Medicinal Chemistry

Chen et al., Med Chem (Los Angeles) 2018, 8:10
DOI: 10.4172/2161-0444.1000524

Research Article Open Access

Synthesis of N-Substituted Benzamide Derivatives and their Evaluation as Antitumor Agents

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Abstract

A series of N-substituted benzamide derivatives designed based on Entinostat (MS-275) were synthesized, and characterized by IR, MS, ^{1}H NMR and ^{13}C NMR. Their anti-proliferative activity *in vitro* against four cell lines including MCF-7, MDA-MB-231, K562 and A549 were also evaluated by the MTT assay, the results showed that several compounds displayed similar inhibitory activity compared with MS-275. Finally, binding affinity of the synthesized compounds towards targets (histone deacetylases, HDACs) was studied using molecular docking simulations, compounds 13h and 13k were observed hydrogen bond, Van der Waals bond and hydrophobic interactions with HDAC2 and HDAC8.

Keywords: N-substituted benzamide derivative; Anti-proliferative activity; Molecular docking study

Introduction

Cancer is traditionally seen as a genetic disease; its expansion is revealed to be associated with mutations resulting in activation of oncogenes or inactivation of tumor suppressor genes [1-3]. With the deep research on gene function, however, the genetic alterations and abnormalities, epigenetic changes which are defined as heritable changes in gene function that do not involve changes in the DNA sequence, are now widely implicated in tumor onset and progression [4]. As for epigenetic regulation mechanism, it mainly contains DNA methylation, post-translational modifications of histone protein and remodeling of nucleosomes [5]. While histone deacetylases (HDACs) play a significant role in epigenetic regulation of gene expression by removing acetyl groups from ε-amino groups of lysine residues in the N-terminal extension of the core histones and controlling other cellular functions, such as inducing cell-cycle arrest, promoting differentiation and stimulating tumor cell death [6,7]. What's more, histone deacetylases inhibitors (HDACIs) with different structures have been developed for cancer therapy, for instance, Chidamide has been approved by FDA to cure peripheral T-cell lymphoma (PTCL), Entinostat (MS-275) is now in the clinical stage III to treat breast cancer, and so on.

Biological efficacy of HDACIs is mainly attributed to the active structure which can coordinate with the zinc ion that exists in histone protein through hydrogen bond [8,9]. Most benzamide derivatives as HDACIs for cancer treatment mainly consist of three parts: zinc binding groups (ZBG), cap and a linker [10]. Based on the strategy of structural modification of drugs, each part mentioned above could be modified to change their biological activity. For instance, Mocetinostat (MGCD0103, shown in Figure 1) which is undergoing clinical phase II for the treatment of lymphoma and bladder cancer with lower toxicity could be seen as a linker modification of MS-275 [11].

Based on modification of the zinc binding group part of MS-275, fourteen N-substituted benzamide derivatives were synthesized, their anti-proliferative activity against MCF-7, MDA-MB-231, K562 and A549 *in vitro* were evaluated, and preliminary structure-activity relationship was also discussed. Molecular docking study was further employed to explain the binding affinity of the synthesized compounds towards targets. The docking data are in agreement with those bioactivity test results.

Experimental

Chemistry

All the reagents were purchased from commercial suppliers without further purification unless otherwise specified. Melting points were determined with a capillary method and are uncorrected. IR spectra were recorded on a Spectrum Two Li10014 spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer. NMR spectra were recorded in DMSO-d6 or $CDCl_3$ solutions at room temperature (20 ± 2°C). ^{1}H and ^{13}C chemical shifts are quoted in parts per million downfield from TMS. ESI-MS spectra were recorded on a Bruker Esquire 3000 instrument. High-resolution mass spectra (HRMS) were obtained on a MicrOTOF-Q II mass spectrometer with an ESI source (Waters, Manchester). All reactions were monitored by thin-layer chromatography (TLC) and using ultraviolet light (254 nm). As for known compounds, only melting point and ^{1}H NMR spectra were confirmed with previously reported literatures and the main intermediates were characterized by IR, ^{1}H NMR, ^{13}C NMR spectra and mass spectra.

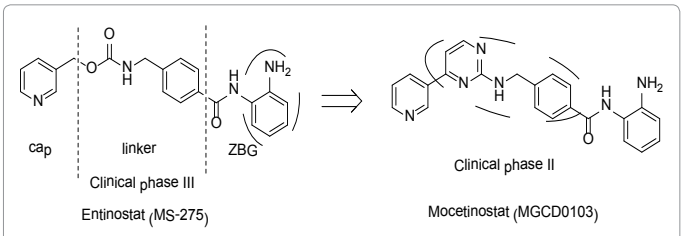

Figure 1: The structure of pre-clinical compounds MS-275 and MGCD0103.

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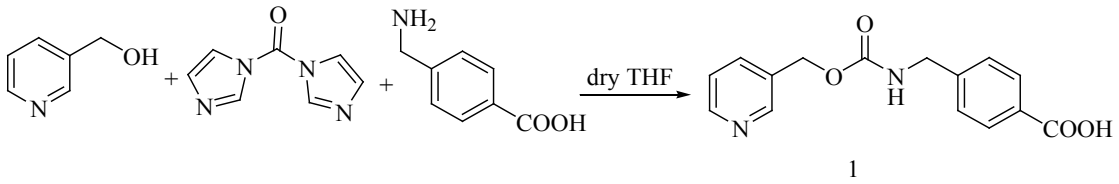
Received October 06, 2018; **Accepted** October 25, 2018; **Published** November 01, 2018

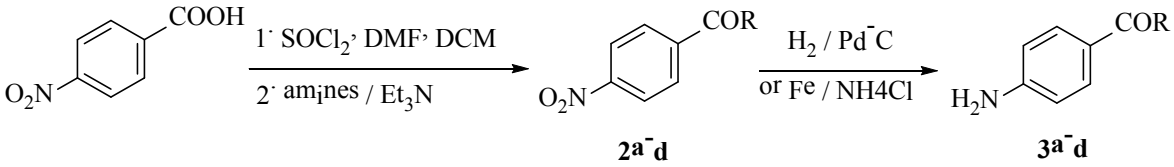
Citation: Chen T, Jiang H, Zhou J, Li Z, Huang W, et al. (2018) Synthesis of N-Substituted Benzamide Derivatives and their Evaluation as Antitumor Agents. Med Chem (Los Angeles) 8: 273-280. doi: 10.4172/2161-0444.1000524

Scheme 1

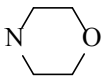
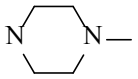


1

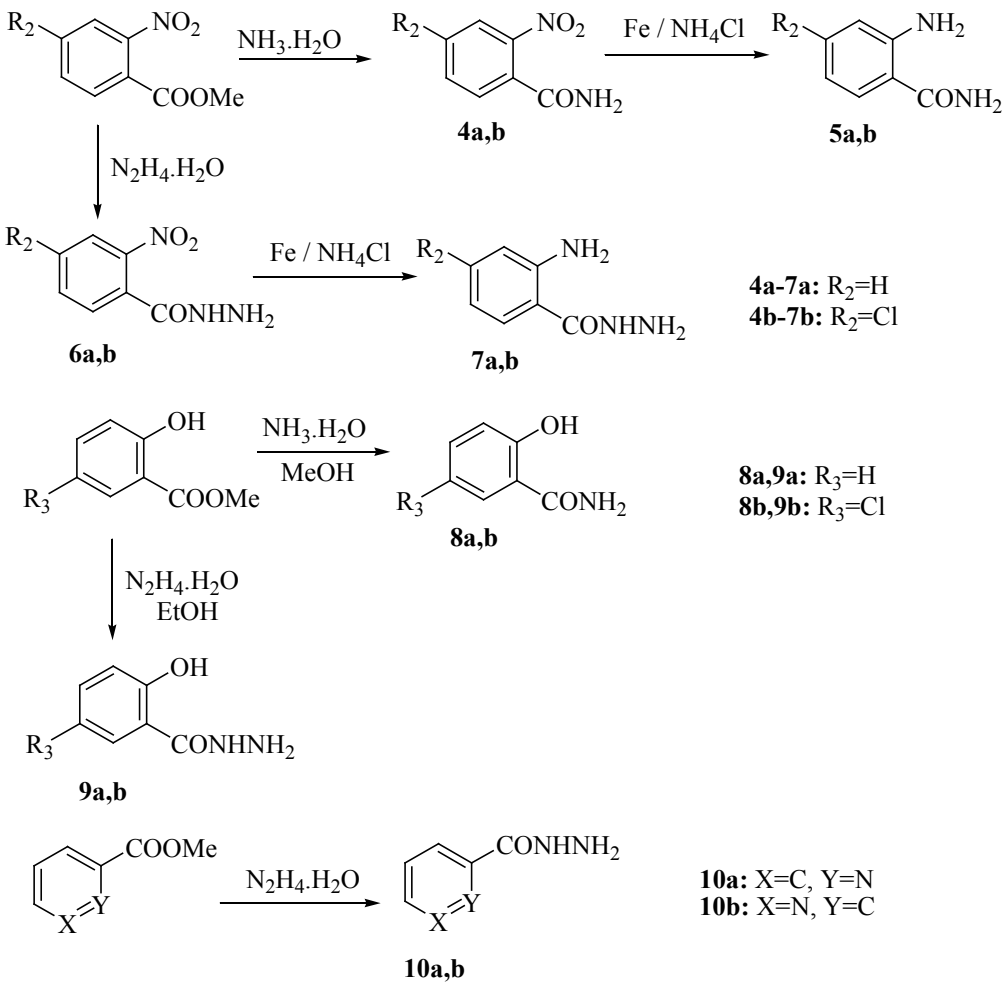
Scheme 2



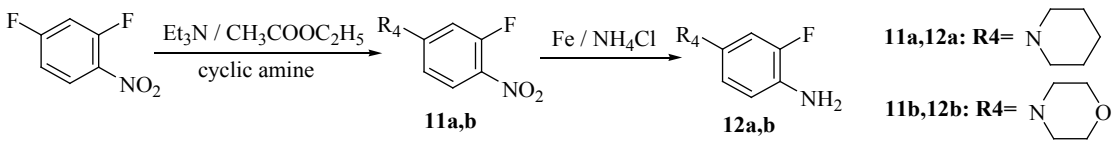
2a-d **3a-d**

a: R= NH_2 **b**: R=  **c**: R=  **d**: R= 

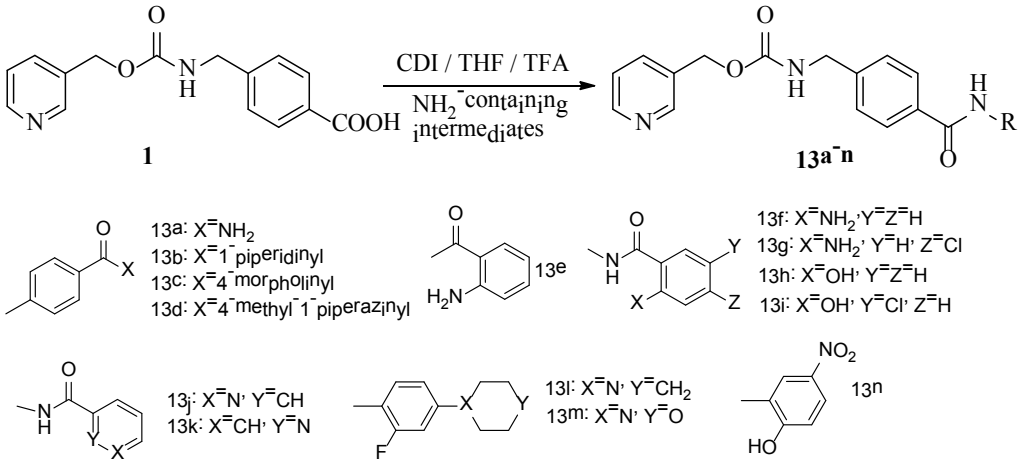
Scheme 3



Scheme 4



Scheme 5



THF distilled off by adding sodium and acetophenone as indicator under N_2 atmosphere and purified from water.

Synthesis of 4-[N-(pyridine-3-methoxycarbonyl) amino methyl] benzoic acid: (1)-To a suspension of N,N'-carbonyldiimidazole (CDI, 10 mmol) in dry THF (10 mL) was added pyridin-3-ylmethanol (10 mmol) dissolved in THF (10 mL) by three portions within 10 min at 0-10°C, the mixture was stirred at that temperature for 1 h. The resulting solution was added into a solution of 4-(aminomethyl)benzoic acid (10 mmol) dissolved in sodium hydroxide solution (10 mL, 1 mol/L) at 0-10°C, after stirring for 5 h at room temperature, the solvent was evaporated and then dissolved in water (25 mL). The solution was acidified with HCl (5 mol/L) to adjust pH 5 to precipitate white solid which was collected by filtration, washed with water twice and dried to give pure 1. Yield: 83%; mp: 206-208°C [14], mp: 207-208°C).

General procedure for the preparation of 4-nitrobenzamide derivatives: (2a-d)-To a mixture of 4-nitrobenzoic acid (10 mmol) in thionyl chloride (10 mL) was added three drops of DMF, the mixture was refluxed for 1.5 h. Then thionyl chloride was evaporated under vacuum, and CH_2Cl_2 (20 mL) was added into the residual to be used next. To a mixture of amine (12 mmol) and triethylamine (30 mmol) in CH_2Cl_2 (50 mL) was dropped slowly the acyl chloride solution prepared above in ice-water bath. The mixture was then stirred for 30 min at room temperature, the organic layer was separated, washed twice with salt water, and dried with anhydrous sodium sulfate, and solvent was concentrated to give the crude product, which was recrystallized from ethanol to give 2.

4-Nitrobenzamide (2a): Creamy-white solid; yield: 77%; mp: 198-200°C ([15], mp: 200-202°C).

(4-Nitrophenyl)(piperidin-1-yl)methanone (2b): Yield: 83%; mp: 119-121°C ([16], mp: 121-122°C).

Morpholino(4-nitrophenyl)methanone (2c): Yield: 81%; mp: 101-102°C ([17], mp: 101-102°C).

(4-Methylpiperazin-1-yl)(4-nitrophenyl)methanone (2d): Yield: 78%; mp: 98-100°C ([18], mp: 98-100°C).

General procedure for the preparation of 4-aminobenzamide derivatives: (3a-d)-Compound 2 (10 mmol) in ethanol (50 mL) was hydrogenated at 0.2-0.4 MPa catalyzed by 10% Pd-C for 5 h and recrystallized from ethanol to obtain compound 3.

4-Aminobenzamide (3a): Brown solid; yield: 73%; mp: 178-180°C ([19], mp: 177-179°C).

(4-Aminophenyl)(piperidin-1-yl)methanone (3b): Yield 75%; mp: 158-160 ([20], mp:158-160°C).

(4-Aminophenyl)(morpholino)methanone (3c): Yield: 76%; mp: 132-134°C ([21], mp: 136-138°C).

(4-Aminophenyl)(4-methylpiperazin-1-yl) methanone (3d): Yield: 70%; mp: 144-146°C ([22], mp: 140°C).

General procedure for the preparation of different kinds of substituted aromatic amides: (5a-b, 9a-b) and aromatic hydrazides (7a-b, 10a-b, 12a-b)-To a solution of methyl 2-nitrobenzoate, methyl salicylate, methyl 5-chlorosalicylate, or methyl 4-chloro-2-nitrobenzoate (10 mmol) in methanol (25 mL) was added ammonia (15 mL) or hydrazine hydrate (30 mmol), the solution was stirred at 50°C for 48 h, the resulting precipitate was filtered to obtain the corresponding aromatic amides or aromatic hydrazides.

The other amides or form hydrazide were similarly prepared from corresponding methyl ester.

2-Nitrobenzamide (4a): Yield: 77%; mp: 173-175°C ([23], mp: 175-176°C).

4-Chloro-2-nitrobenzamide (4b): Yield: 80%; mp: 144-146°C ([24], mp: 145-149°C).

2-Nitrobenzohydrazide (6a): Yield: 78%; mp: 121-123°C ([25], mp: 121-123°C).

4-Chloro-2-nitrobenzohydrazide (6b): Yield: 83%; mp: 178-180°C.

2-Hydroxybenzamide (8a): Yield: 70%; mp: 140-142°C ([26], mp: 140-142°C).

5-Chloro-2-hydroxybenzamide (8b): Yield: 79%; mp: 226-227°C ([27], mp: 226-227°C).

2-Hydroxybenzohydrazide (9a): Yield: 84%; mp: 148-150°C ([28], mp: 149-150°C).

5-Chloro-2-hydroxybenzohydrazide (9b): Yield: 78%; mp: 211-213°C ([29], mp: 208-210°C).

Picolinohydrazide (10a): Yield: 83%; mp: 100-102°C ([30], mp: 100.2-101.0°C).

Nicotinohydrazide (10b): Yield: 78%; mp: 160-162°C ([30], mp: 161.6-162.3°C).

General procedure for the preparation of 4-substituted-2-aminobenzamide and 4-substituted-2-aminobenzohydrazide: To a solution of NH_4Cl (15 mmol) dissolved in water (20 mL) was added iron powder (15 mmol), then compound 4 or 6 dissolved in ethanol (20 mL) was added slowly into the solution with agitating, the mixture was heated to 80°C and stirred for 1.5 h. The resulting mixture was filtered with diatomite and the residue was washed with ethanol (2 × 15 mL), the filtrate was evaporated under vacuum to remove ethanol and the residue was extracted with dichloromethane (2 × 20 mL), the combined organic layers were washed with brine once and dried with anhydrous Na_2SO_4, then evaporated to give the title compound.

2-Aminobenzamide (5a): White solid; yield: 81%; mp: 110-112°C ([31], mp: 110-112°C).

2-Amino-4-chlorobenzamide (5b): Yield: 77%; mp: 180-182°C([32], mp: 180-182°C).

2-Aminobenzohydrazide (7a): Yield: 87%; mp: 119-121°C ([33], mp: 119-121°C).

2-Amino-4-chlorobenzohydrazide (7b): Yield: 84%; mp: 122-123°C([34], mp: 122-123°C).

General procedure for the preparation of 4-substituted-2-fluoronitrobenzene: To a solution of 2,4-difluoronitrobenzene (35 mmol) dissolved in ethyl acetate (50 mL) was added triethylamine (40 mmol) and cyclic amine (40 mmol), the solution was stirred at room temperature for 10 h. The resulting mixture was filtered to remove the formed salt, and filtrate was washed with water twice, the organic layer was separated and dried with anhydrous Na_2SO_4, then evaporated to give the title compound.

1-(3-Fluoro-4-nitrophenyl) piperidine (11a): Yield: 70%; mp: 127-129°C ([35], mp: 128-129°C).

4-(3-Fluoro-4-nitrophenyl) morpholine (11b): Yield: 68%; mp: 146-148°C([36], mp: 146-148°C).

General procedure synthesis of 4-substituted-2-fluoronitroaniline: The reduction reaction was conducted according to the procedure of compound 5.

2-Fluoro-4-(piperidin-1-yl) aniline (12a): Light brown solid; yield: 72%; mp: 85-87°C.

Compound No.	IC_{50}[a,b](μM)			
	MCF-7	A549	K562	MDA-MB-231
13a	>80	>80	>80	37.43
13b	62.17	>80	65.31	71.19
13c	73.46	>80	66.29	>80
13d	>80	18.37	>80	>80
13e	>80	33.65	22.52	>80
13f	>80	22.58	28.11	51.73
13g	>80	>80	>80	>80
13h	19.45	16.91	25.38	32.88
13i	23.19	55.39	>80	35.75
13j	>80	>80	45.35	>80
13k	28.73	36.45	27.66	>80
13l	>80	>80	21.55	40.25
13m	>80	>80	19.83	63.57
13n	>80	>80	>80	>80
MS[c]	17.36	38.79	18.34	21.31

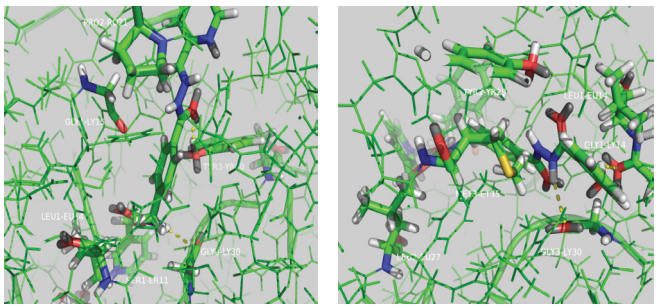
[a]The IC_{50} values represent the concentration that causes 50% growth inhibition; [b]The IC_{50} values were the mean values of three repeated experiments, with a deviation within 20%; [c]MS is Entinostat (MS-275).

Table 1: IC_{50} value of target compounds 13a-n against tumor cells *in vitro*.

HDAC PDB code[a]	Fitness				
	13e	13f	13h	13k	MS-275
3max	73.07	62.14	64.11	62.18	66.29
1t69	59.09	51.06	52.70	61.32	57.13
4bkx	50.56	54.26	51.06	54.95	56.01

[a]HDAC PDB code: 3max refers to HDAC2, 1t69 refers to HDAC8 and 4bkx refers to HDAC1

Table 2: Calculated binding affinity from molecular docking.



(a) 13h binding to HDAC2 (PDB code:3max) (b) 13k binding to HDAC2 (PDB code:3max)

Figure 2: The docking modes of 13h and 13k to HDAC2 respectively.

2-Fluoro-4-(tetrahydro-2H-pyran-4-yl) aniline (12b): Brown solid; yield: 68%; mp: 94-96°C ([37], mp, 95-97).

Preparation of N-(2-aminophenyl)-4-[N-(pyridin-3-yl) methoxycarbonylaminomethyl] benzamide (reference drug, MS-275): To a mixture of compound 1 (1.0 mmol), 1,2-phenylenediamine (1.2 mmol) and HBTU (1.0 mmol) in dichloromethane (10 mL) was slowly added triethylamine (4.0 mmol) cooled with ice-bath, the solution was then stirred at room temperature for 4h, the solid was filtered out, the residue was washed with dichloromethane twice to give the title compound. Yield: 68%; mp: 157-159°C [14]; ^1H NMR (DMSO-d6): δ 9.62 (s, 1H, ArCONH), 8.60 (s, 1H, N=C-H), 8.53 (d, J=4.0 Hz, 1H, N=C-H), 7.92-7.98 (m, 3H, Ar-H), 7.79 (dt, J=8.0 Hz, 1H, OCONH), 7.36-7.43 (m, 3H, Ar-H), 7.17 (d, J=12.0 Hz, 1H, Ar-

H), 6.97 (t, J=16.0 Hz, 1H, Ar-H), 6.78 (d, J=8.0 Hz, 1H, Ar-H), 6.60 (t, J=12.0 Hz, 1H, Ar-H), 5.10 (s, 2H, OCH_2), 4.88 (s, 2H, NH_2), 4.28 (d, J=8.0 Hz, 2H, NCH_2); 13C NMR (DMSO-d6, 100 MHz, ppm): δ 171.63, 164.69, 156.73, 149.65, 149.60, 144.37, 140.62, 136.24, 133.72, 133.06, 129.86, 129.67, 129.22, 127.86, 127.52, 124.00, 123.06, 120.46, 119.55, 63.77, 44.06. These spectral and analytical data are as previously reported.

General procedure for the preparation of N-substituted benzamide derivatives: To a suspension of compound 1 (1.0 mmol) in anhydrous THF (20 mL) was added CDI (1.2 mmol), and the mixture was stirred for 3 h at 60°C. After formation of acylimidazole the clear solution was cooled to room temperature. To this solution was added different kinds of NH2-group contained intermediates (1.1 mmol) and trifluoroacetic acid (1.1 mmol), which was then stirred for 16 h. The reaction mixture was evaporated to remove THF, and the crude product was stirred in a mixture of hexane and water (2:5 v/v) for 1 h, filtered and dried. The obtained solid was triturated in dichloromethane twice to afford pure title compound.

Pyridin-3-ylmethyl 4-(4-carbamoylphenylcarbamoyl) benzylcarbamate (13a): Yield: 55%; mp: 213-215°C; IR(cm^{-1}): 3398, 3313, 1714, 1655, 1609, 850, 716; ^{1}H NMR (DMSO-d_6): δ 10.40 (s, 1H, ArCON-H), 8.61 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 7.98 (t, J=8.0 Hz, 1H, OCON-H), 7.92 (d, J=8.0 Hz, 2H, Ar-H), 7.84-7.89 (m, 4H, Ar-H, $CONH_2$), 7.80 (d, J=4.0 Hz, 1H, Ar-H), 7.57-7.64 (t, 1H, Ar-H), 7.40-7.43 (m, 3H, Ar-H), 7.26 (s, 1H, Ar-H), 5.11 (s, 2H, OCH_2), 4.29 (d, J=4.0 Hz, 2H, NCH_2); 13C NMR (100 MHz, DMSO-d6, ppm): δ 167.85, 166.03, 156.72, 149.59, 144.07, 142.31, 136.25, 133.73, 133.15, 129.56, 128.68, 128.31, 127.34, 124.01, 119.76, 112.90, 63.73, 44.08; ESI-HRMS: Anal. Calcd. for $C_{22}H_{20}N_4O_4$ 13a: m/z 404.1485; Found $[M^+Na]^+$: m/z 427.1386.

Pyridin-3-ylmethyl 4-((4-(piperidine-1-carbonyl)phenyl) carbamoyl)benzylcarbamate(13b): Yield: 50%; mp: 182-185°C; IR (cm^{-1}): 3354, 3306,1724, 1666, 1617, 764, 708; ^{1}H NMR (DMSO-d_6): δ 10.34 (s, 1H, ArCONH), 8.60 (s, 1H, N=C-H), 8.53 (d, J= 4.0 Hz, 1H, N=C-H), 7.85-7.99 (m, 3H, Ar-H), 7.83 (d, J=4.0 Hz, 2H, Ar-H), 7.79 (dt, J=8.0 Hz, 1H, OCON-H), 7.35-7.43 (m, 5H, Ar-H), 5.10 (s, 2H, OCH_2), 4.28 (d, J=8.0 Hz, 2H, NCH_2), 3.43 (m, 4H, CH_2NCH_2), 1.61 (m, 2H, CH_2), 1.51 (m, 4H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 169.18, 165.98, 156.71, 149.62, 149.60, 144.01, 140.59, 136.22, 133.80, 133.13, 131.79, 128.27, 127.96, 127.34, 124.00, 120.16, 63.73, 44.08, 24.57; ESI-HRMS: Anal. Calcd. for $C_{27}H_{28}N_4O_4$ 13b: m/z 472.2111; Found $[M^+Na]^+$: m/z 495.2009.

Pyridin-3-ylmethyl 4-((4-(morpholine-4-carbonyl)phenyl) carbamoyl)benzylcarbamate(13c): Yield: 52%; mp: 178-180°C; IR (cm^{-1})°C3361, 3311, 1721, 1670, 1612, 839, 760, 702; ^{1}H NMR (DMSO-d_6): δ 10.36 (s, 1H, ArCONH), 8.60 (s, 1H, N=C-H), 8.53 (d, J=4.0 Hz, 1H, N=C-H), 7.84-7.99 (m, 5H, Ar-H), 7.79 (dt, J=8.0 Hz, 1H, OCON-H), 7.39-7.44 (m, 5H, Ar-H), 5.10 (s, 2H, OCH_2), 4.28 (d, J=8.0 Hz, 2H, NCH_2), 3.50-3.60 (m, 8H, OCH_2CH_2N); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 169.38, 166.02, 156.72, 149.61, 144.05, 140.94, 136.22, 133.76, 133.13, 130.74, 128.44, 128.29, 127.34, 124.00, 120.13, 66.59, 63.73, 44.08; ESI-HRMS: Anal. Calcd. For $C_{26}H_{26}N_4O_5$ 13c: m/z 474.1903; Found $[M^+Na]^+$: m/z 497.1800.

Pyridin-3-ylmethyl 4-((4-(4-methylpiperazine-1-carbonyl) phenyl)carbamoyl)benzylcarbamate (13d): Yield: 48%; mp: 153-155°C; IR (cm^{-1}): 3360, 3303, 1720, 1660, 845, 710; ^{1}H NMR (DMSO-d_6): δ 10.38 (s, 1H, ArCON-H), 8.61 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 7.98 (t, J=8.0 Hz, 1H, Ar-H), 7.91 (d, J=8.0 Hz, 2H, Ar-H), 7.84 (d, J=12.0 Hz, 2H, Ar-H), 7.78 (dt, J=8.0 Hz, 1H, OCON-H), 7.38-7.43 (m, 5H, Ar-H), 5.11 (s, 2H, OCH_2), 4.29 (d, J=4.0 Hz, 2H, NCH_2), 3.44 (m, 4H, $CONCH_2(CH_2)$), 2.20 (s, 3H,-CH_3), 1.23 (t, J=8.0 Hz, 4H, $NCH_2(CH_2)$); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 169.25, 166.00, 156.72, 149.62, 149.60, 144.04, 140.83, 136.21, 133.78, 133.13, 131.17, 128.29, 128.26, 127.34, 124.00, 120.15, 63.73, 62.99, 52.47, 46.09, 44.08; ESI-HRMS: Anal. Calcd. for $C_{27}H_{29}N_5O_4$ 13d: m/z 487.2220; Found $[M^+H]^+$: m/z 488.2295.

Pyridin-3-ylmethyl 4-((2-aminobenzoyl)carbamoyl) benzylcarbamate(13e): Yield: 46%; mp: 208-210°C; IR (cm^{-1}): 3367, 3283, 1687, 1663, 764; ^{1}H NMR (DMSO-d_6): δ 12.95 (s, 1H, CONHCOAr), 8.70 (d, J=12.0 Hz, 1H, Ar-NH_2), 8.61 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 8.43 (s, 1H, Ar-NH_2), 7.98 (t, J=8.0 Hz, 1H, OCON-H), 7.89-7.93 (m, 3H, Ar-H), 7.85 (s, 1H, Ar-H), 7.81 (d, J=8.0 Hz, 1H, Ar-H), 7.58 (t, J=8.0 Hz, 1H, Ar-H), 7.41-7.46 (m, 3H, Ar-H), 7.18 (t, J=8.0 Hz, 1H, Ar-H), 5.12 (s, 2H, OCH_2), 4.30 (d, J=4.0 Hz, 2H, NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 171.64, 164.69, 156.74, 149.65, 149.60, 144.37, 140.62, 136.24, 133.73, 133.12, 133.05, 129.22, 127.86, 127.52, 124.00, 123.06, 120.48, 119.56, 63.78, 44.07; ESI-HRMS: Anal. Calcd. for $C_{22}H_{20}N_4O_4$ 13e: m/z 404.1485. Found $[M^+Na]^+$: m/z 427.1383.

Pyridin-3-ylmethyl 4-(2-(2-aminobenzoyl) hydrazinecarbonyl) benzylcarbamate (13f): Yield: 43%; mp: 156-158°C; IR (cm^{-1}): 3489, 3361, 3294, 3244, 1726, 1677, 1615, 823, 753; ^{1}H NMR (DMSO-d_6): δ 10.34 (s, 1H, ArCONHNHCO), 10.15 (s, 1H, ArCONHNHCO), 8.61 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 7.96 (t, J=4.0, 8.0 Hz, 1H, Ar-H), 7.87 (d, J=8.0 Hz, 2H, Ar-H), 7.78 (d, J=8.0 Hz, 1H, OCONH), 7.61 (d, J=8.0 Hz, 1H, Ar-H), 7.37-7.43 (m, 3H, Ar-H), 7.20 (m, 1H, Ar-H), 6.76 (d, J=8.0 Hz, 1H, Ar-H), 6.53 (m, 1H, Ar-H), 6.43 (s, 2H, Ar-NH_2), 5.11 (s, 2H, OCH_2), 4.28 (d, J=4.0 Hz, 2H, NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 168.78, 166.23, 156.74, 150.38, 149.63, 149.59, 144.04, 136.22, 133.13, 132.76, 131.73, 128.64, 128.00, 127.39, 124.00, 116.89, 115.09, 113.04, 63.76, 44.09. ESI-HRMS: Anal. Calcd. for $C_{22}H_{21}N_5O_4$ 13f: m/z 419.1594. Found $[M^+Na]^+$: m/z 442.1493.

Pyridin-3-ylmethyl 4-(2-(2-amino-4-chlorobenzoyl) hydrazinecarbonyl)benzylcarbamate (13g): Yield: 49%; mp: 204-206°C; IR (cm^{-1}): 3404, 3340, 3292, 1708, 1682, 1642, 718; ^{1}H NMR (DMSO-d_6): δ 10.37 (s, 1H, ArCONHNHCO), 10.24 (s, 1H, ArCONHNHCO), 8.60 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 7.96 (t, J=4.0, 8.0 Hz, 1H, Ar-H), 7.88 (d, J=8.0 Hz, 2H, Ar-H), 7.78 (d, J=8.0 Hz, 1H, OCONH), 7.61 (d, J=8.0 Hz, 1H, Ar-H), 7.37-7.43 (m, 3H, Ar-H), 6.82 (s, 1H, Ar-H), 6.69 (s, 2H, Ar-NH_2), 6.57 (d, J=8.0 Hz, 1H, Ar-H), 5.11 (s, 2H, OCH_2), 4.28 (d, J=4.0 Hz, 2H, NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 168.01, 166.23, 156.73, 151.67, 149.62, 149.59, 144.10, 137.29, 136.21, 133.12, 131.62, 130.46, 128.00, 127.40, 123.99, 115.65, 114.82, 111.77, 63.76, 44.08. ESI-HRMS: Anal. Calcd. for $C_{22}H_{20}ClN_5O_4$ 13g: m/z 453.1204. Found $[M^+Na]^+$: m/z 476.1108.

Pyridin-3-ylmethyl 4-(2-(2-hydroxybenzoyl)hydrazinecarbonyl) benzylcarbamate (13h): Yield: 43%; mp: 172-174°C; IR (cm^{-1}): 3301, 1720, 1607, 802, 759; ^{1}H NMR (DMSO-d_6): δ 11.95 (s, 1H, Ar-OH), 10.68 (s, 1H, ArCONHNHCO), 10.64 (s, 1H, ArCONHNHCO), 8.60 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 7.93-7.98 (m, 2H, Ar-H), 7.88 (d, J=8.0 Hz, 2H, Ar-H), 7.78-7.81 (m,1H, Ar-H), 7.38-7.49 (m, 4H, Ar-H), 6.94-7.00 (m, 2H, Ar-H), 5.11 (s, 2H, OCH_2), 4.28 (d, J=4.0 Hz, 2H, NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 168.21, 165.82, 159.77, 156.73, 149.62, 149.59, 144.25, 136.22, 134.64, 133.12, 131.35, 128.74, 128.06, 127.44, 124.00, 119.53, 117.87, 115.01, 63.76, 44.09. ESI-HRMS: Anal. Calcd. for $C_{22}H_{20}N_4O_5$ 13h: m/z 420.1434.

Found $[M^+Na]^+$: m/z 443.1328.

Pyridin-3-ylmethyl 4-(2-(5-chloro-2-hydroxybenzoyl) hydrazinecarbonyl)benzylcarbamate (13i): Light yellow solid; Yield: 47%; mp: 212-214°C; IR (cm^{-1}): 3303, 1719, 1601, 1560, 706; ^1H NMR (DMSO-d_6): δ 11.93 (s, 1H, Ar-OH), 10.72 (s, 2H, ArCONHNHCO), 8.61 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 7.95-7.98 (m, 2H, Ar-H), 7.88 (d, J=8.0 Hz, 2H, Ar-H), 7.78-7.81 (m, 2H, Ar-H), 7.50 (dd, J=4.0, 8.0 Hz, 1H, Ar-H), 7.39-7.43 (m, 3H, Ar-H), 7.03 (d, J=8.0 Hz, 1H, Ar-H), 5.11 (s, 2H, OCH_2), 4.29 (d, J=4.0 Hz, 2H, NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 166.38, 165.68, 157.97, 156.73, 149.62, 149.59, 144.29, 136.22, 134.07, 133.12, 131.25, 128.43, 128.09, 127.44, 123.99, 123.22, 119.78, 117.13, 63.76, 44.09. ESI-HRMS: Anal. Calcd. for $C_{22}H_{19}ClN_4O_5$ 13i: m/z 454.1044. Found $[M^+Na]^+$: m/z 477.0942.

Pyridin-3-ylmethyl 4-(2-nicotinoylhydrazinecarbonyl) benzylcarbamate(13j): Yield: 45%; mp: 116-118°C; IR (cm^{-1}): 3426, 3322, 3287, 1699, 1650, 709; ^1H NMR (DMSO-d_6): δ 10.71 (s, 1H, ArCONHNHCO), 10.58 (s, 1H, ArCONHNHCO), 9.08 (s, J=4.0 Hz, 1H, Ar-H), 8.78 (dd, J=4.0, 8.0 Hz, 1H, Ar-H), 8.60 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 8.25-8.28 (m, 1H, Ar-H), 7.96 (t, J=4.0, 8.0 Hz, 1H, Ar-H), 7.88 (d, J=8.0 Hz, 2H, Ar-H), 7.78 (m, 1H, Ar-H), 7.56-7.60 (m, 1H, Ar-H), 7.39-7.43 (m, 3H, Ar-H), 5.11 (s, 2H, OCH_2), 4.29 (d, J=4.0 Hz, 2H, NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 166.10, 164.95, 156.74, 152.99, 149.62, 149.59, 148.89, 144.23, 136.22, 135.68, 133.12, 131.47, 128.71, 128.03, 127.46, 124.19, 124.00, 63.76, 44.08. ESI-HRMS: Anal. Calcd. for $C_{21}H_{19}N_5O_4$ 13j: m/z 4005.1437. Found $[M^+H]^+$: m/z 406.1511.

Pyridin-3-ylmethyl 4-(2-picolinoylhydrazinecarbonyl) benzylcarbamate (13k): Yield: 41%; mp: 178-180°C; IR (cm^{-1}): 3312, 1702, 1657, 1531, 712; ^1H NMR (DMSO-d_6): δ 10.62 (s, 1H, CONHNHCO), 10.53 (s, 1H, CONHNHCO), 8.71 (dt, J=4.0 Hz, 1H, Ar-H), 8.61 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 8.02-8.08 (m, 2H, Ar-H), 7.97 (t, J=4.0, 8.0 Hz, 1H, Ar-H), 7.88 (d, J=8.0 Hz, 2H, Ar-H), 7.79 (m, 1H, Ar-H), 7.65-7.68 (m, 1H, Ar-H), 7.38-7.43 (m, 3H, Ar-H), 5.12 (s, 2H, OCH_2), 4.29 (d, J=4.0 Hz, 2H, NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 165.73, 163.71, 156.75, 149.75, 149.62, 149.59, 149.15, 144.07, 138.32, 136.22, 133.13, 131.62, 128.06, 127.46, 127.37, 124.00, 122.84, 63.76, 44.09. ESI-HRMS: Anal. Calcd. for $C_{21}H_{19}N_5O_4$ 13k: m/z 4005.1437. Found $[M^+H]^+$: m/z 406.1508.

Pyridin-3-ylmethyl 4-((2-fluoro-4-(piperidin-1-yl)phenyl) carbamoyl)benzylcarbamate(13l): Gray solid; Yield: 45%; mp: 176-178°C; IR (cm^{-1}): 3362, 864, 712; $_1H$ NMR (DMSO-d_6): δ 10.21 (s, 1H, ArCONH), 8.60 (s, 1H, N=C-H), 8.53 (d, J=4.0 Hz, 1H, N-CH=C), 7.89-7.97 (m, 3H, Ar-H), 7.80 (d, J=8.0 Hz, 1H, OCONH), 7.69 (dd, J=4.0, 16.0 Hz, 1H, Ar-H), 7.47 (dd, J=4.0, 8.0 Hz, 1H, Ar-H), 7.38-7.43 (m, 3H, Ar-H), 7.01 (t, J=8.0, 12.0 Hz, 1H, Ar-H), 5.10 (s, 2H, OCH_2), 4.28 (d, J=4.0 Hz, 2H, NCH_2), 3.74 (m, 4H, CH_2OCH_2), 2.97 (m, 4H, CH_2NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 165.50, 156.72, 149.61, 143.84, 136.20, 134.41, 134.30, 133.82, 133.14, 128.13, 127.32, 123.98, 119.74, 119.70, 116.69, 108.98, 108.73, 63.74, 52.22, 44.09, 26.21, 24.21. ESI-HRMS: Anal. Calcd. for $C_{26}H_{27}FN_4O_3$ 13l: m/z 462.2067. Found $[M^+H]^+$: m/z 463.2140.

Pyridin-3-ylmethyl 4-((2-fluoro-4-morpholinophenyl) carbamoyl) benzylcarbamate (13m): Yield: 46%; mp: 180-182°C; IR (cm^{-1}): 3337, 1695, 1634, 754, 719; ^1H NMR (DMSO-d_6): δ 10.18 (s, 1H, ArCONH), 8.60 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 7.88-7.98 (m, 3H, Ar-H), 7.80 (d, J=8.0 Hz, 1H, OCONH), 7.69 (dd, J=4.0, 16.0 Hz, 1H, Ar-H), 7.38-7.46 (m, 4H, Ar-H), 7.01 (t, J=8.0, 12.0 Hz, 1H, Ar-H), 5.10 (s, 2H, OCH_2), 4.28 (d, J=4.0 Hz, 2H, NCH_2), 2.92 (m, 4H, CH_2NCH_2), 1.63-1.66 (m, 4H, NCH_2CH_2); 1.50 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 165.55, 156.72, 149.59, 143.89, 136.20, 134.83, 134.72, 133.76, 133.13, 128.15, 127.33, 123.98, 119.42, 119.37, 116.73, 109.06, 108.81, 66.67, 63.74, 51.21, 44.09. ESI-HRMS: Anal. Calcd. for $C_{25}H_{25}FN_4O_4$ 13m: m/z 464.1860. Found $[M^+H]^+$: m/z 465.1934.

Pyridin-3-ylmethyl 4-((2-hydroxy-5-nitrophenyl) carbamoyl) benzylcarbamate (13n): Yellow solid; Yield: 43%; mp: 178-180°C; IR (cm^{-1}): 3400, 3332, 1705, 1656, 1595, 749; ^1H NMR (DMSO-d_6): δ 11.65 (s, 1H, Ar-OH), 9.58 (s, 1H, ArCONH), 8.80 (d, J=4.0 Hz, 1H, Ar-H), 8.61 (s, 1H, N=C-H), 8.54 (d, J=4.0 Hz, 1H, N-CH=C), 7.94-8.02 (m, 4H, Ar-H), 7.80 (m, 1H, Ar-H), 7.40-7.43 (m, 3H, Ar-H), 7.08 (d, J=12.0 Hz, 1H, Ar-H), 5.12 (s, 2H, OCH_2), 4.30 (d, J=4.0 Hz, 2H, NCH_2); ^{13}C NMR (100 MHz, DMSO-d_6, ppm): δ 165.69, 156.73, 156.13, 149.62, 149.58, 144.34, 139.54, 136.23, 133.13, 132.98, 128.18, 127.51, 126.74, 124.00, 122.17, 119.23, 115.69, 63.76, 56.51, 44.09, 19.02. ESI-HRMS: Anal. Calcd. for $C_{21}H_{18}N_4O_6$ 13n: m/z 422.1226. Found $[M^+H]^+$: m/z 423.1300.

Biology

Antitumor activity test

The antitumor activity of all the synthesized compounds (13a-n) against cancer cell lines of MCF-7, A549, K562 and MDA-MB-231 by the MTT assay was carried out in the State Key Laboratory of Biotherapy, Sichuan University. All the data were obtained by the mean values of three repeated experiments. Briefly, Cells (3×10^4/ml) were seeded in 96-well plates and cultured for 24 hours, followed by various concentration of compounds from 50 μM to 1.56 μM treatment for 48 hours at 37°C, 5% CO_2. Then, 20 μl of 5 mg/ml MTT was added per well and incubated for another 4 hours at 37°C, the supernatant fluid was removed, and DMSO was added 150 μl/well for 15-20 minutes. The light absorptions (OD) were measured at 570 nm with SpectraMAX M5 microplate spectrophotometer (Molecular Devices). Entinostat was used as referential drug for anti-proliferative evaluation, respectively, which was also assessed under similar conditions for comparison with the tested compounds. The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability. The data for the anti-proliferative activity are presented in Table 1.

Results and Discussion

Chemistry

The synthetic strategy of N-substituted benzamide derivatives was depicted in Schemes 1-5. Compound 1 was obtained by condensation of pyridin-3-ylmethanol and 4-(aminomethyl) benzoic acid in the presence of CDI in dry THF with 83% yield (Scheme 1), which was used as the starting material to react with amino-containing intermediates. Three kinds of amino-containing intermediates including substituted anilines, substituted aromatic amides and substituted aromatic hydrazides were synthesized. 4-Nitrobenzoic acid was transferred into acyl chloride in $SOCl_2$ and then reacted with different kinds of amines in the presence of triethylamine to abtain 2a-d, which was then reduced under the condition of H_2/Pd-C to give 3a-d (Scheme 2). Substituted aromatic amides 5a-b, 8a-b and substituted aromatic hydrazides 7a-b, 9a-b and 10a-b can be formed from methyl benzoate reacted with ammonia and hydrazine hydrate, respectively. Nitro group compounds were then reduced to amino group in the presence of Fe/NH_4Cl (Scheme 3). 2,4-Difluoronitrobenzene was firstly reacted with cyclic amines to afford 11a-b, which were then similarly reduced to give

12a-b (Scheme 4). Compound 1 was condensed with NH_2-contained intermediates in the presence of CDI in dry THF to form the target compounds (Scheme 5) with a moderate yield.

Biology

The anti-proliferative activities *in vitro* of compounds 13a-n were evaluated by MTT assay and Entinostat (MS-275) as the referential compound. Four different cancer cell lines which are human breast adenocarcinoma cell (MCF-7), human lung cell (A549), Human chronic myeloid leukemia cell (K562) and human breast cancer cell (MAD-MB-231) were chosen to be tested. The results are shown in Table 1.

As can be seen, 13h displayed good inhibitory activity against all four cell lines, even better activity than MS-275 towards A549; 13d, 13e, 13f, 13k also showed good inhibitory activity against A549 compared with MS-275; 13e, 13l, 13m displayed the similar activity against K562 compared with MS-275, 13f, 13k showed good activity against K562; 13i and 13k exhibited moderate activity against MCF-7; 13a, 13h and 13i also displayed moderate activity against MDA-MB-231. Other compounds showed low or no inhibitory activity towards the cancer cell lines.

Among the data reveal that, as for the zinc binding group, 2-NH_2 or 2-OH on phenyl group of R part is necessary for the inhibitory activity, i.e., generally 13a-d vs 13e-i, while chlorine atom existed in the same benzene ring will largely decrease their activity, i.e., 13f vs 13g and 13h vs 13i, the same function seen in nitro-group (13n vs MS-275). Compounds containing 2-F and 4-cyclic amine on phenyl group of R part (131 and 13 m) displayed good inhibitory activity towards K562. Hydrazide or bisamide fragments rarely influence the results, i.e., 13e vs 13f. What's more, it seems that picolinic structure could also bind with zinc ion to exert their biological activity, like 13k displayed; however, 13j lacked activity.

Molecular docking study

In order to achieve the binding modes of the synthesized compounds and provide more insights into the interactions, a molecular docking simulation of the compounds 13e, 13f, 13h, 13k and MS-275 which displayed low IC50 values was performed using Auto dock Vina (ver.1.1.2). HDAC2 and HDAC8 were chosen as the targets, their structures were prepared for docking as described previously [12,13]. Polar hydrogen's were added, ensuring that the protonation states were correct, and AMBER charges were assigned to receptor atoms. The ligands designed as reference ligands henceforth were extracted separately from the proteins. All water molecules were removed from the structures. The binding site was defined using the reference ligands, and all protein atoms within the geometrical constrain of 8 Å of a non-hydrogen atom in any of the reference ligands were included. The structures of HDAC proteins outside of active sites remained unchanged, while active sites were kept partially flexible upon docking. Binding affinity was computed from contributions of internal and external van der Waals (vdW), hydrophobic properties (HP) and hydrogen bond (HB) using Gold score (empirical scoring) function. Finally, binding affinity and bioactivity of ligands were evaluated by calculated fitness. The results are shown in Table 2.

As can be seen, fitness value (the higher the value is, the stronger the interaction between a ligand and a protein is) is quite close for above four compounds (also close to the value of MS-275), i.e., the interaction (binding affinity) between the ligand and the protein is similar. Below is the docking modes of 13h and 13k to HDAC2 respectively (Figure 2). The hydrogen bonds (HB) are shown in dotted yellow lines with HB distance of 2.45~2.97 Å and important residues are shown and labeled. Besides hydrogen bonds shown above, short contacts such as Van der Waals bond and hydrophobic interactions are also found between 13h and HDAC2 (residues HIS180, GLY306 and PHE 210), the same as 13k. These docking results are in agreement with those determined data.

Acknowledgments

We thank State Key Laboratory of Biotherapy, West China Hospital, Sichuan University for ^{1}H NMR, ^{13}C NMR and HRMS determination. We thank Engineering Teaching Experiment Center, School of Chemical Engineering for IR determination. We especially thank Assoc. Prof. Xiang Gao for finishing the molecular docking study.

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