Design, Synthesis and Biological Evaluation of New HDAC1 and HDAC2 Inhibitors Endowed with Ligustrazine as a Novel Cap Moiety

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Introduction: Histone deacetylases (HDACs) represent one of the most validated cancer targets. The inhibition of HDACs has been proven to be a successful strategy for the development of novel anticancer candidates.

Methods: This work describes design and synthesis of a new set of HDAC inhibitors (7a-c and 8a, b) utilizing ligustrazine as a novel cap moiety, and achieving the pharmacophoric features required to induce the desired inhibition.

Results: The newly synthesized derivatives were evaluated for their potential inhibitory activity toward two class 1 histone deacetylases, namely HDAC1 and HDAC2. The tested ligustrazine-based compounds were more potent toward HDAC2 (IC50 range: 53.7–205.4 nM) than HDAC1 (IC50 range: 114.3–2434.7 nM). Furthermore, the antiproliferative activities against two HDAC-expressing cancer cells: HT-29 and SH-SY5Y were examined by the MTT assay. Moreover, a molecular docking study of the designed HDAC inhibitors (7a-c and 8a,b) was carried out to investigate their binding pattern within their prospective targets; HDAC1 (PDB-ID: 4BKX) and HDAC2 (PDB-ID: 6G3O).

Discussion: Compound 7a was found to be the most potent analog in this study toward HDAC1 and HDAC2 with IC50 values equal 114.3 and 53.7 nM, respectively. Moreover, it was the most effective counterpart (IC50 = 1.60 µM), with 4.7-fold enhanced efficiency than reference drug Geltinib (IC50 = 7.63 µM) against SH-SY5Y cells. Whereas, compound 8a (IC50 = 1.96 µM) was the most active member toward HT-29 cells, being 2.5-times more potent than Geltinib (IC50 = 4.99 µM). Collectively, these results suggest that 7a merits further optimization and development as an effective new HDAC1 lead compound.

Keywords: HDAC Inhibitors, Ligustrazine, Anticancer agents, In silico study, Synthesis

Introduction: Health and diseases are always governed by the highly intricate and regulated actions of a wide armory of transcription factors. The fundamental organic structure of DNA is the nucleosome, which consists of strands of DNA wrapped around a core of 8 histone proteins.1 The amino acid residues protruded from the ‘N- and ‘C- on the histone tail are modified by enzyme-driven post-translational acetylation, methylation, and phosphorylation.2,3 These modifications increase the accessibility of transcription factors to gene promoter regions. Deacetylation, demethylation, and dephosphorylation of histones have the opposite effect of decreasing access of transcription factors to promoter regions. Emerging data now implicate histone modification in the pathobiology of cancer and other diseases. Histone acetylation
is mediated by histone acetyltransferases, while acetyl groups are removed by histone deacetylases (HDACs). Recently, the use of HDACs as targeted agents has gained much popularity as a more safe and effective alternative for cancer therapy than classical agents.4–7 Because of the cellular overexpression of HDACs has been reported in the vast majority of human tumors, there is substantial interest to develop more selective and potent inhibitors for these enzymes.6,7

HDAC is known to have 18 isoforms classified into 4 classes (class I-IV) and among these 4 classes, mainly class I is proven to be associated with tumor proliferation.8 It is hypothesized that HDAC-isoform-selective inhibitors would have improved efficacy and minimal adverse effects; thus, isomorf-selective compounds have been developed. To date, few HDAC inhibitors have approved by FDA which include: Vorinostat (Zolinza®) and Romidepsin (Istodax®) for cutaneous T-cell lymphoma,9,10 Panobinostat (Farydak®) for multiple myeloma,11 in addition to Belinostat (Beleodaq®) and Chidamide (Epidaza®) both for use in peripheral T-cell lymphoma.12,13

Generally, the essential topological pharmacophoric features for HDAC inhibitors (HDACIs) contain four basic components. The first one is the zinc-binding group (ZBG) that coordinates the Zn2+ at the bottom of the enzyme cavity; the second one is a cap group (CAP) that interacts with the rim of the catalytic tunnel of the enzyme; the third one is a polar connection unit (CU, apparently dispensable for HDAC8 selective inhibitors) connecting to the fourth pharmacophore which is a linker (hydrophobic spacer) enabling the inhibitor to lie into the aforementioned tunnel (Figure 1).6

Ligustrazine (TMP, 2,3,5,6-tetramethylpyrazine) is originally a natural product extracted from Ligusticum chuanxiong Hort, a Chinese traditional medicinal herb, that is widely used as clinical medication for occlusive cerebrovascular diseases.14 Ligustrazine has recently been reported to be a well-validated adjuvant in the reversal of multidrug resistance (MDR) in tumor cells, in addition to its ability to inhibit growth, invasion and metastasis of tumor cells.15,16 Accordingly, ligustrazine has attracted considerable attention as a privileged scaffold in cancer drug discovery. In this context, several studies reported the development of different ligustrazine-based hybrids such as ligustrazine-curcumin hybrids,17,18 ligustrazine-terpenes hybrids19 and others.20,21

The aforementioned findings have inspired and guided us to design and synthesize a new set of HDAC inhibitors (7a-c and 8a,b) utilizing ligustrazine as a novel cap moiety, and achieving the pharmacophoric features required to induce the desired inhibition (Figure 1). The newly synthesized ligustrazine-based derivatives (7a-c and 8a,b) were evaluated for their potential inhibitory activity toward two class I histone deacetylases, namely HDAC1 and HDAC2. Furthermore, their antiproliferative activities against two HDAC-expressing cancer cell lines, colorectal adenocarcinoma (HT-29) and neuroblastoma (SH-SY5Y) cell lines, were examined by the MTT assay.

Materials and Methods

Chemistry

Melting points were determined on an electrothermal melting point apparatus (Stuart Scientific Co.) and were uncorrected. NMR spectra were recorded on a Bruker Avance III HD NMR spectrometer.1H spectrum was run at 400 MHz and 13C spectrum was run at 100 MHz in deuterated dimethylsulfoxide (DMSO-d6). Chemical shifts were expressed in ppm using the solvent peak as internal standard.22 All coupling constant (J) values were given in hertz. The abbreviations used are as follows: s, singlet; d, doublet; m, multiplet. Analytical thin-layer chromatography (TLC) on silica gel plates containing UV indicator was employed routinely to follow the course of reactions.
Synthesis of 3,5,6-Trimethylpyrazine-2-Carboxylic Acid 2

To a solution of TMP (5 g, 36.8 mmol) in water (200 mL), aqueous KMnO₄ solution (8.6 g KMnO₄; 150 mL water) was added dropwise at room temperature (r.t.) over about 60 min. Upon completion of the addition, the mixture was stirred at 50°C for 12 h, and then the warm reaction mixture was filtered and washed with hot water (300 mL, 90°C). The filtrate and washing liquid were combined, cooled to 0–5°C, and the pH was adjusted to 2.0 with concentrated hydrochloric acid. Extraction was performed with ethyl acetate (200 mL x 3), and the organic phase was treated hydrochloric acid. Extraction was performed with dichloromethane: methanol (20 mL) and the mixture was stirred for 2 h at r.t. Then, the corresponding acid (50 mmol) and trifluoroacetic acid (TFA, 2.4 mL) were added and stirred for additional 10 h at r.t. The reaction mixture was evaporated to afford the key intermediates 4a, b and 6 that used in the next step without further purification.

Synthesis of Key Intermediates 4a, b and 6

3,5,6-Trimethylpyrazine-2-carboxylic acid 2 (1.66 g, 10 mmol) was added to a suspension of 1,1-carbonyldiimidazole (CDI, 50 mmol) in dry tetrahydrofuran (THF, 20 mL) and the mixture was stirred for 2 h at r.t. Then, the corresponding acid (50 mmol) and trifluoroacetic acid (TFA, 2.4 mL) were added and stirred for additional 10 h at r.t. The reaction mixture was evaporated to afford the key intermediates 4a, b and 6 that used in the next step without further purification.

Synthesis of Target HDAC Inhibitors 7a-c and 8a,b

To a suspension of key intermediates 4a, b and 6 (5 mmol) in dry THF (10 mL), CDI (19 mmol) was added, and the mixture was stirred for 3 h at 60°C. The clear solution was cooled to r.t. then hydrazine hydrate hydrochloride or hydroxylamine hydrochloride or hydrazine hydrate hydrochloride (25 mmol) was added. The reaction mixture was stirred for 6 h then evaporated to remove THF, and the crude product was stirred in water for 1 h and filtered and dried. The residue was purified by column chromatography using dichloromethane: methanol (9:1) to furnish target compounds 7a-c and 8a,b.

N-(4-(Hydrazinyl)-3,5,6-Trimethylpyrazine-2-Carboxamide (7b)

Yield 60%, m.p. >250°C; ¹H NMR (DMSO-d₆, 400 MHz) δ ppm: 2.54 (s, 3H, 6-CH₃), 2.56 (s, 3H, 5-CH₃), 2.71 (s, 3H, 3-CH₃), 3.57 (bs, NH₂), 7.08 (s, 1H, NHCO), 7.18–7.76 (m, 4H, Ar-H), 10.38 (s, 1H, NHNO₂); ¹³C NMR (DMSO-d₆, 100 MHz) δ ppm: 21.45 (3-CH₃), 22.09 (5-CH₃), 22.57 (3-CH₃), 29.50 (CH₂CO), 119.50, 120.42, 129.83, 131.28, 131.93, 137.51, 138.11, 141.42, 149.62, 154.28, 167.60 (C=O), 169.63 (C=O).

N-(4-(Hydrazinyl)-3,5,6-Trimethylpyrazine-2-Carboxamide (7c)

Yield 52%, m.p. >250°C; ¹H NMR (DMSO-d₆, 400 MHz) δ ppm: 2.51 (s, 3H, 6-CH₃), 2.54 (s, 3H, 5-CH₃), 2.72 (s, 3H, 3-CH₃), 3.57 (s, 2H, CH₂CO), 4.39 (bs, NH₂), 7.08 (s, 1H, NHNO₂), 7.17–7.74 (m, 4H, Ar-H), 9.12 (s, 1H, NHNH₂), 10.03 (s, 1H, NHCO); ¹³C NMR (DMSO-d₆, 100 MHz) δ ppm: 21.45 (6-CH₃), 22.10 (5-CH₃), 23.20 (3-CH₃), 119.49, 120.41, 129.63, 129.83, 137.52, 138.07, 148.63, 149.62, 164.29 (C=O), 169.50 (C=O).

N-(4-(Hydroxycarbamoyl)phenyl)-3,5,6-Trimethylpyrazine-2-Carboxamide (8a)

Yield 52%, m.p. >250°C; ¹H NMR (DMSO-d₆, 400 MHz) δ ppm: 2.51 (s, 3H, 6-CH₃), 2.55 (s, 3H, 5-CH₃), 2.72 (s, 3H, 3-CH₃), 7.72 (s, 1H, NHNO₂), 7.18–7.76 (m, 4H, Ar-H), 9.10 (s, 1H, NHNO₂), 10.36 (s, 1H, NHCO).

N-(3-Hydrazinecarboxylphenyl)-3,5,6-Trimethylpyrazine-2-Carboxamide (8b)

Yield 45%, m.p. >250°C; ¹H NMR (DMSO-d₆, 400 MHz) δ ppm: 2.50–252 (3, 9H, 3(CH₃)), 4.49 (s, 2H, NH₂), 7.35–7.87 (m, 4H, Ar-H), 8.81 (s, 1H, NHNH₂), 9.69 (s, 1H, NHCO).

Biological Evaluations

Evaluation of Inhibitory Activity Against HDAC1 and HDAC2

All the newly synthesized ligustrazine-based derivatives (7a-c and 8a,b) were evaluated for their potential inhibitory activity toward HDAC1 and HDAC2 as the following. Ten microliters of diluted Trichostatin A was added to two of the positive control wells and to two of each of the sample wells. Trichostatin A eliminated all HDAC activity and was used as a control for generating the sample background values. 10 µL of diluted Assay Buffer was added to the positive control and sample wells that were not treated with Trichostatin A. Reactions were initiated after the addition of...
10 μL of HDAC substrate to all the wells being used including the standard wells. The final concentration of substrate was 200 μM in the wells. The plate was covered and incubated on a shaker for 30 min at 37°C. Then, the plate cover was removed and 40 μL of developer was added and incubated for 15 min at room temperature. Fluorescence was measured by spectrophotometry at an excitation wavelength of 340–360 nm and an emission wavelength of 440–465 nm. The average fluorescence of the Trichostatin-treated samples were subtracted from the average fluorescence of its corresponding samples to yield the corrected sample fluorescence (CSF). Finally, the HDAC activity was calculated using the following equation: HDAC Activity (nmol/min/mL) = [µM/30 min] × sample dilution. One unit is defined as the amount of enzyme that caused the formation of 1.0 nmol of deacetylated compound per minute at 37°C.

In vitro Antiproliferative Activity
Ligustrazine-based derivatives (7a-c and 8a,b) were evaluated for their antiproliferative potency toward colorectal (HT-29) and neuroblastoma (SH-SY5Y) cancer cell lines. Both cell lines were obtained from American Type Culture Collection (ATCC). Cells were cultured using DMEM (Invitrogen, Life Technologies) supplemented with 10% FBS (HyClone), 10 µg/mL of insulin (Sigma), and 1% penicillin-streptomycin. All of the other chemicals and reagents were from Sigma, or Invitrogen. Plate cells (cell density: 1.2–1.8 x 10⁴/well) in a volume of 100-µL complete growth medium + 100 µL of the tested compound per well in a 96-well plate for 24 h before the assay. Antiproliferative activity of compounds (7a-c and 8a,b) was evaluated following the protocol of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay, as reported previously.24–26

Molecular Docking
All molecular docking simulations were performed in the molecular operating environment (MOE).27 The crystal structures of HDAC1 and HDAC2 (PDB ID: 4BKX and 6G3O, respectively) were downloaded from the RCSB Protein Data Bank. The structures were chosen based on crystal structure resolution and 2D similarity of the co-crystallized ligand to the target compounds. Prior to docking simulations, the proteins were prepared according to the following protocol. All water molecules and ligands were removed, except for the Zn ion. Afterward, the hydrogen atoms were added and the protein structure was protonated using the Protonate 3D tool at a temperature of 300K, pH of 7.0 and ion concentration of 0.1 mol/L. The Site Finder tool was used to determine the protein–ligand binding site, which included 19 residues of the ligand-binding pocket. The 2D structures of the ligustrazine-based derivatives (7a-c and 8a,b) and their tautomers were constructed using the Builder tool and energy minimized under the MMFF94x force field and 0.05 rmsd gradient.

The docking method was verified by performing re-docking of the co-crystallized squaramide inhibitor28 from the HDAC2 structure (PDB ID: 6G3O). Under given docking parameters, the predicted dock pose retained the key interactions from the initial crystal structure (Figure 2), and heavy atom RMSD between the co-crystal and dock pose was 0.8784 Å, with most of the deviations occurring in the solvent-exposed region of the binding site.

Result and Discussion
Chemistry
The synthetic pathway adopted for the preparation of the key intermediates and target ligustrazine-based HDACIs (7a-c and 8a,b) is depicted in Scheme 1. Synthesis was initiated by oxidation of ligustrazine 1 with hot KMnO₄ solution to afford 3,5,6-trimethylpyrazine-2-carboxylic acid 2.19 Then, carboxylic acid 2 underwent coupling with p-aminobenzoic acid 3a, p-aminophenylactic acid 3b, and m-aminobenzoic acid 4 via 1’-carbonyldimidazole (CDI) to furnish the key intermediates 4a,b and 6, respectively. Then, the target ligustrazine-based HDACIs (7a-c and 8a,b) were obtained, with 45–60% yield, via coupling of key intermediates 4a,b and 6 with hydrazine hydrate or hydroxylamine by the use of CDI (Scheme 1).

HNNMR spectra of 7a and 7c, and 8b showed new peaks at δ 4.53, 4.39 and 4.49, respectively, equivalent to NH₂ protons.
but at δ 9.67, 9.12 and 8.81 represent NHCN2 consequently. The characteristic OH peak of 7c and 8a appeared down filed at δ 10.58 and 9.10, respectively. New other NH (NHOH) peaks of 7b and 8b appeared at δ 7.08 and 7.72, respectively. The mentioned new HNMR peaks confirmed the structure of 7a-c and 8 a and b, and reactional conversion of 4a and b to 7a-c and 6 to 8a and b.

**Biological Evaluation**

**Evaluation of Inhibitory Activity Against HDAC1 and HDAC2**

All the newly synthesized ligustrazine-based derivatives (7a-c and 8a,b) were evaluated for their potential inhibitory activity toward HDAC1 as well as toward HDAC2. The inhibitory activities were compared to Dacinostat, a clinically used standard HDAC inhibitor. Table 1 displays the half-maximal inhibitory concentration data (IC₅₀ values) for the examined compounds and Dacinostat towards HDAC1 and HDAC2.

The data displayed in Table 1 showed excellent to weak inhibitory activities against both HDAC1 and HDAC2. Investigation of the obtained IC₅₀ values revealed that the tested ligustrazine-based compounds were more potent toward HDAC2 (IC₅₀ range: 53.7–205.4 nM) than HDAC1 (IC₅₀ range: 114.3–2434.7 nM). In particular, compound 7a was found to be the most potent analog in this study toward HDAC1 and HDAC2 with IC₅₀ values equal 114.3 and 53.7 nM, respectively. In addition, compounds 7b and 8b displayed more potent HDAC2 inhibitory activity (IC₅₀ = 112.9 and 132.3 nM, respectively) than the standard drug Dacinostat (IC₅₀ = 112.86 nM). On the other hand, compounds 7c and 8a showed moderate inhibitory activity toward HDAC1 with IC₅₀ = 263.5 and 468.1 nM, respectively, whereas, compounds 7b and 8b exhibited weak HDAC1 inhibitory activity (IC₅₀ = 2434.7 and 1800.1 nM, respectively)

It is noteworthy that shifting of hydrazide ZBG from para (7a) to meta (8b) position resulted in a 15.7-fold and 2.1-fold...
worsening of effectiveness against HDAC1 and HDAC2, respectively. Analogously, extending the hydrophobic spacer from phenyl (7a) to benzyl (7c) moiety resulted in a reduced inhibitory activity toward both HDAC1 and HDAC2.

In vitro Antiproliferative Activity

Ligustrazine-based derivatives (7a-c and 8a,b) were evaluated for their antiproliferative potency toward two HDAC-expressing cancer cell lines;\textsuperscript{29,30} colorectal (HT-29) and neuroblastoma (SH-SY5Y) cell lines, utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [24]. The clinically used anticancer drug Gefitinib was co-assayed as a reference drug. The growth inhibitory activities of the tested ligustrazine-based derivatives against the two cancer cell lines were measured as IC\textsubscript{50} values (μM), which...
represent the compound concentration required to produce a 50% inhibition of cell growth after 48 h of incubation, compared to untreated controls, and listed in Table 2.

Investigations of the results of the MTT assay highlighted that the tested ligustrazine-based derivatives (7a-c and 8a,b) were more effective toward colorectal HT-29 cells than neuroblastoma SH-SY5Y cells, except compound 7a which possessed much enhanced growth inhibitory activity against SH-SY5Y (IC$_{50}$ = 1.60±0.04 µM) than HT-29 cells (IC$_{50}$ = 15.10±0.43 µM). Regarding the antiproliferative activity against colorectal HT-29 cells, compound 8a (IC$_{50}$ = 1.96±0.05 µM) was found to be the most active member in this study, being 2.5-times more potent than reference drug Gefitinib (IC$_{50}$ = 4.99±0.14 µM). Also, compounds 7b and 8b displayed higher antiproliferative activities (IC$_{50}$ = 4.57±0.13 and 3.25±0.09 µM, respectively) than reference drug Gefitinib in HT-29 cell line.

On the other hand, exploring the antiproliferative activity toward neuroblastoma SH-SY5Y cells revealed that compound 7a was the most effective counterpart (IC$_{50}$ = 1.60±0.04 µM), with 4.7-fold enhanced efficiency than Gefitinib (IC$_{50}$ = 7.63±0.21 µM) against SH-SY5Y cells. Moreover, compound 8a possesses higher activity to Ge$_{31}$ (IC$_{50}$ = 1.96±0.05 µM) was found to be the most effective counterpart (IC$_{50}$ = 15.10±0.43 µM). Regarding the IC$_{50}$ values equal 4.57±0.13, 6.11±0.17 and 3.25±0.09 µM, respectively.

**Molecular Modeling**

Comparison of HDAC1 and HDAC2 Sequences and Structure Overlap

The sequences of HDAC1 and HDAC2 are highly conserved (sequence identity: 93.8% and sequence similarity: 98.1%). Within 8 Å radius from the center of the active site, there is only 1 sequence difference: Ser263 (HDAC1) vs Ala268 (HDAC2), which does not cause significant structural changes in the backbone position of active site residues. Most structural deviation occurs in residues Asp104, His146, Phe155, His183, Tyr209, Phe210 according to HDAC2 numbering. Three-dimensional figures viewed from different angles (A, B, C) were grouped and depicted clearly in Figure 3. The resolution of the HDAC1 crystal structure (3 Å) is a limitation that does not allow comparing the structures with full confidence.

**Molecular Docking Studies**

The binding orientations of the ligustrazine-based derivatives (7a-c and 8a,b) and their possible tautomeric forms were predicted using MOE under default docking placement parameters, followed by the force field pose refinement stage and re-scoring by the Affinity dG scoring function. The capability of the chosen docking protocol to correctly rank the compounds by their binding affinity (scoring power) is illustrated in Figure 4. In the cases of both HDAC1 and HDAC2 docking, the most and least potent compounds were correctly ranked with sufficient difference in dock score (S). The overall correlation coefficient R$_{2}$ (0.61 and 0.74 for HDAC1 and HDAC2, respectively) is as well- or higher-performing than the recent evaluation of scoring power done by Huo et al$^{13}$ on a diverse set of protein-ligand complexes for 10 commercially available and academic docking programs. Together with the ability to correctly reproduce the binding pose of the known squaramide inhibitor (see details in Methods section), these findings indicate that the generated 3D ligand binding poses are trustworthy and can be used to explain the true protein–ligand binding interactions. The dock scores (S) and a list of the highly contributing

### Table 1 HDAC1 and HDAC2 Inhibitory Activities of the Newly Synthesized Ligustrazine-Based Derivatives 7a-c and 8a, b

| Compound | HDAC1 (nM) | HDAC2 (nM) |
|----------|------------|------------|
| 7a       | 114.3 ± 0.44 | 53.7 ± 0.05 |
| 7b       | 2434.7 ± 1.25 | 132.3 ± 0.12 |
| 7c       | 263.5 ± 1.27  | 205.4 ± 0.43 |
| 8a       | 468.1 ± 0.08  | 168.8 ± 0.13 |
| 8b       | 1800.1 ± 0.45 | 112.9 ± 0.09 |
| Dacinostat | 129.4 ± 0.07 | 136.3 ± 0.11 |

**Note:** Data were expressed as mean ± standard error (S.E.) of three independent experiments.

### Table 2 In vitro Antiproliferative Activities of Compounds 7a-c and 8a, b Against HT-29 and SH-SY5Y Cancer Cell Lines

| Compound | IC$_{50}$ (µM) |
|----------|----------------|
|          | HT-29 | SH-SY5Y |
| 7a       | 15.10 ±0.43 | 1.60 ±0.04 |
| 7b       | 4.57 ±0.13  | 67.00 ±1.91 |
| 7c       | 6.11 ±0.17  | 43.45 ±1.21 |
| 8a       | 1.96 ±0.05  | 4.27 ±0.12 |
| 8b       | 3.25 ±0.09  | 28.50 ±0.8 |
| Gefitinib | 4.99 ±0.14  | 7.63 ±0.21 |

**Note:** IC$_{50}$ values are the mean ± SD of three separate experiments.
interactions of the ZBG for each of the ligustrazine-based derivatives (7a-c and 8a,b) are presented in Tables 3 and 4.

The most active compound 7a demonstrated the best binding affinity with S equal −7.2309 (HDAC2) and −7.9092 (HDAC1) with similar dock poses in both proteins (Figure 5). The ZBG of 7a makes several strong ionic interactions with Zn$^{2+}$ ion, as well as numerous H-bonds with His145, His183, Tyr308, and Asp181 residues. The phenyl linker group is sandwiched between 3 aromatic residues Phe155, His183 and Phe210 by pi-pi stacking interactions. The ligustrazine cap moiety and amide linker are capable of pinching the Asp104 via a pi-cation and H-bond, respectively, thus resembling the known squaramide HDAC2 inhibitor.

The docking pose and binding interactions of compounds 7b, 7c, 8a, and 8b in HDAC2 are similar to that of 7a; however, they have less favorable interactions and lower dock scores, indicating a lower value of the free energy of binding for these compounds (Table 4). Generally, hydrazine ZBG-based derivatives show stronger ionic binding interactions,

![Figure 3](#) Comparison of HDAC1 (blue) and HDAC2 (purple) structures from different angles (A–C). Residues are labeled according to HDAC2 numbering, co-crystal ligand represented in stick view, Zn atom – as red bead. Images prepared in Discovery Studio Visualizer.

![Figure 4](#) Correlation between pIC$_{50}$ and S Score for target compounds in HDAC1 and HDAC2.
as well as the dock scores are higher for para-substituted aryl linker derivatives.

The comparison of the 3D ligand docking poses provides a possible explanation for the selectivity of 7b for HDAC2 over HDAC1 and the corresponding decrease in the estimated free binding energy. Although the sequences of HDAC1 and HDAC2 are highly conserved, the slight differences in the published crystal structures resulted in the 3D ligand binding poses of 7a, 7b, and 7c compounds to show different patterns when overlapped (Figure 6). In HDAC2, the complex of interactions with Zn$^{2+}$ ion is maintained by all three compounds, and the decrease of docking score is small, coming from slightly less favorable hydrophobic interactions. Whereas in HDAC1, the most

| Compound | IC$_{50}$ (nM) | Docking Score ($) | Bonding Interaction | Bond Length | Bond Type |
|----------|----------------|-------------------|---------------------|-------------|-----------|
| 7a       | 114.3286       | −7.9092           | Zn                  | 1.95        | Ionic     |
|          |                |                   | His140              | 3.14        | H-donor   |
|          |                |                   | Tyr308              | 2.63        | H-donor   |
| 7b       | 2434.738       | −5.2063           | Zn                  | 2.28        | Ionic     |
|          |                |                   | Gly149              | 1.84        | H-donor   |
|          |                |                   | His178              | 2.69        | H-donor   |
| 7c       | 263.3337       | −5.8862           | Zn                  | 1.93        | Ionic     |
|          |                |                   | Asp176              | 1.96        | Ionic     |
|          |                |                   | His178              | 2.28        | Ionic     |
| 8a       | 468.1426       | −5.7948           | Zn                  | 2.49        | Ionic     |
|          |                |                   | Asp176              | 1.96        | Ionic     |
|          |                |                   | His141              | 2.33        | Ionic     |
| 8b       | 1800.053       | −5.9971           | Zn                  | 1.86        | Ionic     |
|          |                |                   | Asp176              | 1.95        | Ionic     |
|          |                |                   | His178              | 2.28        | Ionic     |

| Compound | IC$_{50}$ (nM) | Docking Score ($) | Bonding Interaction | Bond Length | Bond Type |
|----------|----------------|-------------------|---------------------|-------------|-----------|
| 7a       | 53.70386       | −7.2309           | Zn                  | 1.86        | Ionic     |
|          |                |                   | His145              | 3.2         | H-donor   |
|          |                |                   | Tyr308              | 2.66        | H-donor   |
| 7b       | 132.273        | −6.9653           | Zn                  | 2.05        | Ionic     |
|          |                |                   | His145              | 2.6         | H-donor   |
|          |                |                   | Asp181              | 1.97        | Ionic     |
| 7c       | 205.3559       | −5.8036           | Zn                  | 1.83        | Ionic     |
|          |                |                   | Asp181              | 1.97        | Ionic     |
|          |                |                   | Tyr308              | 2.73        | H-acceptor|
| 8a       | 168.7801       | −6.3261           | Zn                  | 2.29        | Ionic     |
|          |                |                   | Asp181              | 1.97        | Ionic     |
|          |                |                   | His146              | 2.84        | H-donor   |
| 8b       | 112.8581       | −6.5720           | Zn                  | 1.89        | Ionic     |
|          |                |                   | Tyr308              | 2.81        | H-acceptor|
|          |                |                   | Asp181              | 1.97        | Ionic     |

Table 3 Docking Results and Characteristic of the Strongest Interactions of ZBG of Target Compounds Within HDAC1

Table 4 Docking Results and Characteristic of the Strongest Interactions of ZBG of Target Compounds Within HDAC2
favorable docking poses showed such a reorientation of the ligand in the binding site that the Pi-Pi stacking interactions with Phe155, His183, and Phe210 are conserved, but there is a significant loss in interactions of the ZBG, which is more critical for inhibitor potency.

A similar tendency appears when the 3D ligand binding poses for compounds 7a, 8a and 8b are overlapped (Figure 7). In the HDAC2 crystal structure, the ZBG orientation is well overlapped in the poses of 7a, 8a and 8b. Whereas in HDAC1, the docking results show that both 8a and 8b share the hydrophobic Pi-Pi stacking interactions of compound 7a; however, both fail to repeat the same interactions of the ZBG, which results in a higher penalty for the docking score and possibly explains why the changes in inhibition potency for these compounds in HDAC1 and HDAC2 are different.
Conclusion
In summary, herein we report the design and synthesis of a new set of HDAC inhibitors (7a-c and 8a, b) utilizing ligustrazine as a novel cap moiety. All the newly synthesized derivatives were evaluated for their potential inhibitory activity toward two class I histone deacetylases, namely HDAC1 and HDAC2. The tested ligustrazine-based compounds were more potent toward HDAC2 (IC_{50} range: 53.7–205.4 nM) than HDAC1 (IC_{50} range: 114.3–2434.7 nM). Compound 7a was found to be the most potent analog in this study toward HDAC1 and HDAC2 with IC_{50} values equal 114.3 and 53.7 nM, respectively. The SAR outcomes hinted that shifting of hydrazide ZBG from para (7a) to meta (8b) position resulted in a 15.7-fold and 2.1-fold worsening of effectiveness against HDAC1 and HDAC2, respectively. Analogously, extending the hydrophobic spacer from phenyl (7a) to benzyl (7c) moiety resulted in a reduced inhibitory activity toward both HDAC1 and HDAC2. Furthermore, the antiproliferative activities against two HDAC-expressing cancer cell lines; HT-29 and SH-SY5Y were examined by the MTT assay. Compound 7a was the most effective molecule (IC_{50} = 1.60 µM), with 4.7-fold enhanced efficiency than reference drug Gefitinib (IC_{50} = 7.63 µM) against SH-SY5Y cells. Whereas, 8a (IC_{50} = 1.96 µM) was the most active compound toward HT-29 cells, being 2.5-times more potent than Gefitinib (IC_{50} = 4.99 µM).

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Disclosure
The authors report no conflicts of interest in this work.

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Figure 7 Overlay of the 3D ligand binding poses of compounds 7a (purple), 8a (orange) and 8b (yellow) in the HDAC1 (A) and HDAC2 (B) ligand-binding sites. The interacting residues are shown in line view, Zn ion is shown as a sky blue sphere. Dotted lines are used to visualize the protein–ligand interactions. Image prepared in Discovery Studio Visualizer.
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