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Discovery of Multi-target Anticancer Agents Based on HDAC Inhibitor MS-275 and 5-FU

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Abstract: Histone deacetylases (HDACs) inhibitors have multiple effects targeting the cancer cells and have become one of the promising cancer therapeutics with possibly broad applicability. Combination of HDAC inhibitors with the cytotoxic fluorouracil (5-FU) showed additive and synergistic effects both *in vitro* and *in vivo*. To explore the possibility in cancer therapy of a bivalent agent that combines two bioactive groups within a single molecular architecture, we designed and synthesized new dual-acting compounds by combining the bioactive fragment of MS-275, a clinical HDACs inhibitor, with cytotoxic agent 5-FU. The target compounds 9a and 9b showed comparable HDACs inhibition with MS-275 and moderate antiproliferative acitivities against six cancer cells lines.

Keywords: Anticancer, HDAC, MS-275, Multitarget, 5-Fluorouracil.

1. INTRODUCTION

Histone deacetylases (HDACs) are a family of hydrolytic enzymes that catalyze the removal of acetyl groups from the side chain of lysines in histone. Recent studies have reported that HDACs play a significant role in epigenetic control of gene expression. Aberrant histone acetylation is associated with the development of numerous malignancies [1-3]. HDACs are well conserved enzymes and there are 18 members of human HDACs which are classified into 4 classes according to their homology to yeast prototypes, subcellular localization and function: Class I (HDACs 1, 2, 3, and 8), II (HDACs 4, 5, 6, 7, 9, and 10), IV (HDAC 11), Class III HDACs (SIRT 1-7). All the members of Classes I, II, and IV HDACs are $zinc^{2+}$-dependent enzymes, whereas class III HDACs are NAD^{+}-dependent [4].

Over the past few years, over 490 clinical trials of more than 20 HDAC inhibitors (HDACIs) have been initiated, among which, MS-275 is an orally active synthetic benzamide derivative that functions as a selective inhibitor of primary class I (HDACs 1 and 3) [5-7]. MS-275 has been evaluated in multiple Phase I and II trials as therapy for advanced and/or refractory solid tumors and hematologic malignancies [8-13]. In the meanwhile, combinational therapy of MS-275 with other agents is now being further explored in preclinical and clinical trials, such as, exemestane [14], erlotinib [15], 5-azacitidine [16, 17], 13-cis-retinoic acid [18, 19], 5-fluorouracil [20] and so on.

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5-Fluorouracil (5-FU) is one of the clinical antitumor drugs most frequently used for treating a wide range of solid tumors, such as colorectal cancer, stomach and breast cancer. However, the clinical applications of 5-FU are subjected to great limitations because of its short plasma half-life, poor tumor selectivity and high incidences of toxicity in gastrointestinal tract, the bone marrow central, nerve system, skin and so on [21]. Therefore, to overcome these problems, a lot of novel 5-FU derivatives have been developed with high efficiency and much less toxicity, such as Floxuridine®, Carmofur®, Doxifluridine®, Capecitabine®, Atofluding® and so on [22, 23]. The common feature of these derivatives is that they are all N^1-modi ed or N^3-modi ed derivatives through different biodegradable linkers [24].

In recent study, Sylwia Flis, *et al.* have con rmed that combination of 5-FU with MS-275 could induce cell cycle perturbation and caspase-dependent apoptosis of colorectal carcinoma (CRC) cells [20]. Additionally, they also indicated that MS-275 synergistically potentiated cytotoxic effects of 5-FU in SW48, HT-29 and Colo-205 cell lines [20].

On the basis of these premises, following multi-target approach [25, 26], we designed and synthesized a novel multi-target antitumor agent **9a** by replacing the pyridine cap group of MS-275 with its bioisostere, the cytotoxic agent 5-FU (Fig. **1**). Compared with the parent compound MS-275, incorporation of the more hydrophilic 5-FU group might dramatically increase the solubility of compound **9a**. Moreover, the carbamate linker has been successfully used for prodrugs of norfloxacin [27], entacaponeo [28], pseudomycins [29] and so on, as this linker was labile and could be cleaved by the enzyme *in vivo*. Therefore, we assumed that compound **9a** could not only perform HDACs inhibition as a single molecule, but also act as a prodrug, of which the carbamate bond cleavage *in vivo* could release 5-FU and an-

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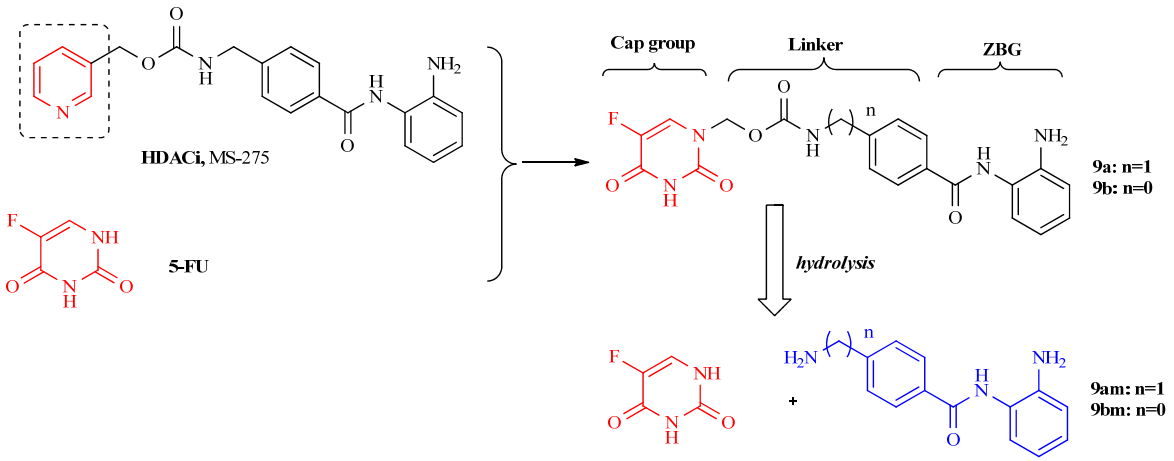
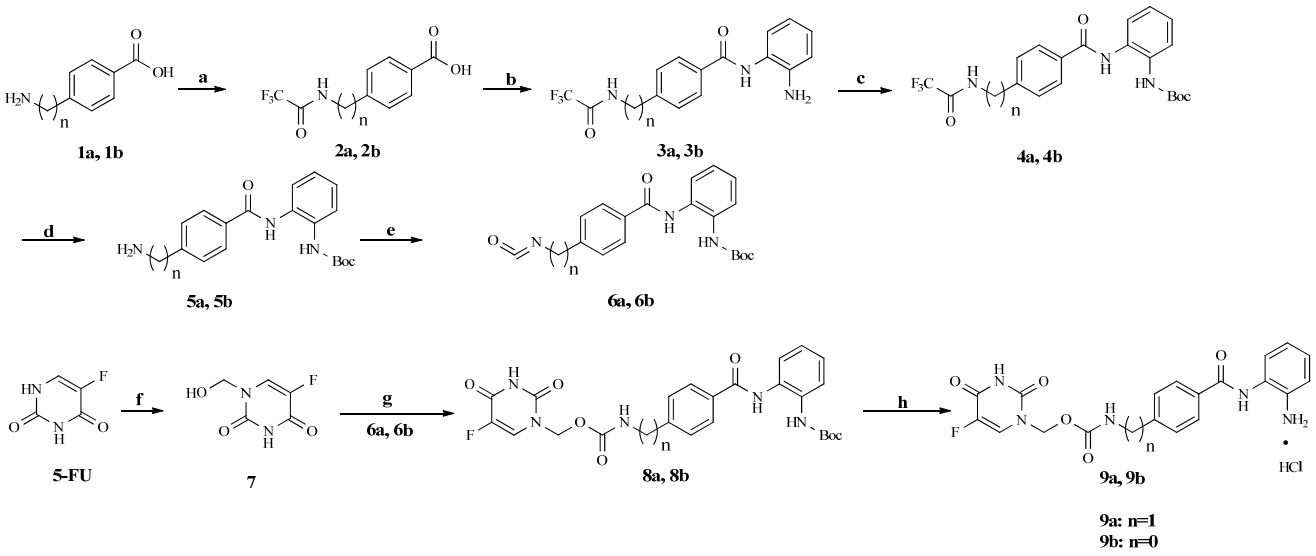


Fig. (1). Schematic representation of the design and chemical structure of **9a**, **9b**. To create **9a**, **9b** we introduced 5-FU with a flexible side chain onto the backbone of the HDAC inhibitor (MS-275).



[a]Reagents and conditions: (a) TFAA, 25°C, 2h; (b) TBTU, TEA, DCM. ; (c) $(Boc)_2O$, TEA, THF; (d) K_2CO_3, aqueous MeOH, 25°C, 10h ; (e) $NaHCO_3$, BTC, 2h; (f) 37% oxymethylene; (g) TEA, 1,4-dioxane; (h) dry HCl in EtOAc.

Scheme 1. Synthesis of Compound **9a**, **9b**[a].

other HDACs inhibitor 9am to exert synergistic antitumor effects (Fig. **1**). In order to investigate the effects of different linker length on compound property, compound 9b was designed and synthesized.

2. RESULTS AND DISCUSSION

2.1. Chemistry

Compounds **9a** and **9b** were prepared following the synthetic route illustrated in Scheme **1**. The starting materials **1a** (p-aminomethylbenzoic acid) and **1b** (p-aminobenzoic acid), were protected by trifluoroacetic anhydride (TFAA), followed by condensation reaction to give compounds **3a** and **3b**, respectively. Boc-protected products **4a** and **4b** were treated with K_2CO_3 in aqueous MeOH to afford the intermediates **5a** and **5b**, respectively. The isocyanates **6a** and **6b** were synthesized from **5a** and **5b** by reaction with triphosgene (BTC) in the presence of $NaHCO_3$. Synthesis of **7** was accomplished using **5-FU** in the presence of 37% oxymethylene. Reaction of **7** with **6a** and **6b** in the presence of TEA gave the compounds **8a** and **8b**. Subsequent deprotection gave the target products **9a** and **9b**.

2.2. HeLa Cell Nuclear Extract Inhibition of the Target Compounds 9a, 9b

We used HeLa cell nuclear extract as the HDACs enzyme source to efficiently screen our compounds 9a and 9b. In this assay, **MS-275** was used as positive control. The IC_{50} values (μmol/L) towards HeLa extract are shown in Table **1**. The results showed that the inhibitory activities of **9a** and **9b** were comparable with that of **MS-275**. The IC_{50} values were 5.92 ± 0.75, 2.31 ± 0.24 and 2.09 ± 0.11 μM for **9a, 9b** and **MS-275**, respectively, which indicated that replacement of the pyridine ring of **MS-275** with 5-FU almost had no effect to its inhibitory potency against HDACs.

Table 1. Inhibitory activities of 9a, 9b, and MS-275 against HDACs.

Compound	IC_{50} against HeLa Extract (μM)[a]
9a	5.92±0.75
9b	2.31±0.24
MS-275	2.09±0.11

[a]Assays were performed in three times; values are shown as mean ± SD.

2.3. Molecular Docking

In order to explore the interaction between our target compounds and HDAC, **9a** and **MS-275** [30, 31] were chosen to be constructed using a Sybyl/Sketch module. Fig. (**2**) showed the docked conformation of compounds **9a** and **MS-275**. The conformations showed in Fig. (**2a** and **2b**) demonstrated the docking modes of the two compounds were similar in the linker and ZBG fragment, while they seemed different in the pyrimidine ring and pyridine ring of cap group. Detailed interactions between compounds and HDAC3 were shown in Fig. (**2c**) (9a) and (**2d**) (MS-275). Comparing the two figures, we could find in the cap group, both 9a and MS-275 could not form hydrogen bonds with HDAC3. However, in the linker and ZBG fragment, 9a could form two hydrogen bonds with amide N−H of Asp104 and amide N−H of Gly154, while MS-275 could form three hydrogen bonds with amide N−H of Asp104, amide N−H of Gly154 and amide N−H of Asp181. Therefore, we postulated that the lost hydrogen bond with Asp81 might be the reason why 9a was less potent than MS-275.

2.4. Antiproliferative Activity Assay

Aiming to investigate the antiproliferative activities of **9a** and **9b**, we selected 6 types of hematological or solid tumor cell lines which were most frequently used in evaluating HDACs to test our compounds (Table **2** and Fig. **3**). Overall, **9a** and **9b** were less potent than MS-275 in the examined cell lines, which did not meet our expectations. We inferred that

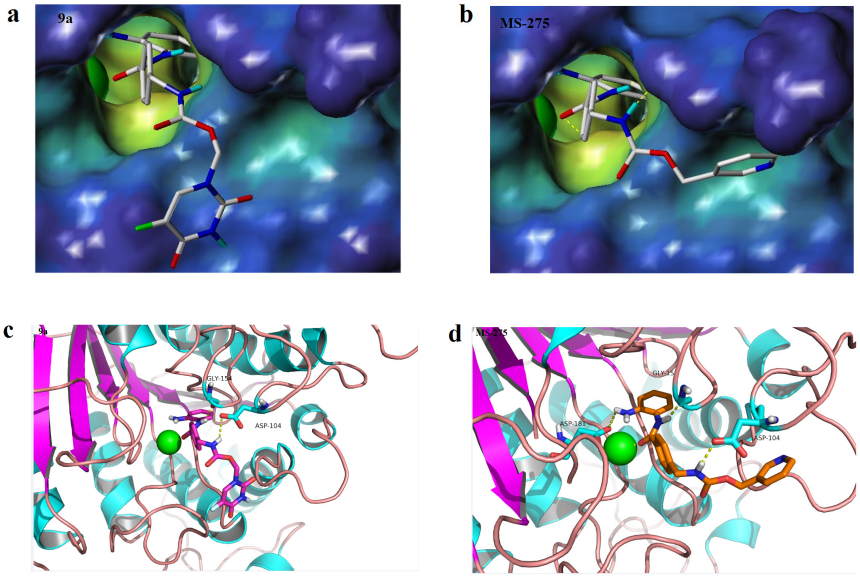


Fig. (2). (**a-d**) Proposed binding mode of compounds 9a (a,c) and MS-275 (b,d) with HDAC3. The green sphere is zinc ion, and the dashed lines represent the hydrogen bonds (atom types: H = white; N = blue; O = red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper).

Table 2. Antiproliferative activities of 9a, 9b, and MS-275 against six tumor cell lines (IC_{50} in μM[a]).

Cell Lines	9a	9b	MS-275
K562	5.70±1.06	7.83±0.64	1.02±0.25
A549	16.50±1.43	19.99±2.59	4.45±0.92
U266	7.48±1.01	8.54±0.87	1.54±0.63
PC-3	26.54±1.07	15.49±1.20	6.18±1.02
HCT-116	23.79±5.51	17.30±1.91	16.34±2.03
ES-2	53.74±5.71	15.47±2.43	4.98±0.89
HL-7702	100.20±16.32	ND[b]	30.43±3.86

[a]Assays were performed in three times; values are shown as mean ± SD. [b]Not determined.

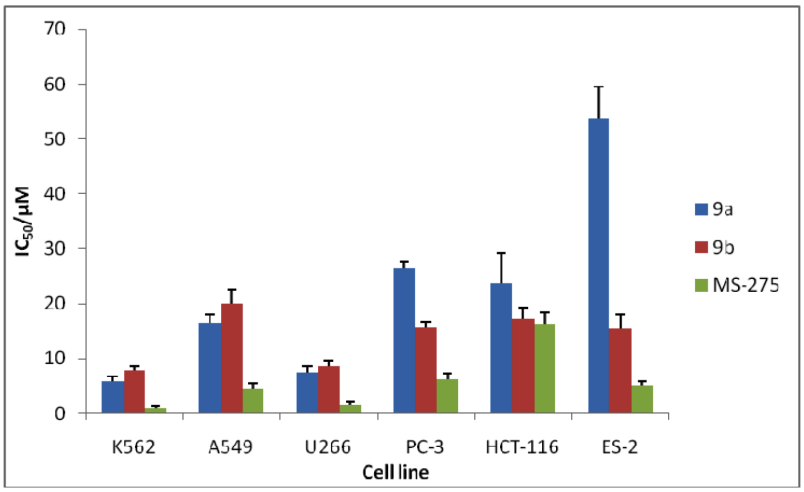


Fig. (3). Antiproliferative activities of **9a**, **9b**, and MS-275 against six tumor cell lines.

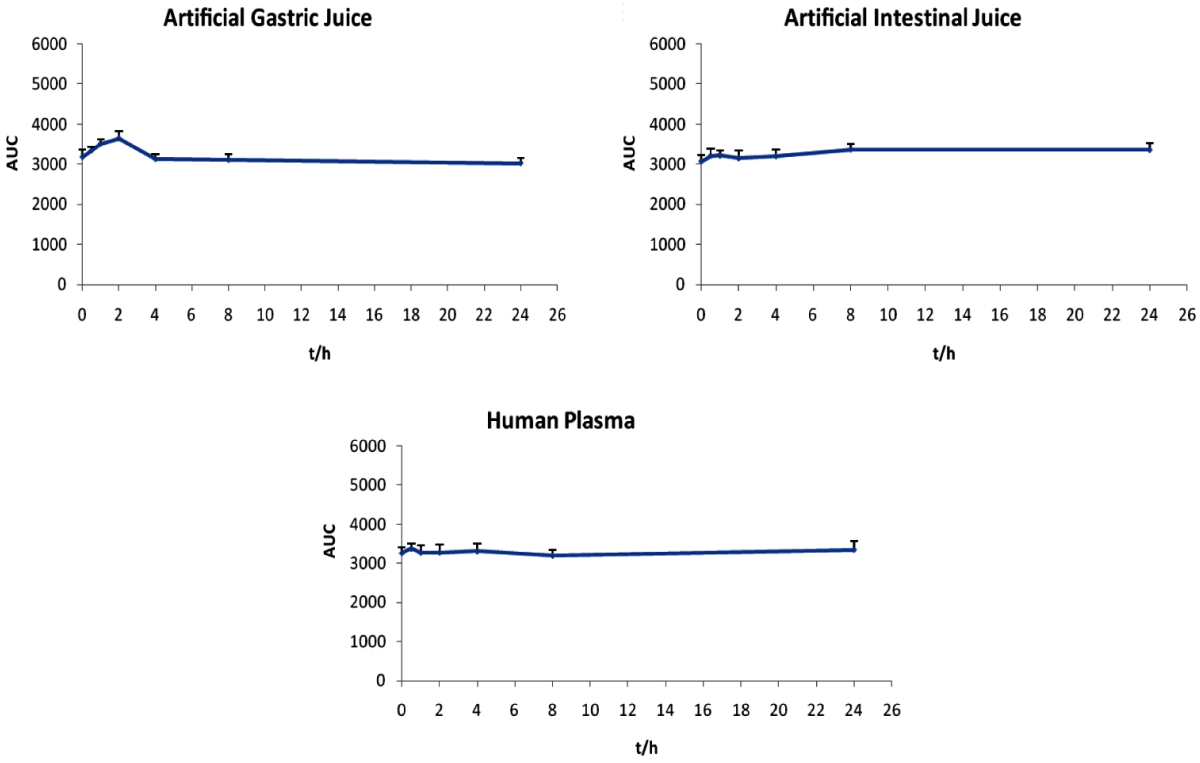


Fig. (4). Stability of compound **9a** *in vitro*. Points were achieved after 0, 0.5, 1, 2, 4, 8, and 24 h, respectively, and values are shown as mean + SD.

the modest potency of these two compounds only came from their HDACs inhibition as single molecules, and their carbamate bonds could not been hydrolyzed to release 5-FU and another HDACs inhibitor. However, MS-275 inhibited non-malignant HL-7702 cell, but **9a** had relatively lower cytotoxicity (Table **2**).

2.5. Stability of Compound 9a *in vitro*.

To validate our aforementioned inference, the stability study of 9a in arti cial gastric juice, arti cial intestinal juice, and human plasma was studied using a HPLC method. Brie y, the hybrid 9a was incubated into each condition at 37°C for 24 h. At predetermined time points, a sample of the mixture was precipitated, extracted and analyzed by HPLC. Unfortunately, **9a** was very stable in arti cial gastric juice, arti cial intestinal juice, and human plasma (Fig. **4**). This may explain why compounds **9a** and **9b** displayed less potent activities than MS-275 in *in vitro* antiproliferative assay. However, the stability *in vitro* could not be the evidence that **9a** would not release 5-FU *in vivo*, the pharmacokinetic study of **9a** *in vivo* is underway in our lab.

CONCLUSION

In this study, we designed and synthesized two dual-acting compounds 9a and 9b following multi-target approach. In HDAC inhibitory assay, 9a and 9b showed similar

HDAC inhibitory activity with MS-275. Though their *in vitro* antiproliferative activities were disappointing due to their unexpected *in vitro* stability and disability of releasing 5-FU, their detailed *in vivo* pharmacokinetic profiles deserve further investigation. Moreover, considering there were many successful examples of carbamate-based prodrugs, we hoped that structural modification of our compounds **9a** and 9b using medicinal chemistry methods could lead to promising analogues with ideal stability which could release 5-FU *in vitro* and *in vivo*. The proof of concept described in this research could also be used in other compound design and synthesis.

3. EXPERIMENTAL SECTION

3.1. Chemistry

All materials, reagents and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. All reactions were monitored *via* thin-layer chromatography with 0.25 mm silica gel plates (60GF-254), while the UV light was used to visualize the spots. The compounds were puri ed *via* column chromatography which was performed using silica gel or C18 silica gel. NMR spectra were recorded with a Bruker DRX spectrometer at 400 MHz, which use the TMS as an internal standard. High-resolution mass spectra were performed by Shandong Analysis and Test Center in Ji'nan, China. ESI-MS spectra were determined on an API 4000 spectrometer. Melting points were determined with an electrothermal melting point apparatus and were uncorrected.

3.1.1. General Procedure for the Preparation of 2a and 2b [32]

4-((2,2,2-trifluoroacetamido)methyl)benzoic acid (2a). Tri uoroacetic anhydride (5.9 mL, 41.3 mmol) was added in small portions to solid 4-(aminomethyl) benzoic acid (2.5 g, 16.5 mmol) at 4°C. Upon completion of addition, the reaction mixture was homogeneous. Stirring was continued at room temperature for 2 h, and then ice water was added to precipitate the product. The white solid **2a** was precipitated and collected by ltration. (3.3 g, 81% yield). mp:214-215ºC, ^{1}H-NMR(400 MHz DMSO-d_6): δ 4.47 (d, J = 6.0 Hz, 2H), 7.40 (d, J = 8.2 Hz, 2H), 7.94 (d, J = 8.2 Hz, 2H), 10.07 (s, 1H), 12.94 (s, 1H). ESI-MS m/z: 248.5 $[M + H]^+$.

3.1.2. General Procedure for the Preparation of 3a and 3b

N-(2-aminophenyl)-4-((2,2,2-trifluoroacetamido)methyl)benzamide (3a). To a solution of **2a** (2.5 g, 10 mmol) in anhydrous THF was added 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tet-ra uoroborate (TBTU, 3.5 g, 11 mmol), followed by TEA (1.5 mL, 11 mmol). After 30 min, 1,2-diaminobenzene (1.0 g, 9 mmol) was added. After 5h, the solution of THF was evaporated with the residue taken up in EtOAc. The organic phase was washed with 10% $NaHCO_3$ solution (3 × 30 mL), and brine (3 × 30 mL), dried over anhydrous sodium sulfate overnight, and the solvent was evaporated under vacuum. The crude product **3a** was puri ed by recrystallization with saturated chloride hydrogen in dry ethyl acetate to get a white pure hydrochloride solid (1.9 g, 56% yield).

3.1.3. General Procedure for the Preparation of 4a and 4b

tert-butyl-(2-(4-((2,2,2-trifluoroacetamido)methyl)benzamido)phenyl)carbamate(4a). The solution of **3a** (1.8 g, 5 mmol) in CH_2Cl_2 was added TEA (2.1 mL, 15 mmol) for neutralization, followed by the addition of $(Boc)_2O$ (2.2 g, 10 mmol) under ice bath. After 0.5h, the reaction mixture was stirred at 25ºC for another 12h. The resulting solution was washed by 1 M aqueous citric acid (3 × 30 mL), 10% $NaHCO_3$ solution (3 × 30 mL), and saturated brine (3 × 30 mL) and dried over anhydrous sodium sulfate overnight. After evaporating the solvent, the crude material **4a** was puri ed by ash chromatography to afford a white solid (1.7 g, 78% yield). mp: 162-164ºC, ^{1}H-NMR(400 MHz DMSO-d_6): δ 1.44 (s, 9H), 4.45-4.49 (m, 2H), 7.15-7.20 (m, 2H), 7.40-7.45 (m, 2H), 7.53-7.55 (m, 2H), 7.91-7.95 (m, 2H), 8.67 (s, 1H), 9.81 (s, 1H), 10.03-10.09 (m, 1H). ESI-MS m/z: 438.5 $[M + H]^+$.

3.1.4. General Procedure for the Preparation of 5a and 5b

tert-butyl-(2-(4-(aminomethyl)benzamido)phenyl)carbamate (5a). A solution of **4a** (1.3 g, 3 mmol) and K_2CO_3 (1.1 g, 7.5 mmol) in MeOH-H_2O (1:1) was maintained at 25°C for 10h. The solution was concentrated under reduced pressure. The EtOAc (3 × 40 mL) was added to the residue. The solution of EtOAc was washed with saturated $NaHCO_3$ solution (3 × 40 mL), and saturated brine (3× 40 mL), dried with anhydrous sodium sulfate over night, and evaporated under vacuum to obtain 0.8 g of white solid powder **5a** (0.8 g, yield: 78%).

3.1.5. General Procedure for the Preparation of 6a and 6b

tert-butyl-(2-(4-(isocyanatomethyl)benzamido)phenyl) carbamate (6a). To a solution of $NaHCO_3$ (0.4 g, 4.8 mmol) in H_2O was added CH_2Cl_2 and **5a** (0.7 g, 1.9 mmol), then the solution was cooled to 0°C. Triphosgene (0.3 g, 1.0 mmol) was charged in one portion. After stirred at room temperature for 1h, the organic phase was separated and the aqueous phase was extracted with CH_2Cl_2. The combined organic phase were washed with water and saturated brine, dried with anhydrous sodium sulfate, and evaporated under vacuum to give the **6a** as a liquid, which was used for next step directly.

3.1.6. General Procedure for the Preparation of 7 [33]

5-fluoro-1-(hydroxymethyl)pyrimidine-2,4(1H,3H)-dione (7). 5-FU (1.1 g, 8.5 mmol) was dissolved in 37% formaldehyde solution (1.53 g, 18.9 mmol) and the reaction mixture was kept at 60°C for 2 h. After evaporating the solvent, the residue was dried under vacuum to get colorless oil **7** which was directly used without further purification.

3.1.7. General Procedure for the Preparation of 8a and 8b

(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl-4-((2-tert-butyl-aminop-henyl)carbamoyl) benzylcarbamate (8a). The solution of **7** (0.5 g, 3 mmol) in 1,4-dioxane was added TEA (0.4 mL, 3 mmol) followed by the addition of **6a** (0.7 g, 2 mmol) under ice bath. The ice bath was removed. After stirred at 30°C for 5h, 1,4-dioxane was evaporated under vacuum and the residue was dissolve in

EtOAc (3 × 40 mL). The organic phase was washed with 1 M aqueous citric acid, saturated $NaHCO_3$ solution and saturated brine for 3 times, dried with anhydrous sodium sulfate over night, evaporated and the residue chromatographed, using CH_2Cl_2/EtOAc(1:3) as the mobile phase, to obtain 0.4 g of white solid **8a** (0.4 g, yield: 38%).

3.1.8. General Procedure for the Preparation of 9a and 9b

(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl-4-((2-aminopheny- l)carbamoyl)benzylcarbamate hydrochloride (9a). **8a** (0.4 g, 0.7 mmol)was dissolved in 20 mL saturated chloride hydrogen in dry ethyl acetate. Let the solution stirring at room temperature overnight. 0.3 g white powder **9a** was obtained after removing the solvent under vacuum. (0.3g, yield: 92%). mp:160-162ºC, ^{1}H-NMR(300 MHz DMSO-d_6): δ 4.29 (d, J = 6.0 Hz, 2H), 5.54 (s, 2H), 5.76 (s, 1H), 6.68 (t, J = 7.2 Hz, 1H), 6.84 (d, J = 7.2 Hz, 1H), 7.03(dt, J_1 = 1.2 Hz, J_2 = 8.1 Hz, 1H), 7.20 (d, J = 7.5 Hz, 1H), 7.39 (d, J = 8.1 Hz, 2H), 7.95 (d, J = 8.1 Hz, 2H), 8.12 (d, J = 6.6 Hz, 1H), 8.19 (d, J = 6.0 Hz, 1H), 9.68 (s, 1H), 11.98 (d, J = 4.2 Hz,1H). HRMS (AP-ESI) m/z calcd for $C_{20}H_{18}FN_5O_5$ $[M + H]^+$ 428.1685, found 428.1688.

Compound **2b-9b** was prepared following the general procedure as described above.

(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl) methyl-(4-((2-aminopheny-l)carbamoyl)phenyl)carbamate hydrochloride (9b) White powder, 87% yield mp:179-181ºC, ^{1}H-NMR(400 MHz DMSO-d_6): δ 5.68 (s, 2H), 7.35 (t, J = 7.6 Hz, 1H), 7.41 (t, J = 7.6 Hz, 1H), 7.47 (d, J = 7.6 Hz, 1H), 7.57 (d, J = 7.6 Hz, 1H), 7.63 (d, J = 8.6 Hz, 2H), 8.11 (d, J = 8.7 Hz, 2H), 8.21 (d, J = 6.5 Hz, 1H), 10.39 (s, 1H), 10.45 (s, 1H), 12.04 (d, J = 4.9 Hz, 1H). HRMS (AP-ESI) m/z calcd for $C_{19}H_{16}FN_5O_5$ $[M + H]^+$ 414.1359, found 414.1358.

3.2. *In Vitro* HDAC Inhibition Assay

In particular, 10 μL of enzyme solution (HeLa nuclear extract) was added to different concentrations of test compounds (50 μL) and incubated for 5 min at 37°C, then the specific fluorogenic substrate (Boc-Lys-(acetyl)-AMC) was used at 40 μL. Samples were incubated for 1 h at 37°C and stopped by the addition of 100 μL of 2 × HDAC developer in present of trypsin and TSA. After incubation for 20 min, the fluorescence intensity was detected with excitation-emission wavelengths of 390-460 nm, respectively. The HDAC inhibition ratios were calculated as a percentage of activity compared with the control group and the IC_{50} values for the test compounds were calculated using a regression analysis of the concentration/inhibition data.

3.3. Molecular Docking Analysis

The docking study of Compounds and the active site of HDAC3 were performed using Sybyl/FlexX module. Other docking parameters used in the program were remained the default values. The protein structure utilized was PDB code 4A69. During the first step, the protein structure was treated by removing water molecules, adding hydrogen atoms, regulating atom types, and assigning AMBER7 FF99 charges. Then, the protein structure was further optimized by performing a 100-step minimization process. The molecular structures were constructed using the Sybyl/Sketch module and optimized *via* Powell's method by the Tripos force field with convergence criterion set at 0.05 kcal/ (Å mol), and assigned charges with the Gasteiger–Hückel method.

3.4. *In Vitro* Antiproliferative Assay

All cell lines were grown in medium (RPMI1640) containing 10% FBS at 37°C in a 5% CO_2 humidified incubator. Cell proliferation assay was studied using the MTT ((3-[4, 5-dimethyl-2-thiazolyl]-2,5-diphe-nyl-2H-tetrazolium bromide)) method. Briefly, cells were seeded into a 96-well cell plate. After incubation for 12 h, different concentrations of compound sample were added in complete medium and incubated for a further 48 h. Then, a 0.5% MTT solution was added to each well and cultured for 4 h. After the media were removed, formazan formed from MTT were dissolved in 150 μL of DMSO. Absorbance was then measured using an ELISA reader at 570 nm.

3.5. Stability of Test Compound *in vitro*

Preparation of artificial gastric juice, artificial intestinal juice, and Human plasma were conducted as previously described [30]. Artificial gastric juice, artificial intestinal juice, and human plasma added to the stock solution of 9a (4 mg/mL in CH_3CN) and incubated at 37°C for 24 h. At scheduled times sample aliquots were collected and the enzymatic reaction was quenched by adding acetonitrile. The samples were extracted with 600 μL acetonitrile and were filtered (0.22 μm) after shocking 30s and centrifugation at 12,000 rpm for 10 min. Analytical HPLC was performed on Agilent 1200 HPLC instrument using a ODS HYPERSIL column (5 μm, 4.6 mm × 250 mm), compound was eluted with 22% acetonitrile/78% Phosphate Buffered Saline (PH3.0) over 20min. The absorbance was measured at 233 nm, the flow rate was 1 mL/min and the quantity of injection was 40 μM.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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