhibitors with the 4-aminopiperidine-1-carboxamide as the core structure, some of them were identified as potent HDAC6 selective inhibitors. Antiproliferative activities and drug combination effect of representative compounds were also evaluated.

2. Materials and Methods

2.1. *Chemistry*

All of the chemical solvents and reagents, which were analytically pure without further purification, were commercially available. Thin-layer chromatography was performed on 0.20 mm Silica Gel 60 F254 plates (Qingdao Haiyang Chemical, China). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (Bruker Company, Germany) or a Varian spectrometer (Varian, Palo Alto, CA, USA), using tetramethylsilane as an internal standard. Chemical shifts were given in parts per million. Mass spectra were recorded on a Q-TOF Premier mass spectrometer (Micromass, Manchester, U.K.).

2.2. *Cell line and cell culture*

Breast carcinoma cell MCF-7 was employed in the present study. The cell line was obtained from China Cell Bank (Shanghai, China) and maintained in Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; PAN Biotech) at 37°C in a humid atmosphere (5% CO_2-95% air).

2.3. *In vitro HDAC inhibition fluorescence assay*

In brief, 10 μL of enzyme solution (HeLa cell nuclear extract, HDAC1, or HDAC6) was mixed with different concentrations of tested compounds (50 μL). The mixture was incubated at 37°C for 5 min, followed by adding 40 μL fluorogenic substrate (Boc-Lys(acetyl)-AMC). After incubation at 37°C for 30 min, the mixture was quenched by addition of 100 μL of developer containing trypsin and trichostatin A (TSA). Over another incubation at 37°C for 20 min, fluorescence intensity was measured using a microplate reader at excitation and emission wavelengths of 390 and 460 nm, respectively. The inhibition ratios were calculated from the fluorescence intensity readings of tested wells relative to those of control wells, and the IC_{50} values were calculated using a regression analysis of the concentration/inhibition data.

2.4. *Cell proliferation assay*

Cells (5×10^3 per well) seeded in 96-well plates were exposed to HDACis, bortezomib, or their combination for 72 h. Then the medium was removed and the wells were washed with phosphate-buffered saline (PBS). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μL of MTT (5 mg/mL, Sigma-Aldrich) for 4 h. Light absorbance of the solution was measured at 490 nm on a microplate reader (TECON, Swiss) (*12,13*).

2.5. *Statistical analyses*

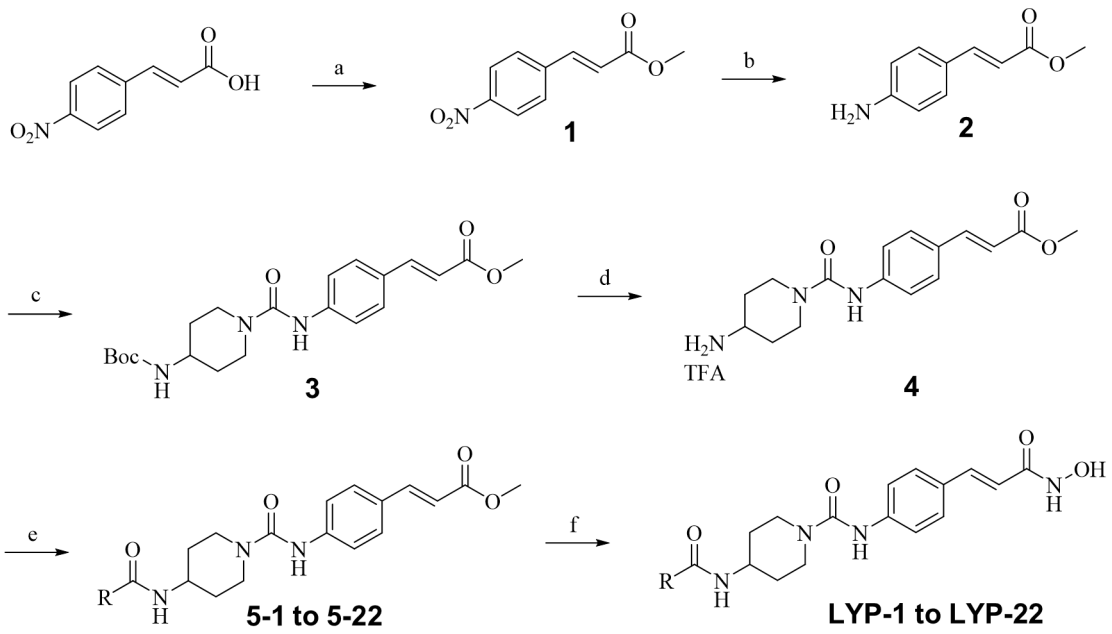
Data were expressed as mean ± S.D. for three different determinations. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests. The value of $p < 0.05$ was considered as statistically significant. Statistical analysis was performed using the SPSS/Win 16.0 software (SPSS, Inc, Chicago, IL, USA).

3. Results and Discussion

3.1. *Synthesis of compounds*

The general procedure to synthesize the target molecules was depicted in Scheme 1. Commercially available starting material 4-nitrocinnamic acid was refluxed with drops of concentrated sulfuric acid in methanol to give methyl 4-nitrocinnamic acid (**1**). The nitro group of compound **1** was then reduced to amino group to give compound **2**. Under nitrogen atmosphere protection, compound **2** was mixed with triphosgene and 4-(*N*-tert-butoxycarbonylamino)piperidine sequentially to give the compound **3**. The tert-butoxycarbonyl (Boc) protecting group was removed by treating with 20% (v/v) DCM solution of trifluoroacetic acid (TFA) to give the key intermediate **4**. Then, different aryl groups were introduced by treatment with the mixture of corresponding aromatic acid, HATU and DIPEA in anhydrous DMF to get the compounds **5-1** to **5-22** which were directly converted into target molecules **LYP-1** to **LYP-22** by condensed with NH_2OH in anhydrous methanol. Specific synthesized methods and spectroscopy data of the above compounds see supplementary data (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=38*).

3.2. *Inhibitory effect of target compounds on HDAC activity*



Scheme 1. Reagents and conditions. a. conc. H_2SO_4, MeOH; **b**. Fe powder, NH_4Cl, EtOH and H_2O, reflux, 4 h; **c**. triphosgene, 4-(*N*-tert-butoxycarbonylamino)piperidine, anhydrous DCM; **d**. TFA:DCM = 1:4, r.t.; **e**. aromatic acid, HATU, DIPEA, dry DMF, r.t.; **f**. NH_2OH, KOH, MeOH.

All the synthesized compounds were first screened for their inhibitory activity against Hela cell nucleus extracts (HDACs). The data are summarized in Table 1. Vorinostat (SAHA) was tested as a pan-HDAC reference inhibitor. Among all 22 compounds, four of them, LYP-2, 3, 6 and 15 displayed stronger activities to HDACs with the inhibition rate more than 75%, and the inhibition rate of SAHA was 96.8%.

With four potent HDACs inhibitors in hand, we go further to evaluate their selectivity toward different HDAC isoforms belonging to different classes. Majority of HDACis share a common pharmacophore model consisting of three parts: a hydrophobic cap structure that can interact with the rim at the entrance of the active pocket of HDACs; a zinc ion binding group (ZBG); and a linker responsible for the connection between the cap and the ZBG and for interaction with the hydrophobic tunnel of the active site (*14*). By analyzing the structure features, it was found that our compounds possessed a bigger "cap" part than the pan-HDACi SAHA. So, we envisioned that our compounds may have selective inhibitory activities toward the HDAC6, because HDAC6 has a relative wider surface of the active pocket than other HDAC isoforms which can accommodate huge cap groups and this makes it possible to design highly selective HDAC6 inhibitors such as tubastatin-A (*15*). So, these four representative compounds were evaluated for their inhibitory activity against HDAC1 and HDAC6. Results in Table 2 showed that compared with SAHA, compounds LYP-2, -3, and -6 exhibited different degrees of selectivity for HDAC6 over HDAC1 isoform except for LYP-15. Among these four compounds, LYP-2 displayed the most potent HDAC6 inhibitory activity (IC_{50} = 12.3 nM) and LYP-6 possessed best selectivity over HDAC1 even better than that of ACY-1215, a well-known HDAC6 inhibitor currently under clinical trials, which validate our compound design strategy.

3.3. *Cell proliferation inhibition*

The effect of LYP-2 and LYP-6 on cell viability was determined using MTT assay. Breast cancer cell MCF-7 was treated with different concentrations of LYP-2 or LYP-6 for 72 h. As shown in Figure 1A, either LYP-2 or LYP-6 dose-dependently suppressed the growth of MCF-7 cells. However, both the compounds demonstrated moderate growth inhibitory effect on this cell line, with IC_{50} values more than 30 μM. Previously studies showed that selective inhibition of HDAC6 increases α-tubulin acetylation and accumulation of ubiquitinated proteins in cancer cells, with synergistic cytotoxicity in combination with proteasome inhibitor bortezomib (*16*). We therefore examined the effect of LYP-2 or LYP-6 plus bortezomib on the proliferation of MCF-7 cells. Cells were treated with different concentrations of bortezomib in presence or absence of LYP-2 or LYP-6 for 72 hours. As shown in Figures 1C and 1D, LYP-2 and LYP-6 dose-dependently enhanced the growth inhibitory effect of bortezomib. To explore the interactive effect between the compounds with bortezomib, we calculated the combination index (CI) values of bortezomib plus LYP (10 μM) using the CompuSyn software and determined interaction

Table 1. Structures of all 22 compounds and their inhibition rates against HDACs (Hela cell nucleus extracts) at 1 μM concentration

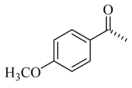
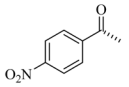
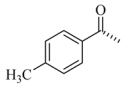
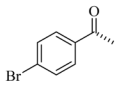
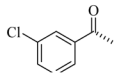
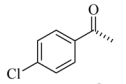
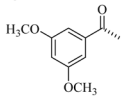
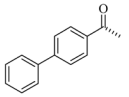
Compound	R	Inhibition rate
LYP-1		69.5%
LYP-2		**83.1%**
LYP-3		**78.4%**
LYP-4		68.6%
LYP-5		58.3%
LYP-6		**75.5%**
LYP-7		69.5%
LYP-8		72.9%
LYP-9		72.9%
LYP-10		73.8%
LYP-11		37.0%

Table 1. Structures of all 22 compounds and their inhibition rates against HDACs (Hela cell nucleus extracts) at 1 μM concentration (continued)

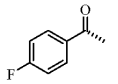
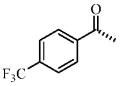
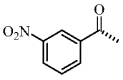
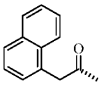
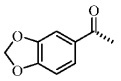
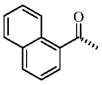
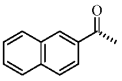
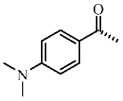
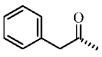
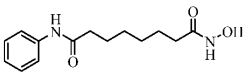
LYP-12		66.5%
LYP-13		67.6%
LYP-14		65.3%
LYP-15		**78.9%**
LYP-16		63.1%
LYP-17		66.1%
LYP-18		67.8%
LYP-19		61.6%
LYP-20		25.8%
LYP-21		71.1%
LYP-22		68.5%
SAHA		96.8%

Table 2. Inhibitory activity of four representative compounds toward HDAC1 and 6

Compound	IC_{50}[a], nM		SF(6/1)[b]
	HDAC1 (class I)	HDAC6 (class IIb)	
LYP-2	177.3	12.3	14.41
LYP-3	408.1	31.7	12.87
LYP-6	775.8	21.0	36.94
LYP-15	107.0	49.9	2.14
SAHA	44.6	21.6	2.06
ACY1215	66.8	5.3	12.6

[a]The IC_{50} values are the means of two experiments, with intra- and inter-assay variations of < 10%. [b]SF(6/1): selectivity factor for HDAC6 over HDAC1 (SF6/1 = IC_{50} (HDAC1)/IC_{50} (HDAC6)).

types according to the Chou-Talalay Method (*17*). The analysis revealed that combining LYP-2 or LYP-6 and bortezomib gave rise to an additive or synergistic effect when bortezomib was used at relatively low concentrations (< 100 nM) (Figures 1E and 1F). These results implied that HDAC6 inhibition may improve the efficacy of proteasome inhibitor bortezomib in breast cancer.

HDACs as an important anti-cancer drug targets have attracted many attentions. Till now, four HDACis, vorinostat, romidepsin, belinostat, and panobinostat, have been approved by the FDA for treatment of cancers including cutaneous T-cell lymphoma, peripheral T-cell lymphoma (PTCL), and multiple myeloma (*18*). The benzamide-based Class I HDAC-selective inhibitor chidamide has been approved in China for the treatment of relapsed or refractory PTCL (*19*). Apart from these five approved, there are several other HDACis at different stages of clinical trials against cancers. That said, shortcomings of HDACis

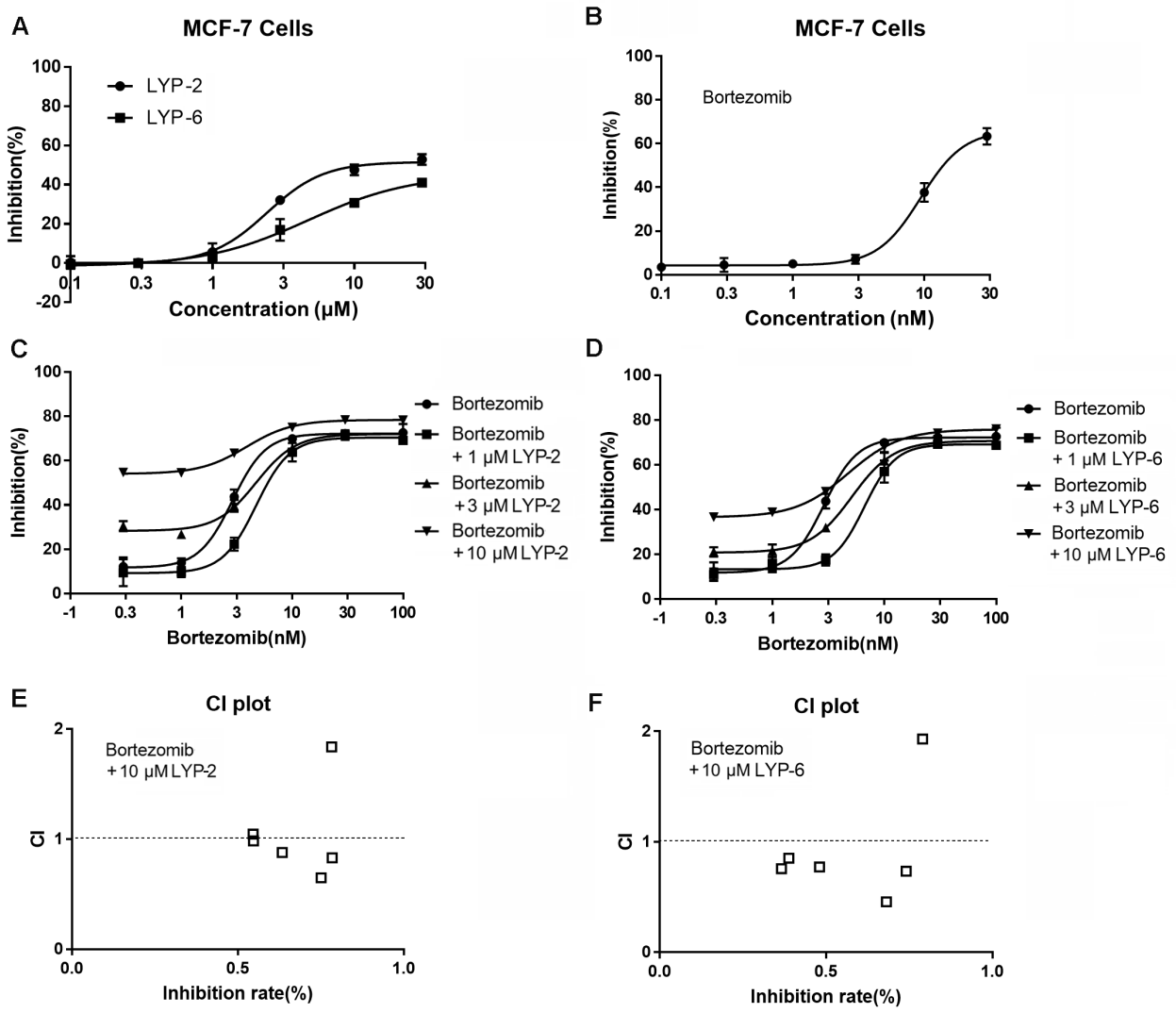


Figure 1. Inhibition of breast cancer cell MCF-7 proliferation. (A and **B)** Cells were incubated with different concentrations of LYP-2, LYP-6, or bortezomib for 72 hours and then subjected to MTT assay. **(C** and **D)** Cells were exposed to bortezomib alone or in combination with LYP-2 or LYP-6 at a fixed concentration (1, 3, and 10 μM, respectively) for 72 h before subjected to MTT assay. **(E** and **F)** Combination indexes (CI) of bortezomib plus LYP-2 or LYP-6 (10 μM) were calculated using the CompuSyn software and were plotted against cell inhibition rates.

still exist, one of which is the toxicity (*18*). In order to avoid unwanted toxicities, isoform selective HDACis are developed, especially the HDAC6 selective inhibitors, many of which have exhibited promising application values.

A number of HDAC6-selective inhibitors with diverse structural skeletons have been reported. The first HDAC6-selective inhibitor, tubacin, achieved selectivity through a bulky, relatively complex capping group (*20*). Unfortunately, the poor pharmacokinetic properties limited its application. In contrast, another well-known HDAC6 selective inhibitor ACY-1215 has a rigid and less cumbersome capping group contributing to its HDAC6 selectivity; nevertheless, it exhibited a selectivity index of only about 10 against HDAC1-3 (*21*). We designed and synthesized a series of novel HDACis with the N-phenylpiperidine-1-carboxamide as the skeleton in this work. Most of them displayed potent HDAC inhibitory activity and three of them were HDAC6 selective inhibitors. The selective index of compound LYP-6 was 36.94, much higher than the reference compound ACY-1215 which validates our design strategy.

Previous studies suggested that HDAC6 expression could be upregulated by estrogen, suggesting that there might be a link between the levels of HDAC6 expression and progression of breast cancer (*22*). We tested whether our newly discovered HDAC6 inhibitors have growth inhibitory ability on breast cancer cells. We found that although LYP-2 and LYP-6 dose-dependently inhibited the proliferation of MCF-7, their efficacy is moderate with IC_{50} values more than 30 μM. This result is consistent with a previous study in which HDAC6 selective inhibitor 4-hydroxybenzoic acid failed to induce significant cell death in MCF-7 cells at concentrations below 20 μM (*23*). On the other hand,

several studies demonstrated that HDAC6 inhibition sensitized the antitumor efficacy of chemotherapeutic drugs such as adriamycin and 5-fluorouracil (*23,24*). In the present study, we found that LYP-2 and LYP-6 enhanced the antiproliferative effect of bortezomib in MCF-7 cells and this combined treatment demonstrated an additive or synergistic effect. The safety and efficacy of bortezomib plus other chemotherapeutic drugs against breast cancer is currently under clinical trials. Our results suggested HDAC6 inhibitor might be an option in bortezomib based combined regimen in treatment of breast cancer in the future.

4. Conclusion

We designed and synthesized 22 novel HDAC inhibitors with the 4-aminopiperidine-1-carboxamide as the core structure. Within these compounds, LYP-2, LYP-3, and LYP-6 were identified as potent HDAC6 selective inhibitors. LYP-2 and LYP-6 showed moderate efficacy in suppressing the proliferation of breast cancer cells MCF-7, however, both compounds additively or synergistically enhanced the growth inhibitory effect of bortezomib. Combined HDAC6 inhibitor and bortezomib for treatment of breast cancer warrants further study in the future.

Acknowledgements

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