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Discovery of a Novel HDAC2 Inhibitor by a Scaffold-Merging Hybrid Query

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Abstract

Histone deacetylases (HDACs) are part of a vast family of enzymes with crucial roles in numerous biological processes, largely through their repressive influence on transcription, with serious implications in a variety of human diseases. Among different isoforms, human HDAC2 in particular draws attention as a promising target for the treatment of cancer and memory deficits associated with neurodegenerative diseases. Now the challenge is to obtain a compound that is truly selective to HDAC2 and down-regulate this enzyme, while most current HDAC2 inhibitors do not show selectivity and suffer from metabolic instability. In order to identify novel, and selective inhibitors for human HDAC2, we designed a shape-based hybrid query from multiple scaffolds of known chemical classes and validated it to be a more effective approach to discover diverse scaffolds than single-molecule query. The hybrid query-based screening rendered a hit compound with the N-benzylaniline scaffold which showed micromolar inhibitory activities against HDAC2, and its chemical structure is novel compared to known HDAC2 inhibitors. Notably, this compound shows the selectivity against the HDAC6, a Class II enzyme, thus has the potential to further develop into the class- and even isoform-selective inhibitors. Our present study supplies an useful approach to identifying novel HDAC2 inhibitors, and can be extended to the inquires of other important biomedical targets.

Graphical abstract

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CONFLICT OF INTEREST

The authors confirm that they do not have any conflicts of interest.

SUPPORTIVE/SUPPLEMENTARY MATERIAL

Supportive Material associated with this article can be found, in the online version, at the site of Bentham Science Publishers.

Keywords

shape-based screening; hybrid query; scaffold merging; HDAC2 inhibitor; N-benzylaniline

INTRODUCTION

Post-translational histone modifications are implicated in various cellular processes, including chromatin remodeling, gene expression, and DNA repair.¹ Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes responsible for the reversible acetylation/deacetylation of the ϵ -amino groups of lysine residues on histones as well as other cellular proteins.² Aberrant histone deacetylation has been shown to be associated with a variety of human diseases, including cancer, neurodegeneration, rheumatoid arthritis, and cardiac hypertrophy.³ Inhibition of HDACs could induce histone hyperacetylation, which is now believed to be a promising way to improve the treatment of these diseases.⁴ So far, eighteen human HDACs members including the NAD⁺-dependent sirtuins (class III, SIRT 1–7) and the Zn²⁺-dependent HDACs composed of Class I (HDACs 1, 2, 3, and 8), Class IIa (HDACs 4, 5, 7, and 9), Class IIb (HDACs 6 and 10), and Class IV (HDAC11) have been identified and categorized based on their structural homology to yeast proteins.⁵ Among them, Class I and II enzymes have been subject to intense research, whereas only recently Class III enzymes have been found to be implicated in proliferation control and Class IV enzyme (HDAC11) has been shown to regulate the balance between immune activation and immune tolerance in CD4⁺ T-cell.⁶ Moreover, current studies suggested that the Class I rather than the Class II HDACs were more significant in regulating cell proliferation.⁷ Class I HDACs (HDAC1, 2, 3 and 8) are mostly located within the nucleus and play an important role in cell survival and proliferation, and are ubiquitously expressed in all tissue types.⁸ Inside Class I, HDAC1 and HDAC2 are highly homologous proteins, and have crucial roles in human development and physiology, especially in the heart and central nervous system (CNS).⁹ It was recently shown that HDAC2, but not HDAC1, conferred therapeutic resistance towards the topoisomerase II inhibitor etoposide in PDAC cells by negative regulation of the pro-apoptotic BH3-only protein NOXA.¹⁰ Similarly, the depletion of HDAC2 rather than HDAC1 in the pancreatic cancer cell lines resulted in a marked sensitization towards the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).¹¹ In addition, HDAC2 has been found to play a key role as a negative regulator of long term memory formation. In mice with Alzheimer's symptoms, HDAC2 instead of other HDACs is overly abundant in the hippocampus where new memories are formed, and it was most commonly found clinging to genes involved in synaptic plasticity.^{3b, 3c} Therefore, these findings indicated that HDAC2 can be regarded as a promising target for the treatment of cancer and memory deficits associated with neurodegenerative diseases.

Since the isolation of trichostatin A (TSA) in the 1970's, several bioactive small molecules of natural or synthetic origin, have been investigated as HDACs inhibitors (HDACIs).¹² With few exceptions, these can be divided into a few structural classes including short-chain fatty acids, hydroxamic acids, benzamides, and cyclic peptides.¹³ They are designed as structural mimics of acetyl-lysine and often contain a zinc-binding group, a linker, and a cap group.⁵ With respect to the zinc-binding functionality, the hydroxamates and benzamides are quite common in HDACIs. Previous studies proved that the benzamides and hydroxamates show better potencies against class I HDACs. However, benzamides could cause side effects of neuroleptics such as tardive dyskinesia, which may be irreversible,¹⁴ while hydroxamate group was considered unattractive to be druggable compounds due to relatively short metabolic half-life, difficulties associated with its synthesis, and low stability.^{3a, 15} These deficiencies promoted us to discover new inhibitors of HDAC2, especially selective inhibitors with novel scaffolds, albeit it is difficult and challenging.

Although various virtual screening methods have been established to identify selective HDACs inhibitors to date,¹⁶ there were only few reports relating to the discovery of novel scaffolds. Thus, this letter focuses on a new screening tool for discovering HDAC2 inhibitors with scaffold diversity. The crystal structure of human HDAC2 (PDB ID: 3MAX¹⁷) provides an unprecedented opportunity for the rational inhibitor design. Interestingly, it shows an additional foot pocket adjacent to the zinc binding site which provides large space for structural modification (cf. Fig. (S1) in Supportive Material). To achieve this aim, we built a shape-based hybrid query and validated it by actives/decoys benchmarking set, which in turn was applied to search for novel HDAC2 inhibitors against our in-house screening databases collection. Subsequently, the top-ranked hits obtained were validated experimentally by specific bioassays. Finally, the structural novelties of confirmed hits were analyzed against known HDAC2 inhibitors, and molecular docking studies were further conducted to evaluate the structural requirements of these inhibitors (cf. Fig. (1)). The current shape-based hybrid query highlights the structural diversity of HDAC2 inhibitors and can provide guidance for further lead optimization for selective HDAC2 inhibitors.

MATERIALS AND METHODS

Preparation of Actives and Decoys

We searched the ChEMBL database¹⁸ to collect molecules with known binding affinities/functional activities to human HDAC2 isoform. A generic dataset of 1000 drug-like molecules from Schrodinger, LLC were employed as decoys. The initial structural curation, protonation, and partial charges assignment were conducted using MOE (Chemical Computing Group Inc.) package¹⁹. The molecules were further minimized by the Merck Molecular Force Field 94 (MMFF94)²⁰ subsequently. We used OMEGA²¹ (OpenEye Scientific Software, Inc.) to generate conformers with the parameters as follows: number of allowed conformations (nconfs) of 300, root-mean-square distance (RMSD) of 0.5 Å, and the energy window of 15.0 kcal/mol. The maximum number of conformations allowed per compound was set to a higher value to ensure the exhaustive conformational coverage. The

energy window is the threshold value used to discard high-energy conformers. The MMFF94 force field was also used during the process of conformation generation.

Shape-based Query Search

The shape-based query for the purpose of database mining was conducted within the Rapid Overlay of Chemical Structures²² (ROCS, OpenEye Scientific Software, Inc.) using the default Implicit-Mills force field. The molecules were scored using the TanimotoCombo score, which considers not only the shape complementarity but also the pharmacophoric features as defined inside the queries. The interpretation and comparison of results was performed using statistical metrics such as the Receiver Characteristic Operating Curve (ROC) along with the Area Under the Curve (AUC) values and the enrichment factors at the initial 0.5%, 1% and 2% of database screening. Once the queries, screening parameters and procedure were optimized and validated, we carried out the searches against our in-house chemical databases collection for novel and selective HDAC2 inhibitors.

Molecular Docking Protocol

The co-crystal complex of human HDAC2 (PDB code: 3MAX) was retrieved from the PDB database. The receptor was prepared by the standard protocol in Discovery Studio v2.5 (Accelrys Software Inc., USA). The water molecules were removed, and hydrogen atoms were added to the whole protein structure. The active site was defined as all residues within 10 Å of the cognate ligand. The molecular docking was carried out on GOLD 3.0.1,²³ using the default settings of 10 GA runs. The non-bond parameters were taken from the default cutoff values, i.e. 2.5 Å for hydrogen bonds and 4.0 Å for van der Waals interactions. When the top three solutions attained RMSD values of less than 1.5 Å, the docking calculation was terminated. We took the GoldScore fitness function as the default scoring function provided by GOLD, which takes into account factors such as hydrogen bonding interaction energy, van der Waals energy, and ligand torsion strain.

HDAC2 Inhibitory Activity Assay

The HDAC2 assay kit²⁴ was purchased from BPS Bioscience (San Diego, CA). The assays were carried out in black background. Each well contained a volume of 50 µL including 30 µL buffer (BPS Bioscience, Catalog No. 50031), 5 µL BSA (1 mg/mL, Sigma-Aldrich), 5 µL inhibitor (1000, 631, 398, 251, 158, and 100 µM), 5 µL HDAC2 (1.8 ng/µL, BPS Bioscience, Catalog No. 50002), and 5 µL HDAC substrate (200 µM, BPS Bioscience, Catalog No. 50037). Prior to adding substrate, the plate was pre-incubated at 37 °C for 30 minutes. Upon the addition of substrate the plate was incubated at the same temperature for another 30 minutes. The HDAC assay developer (50 µL, BPS Bioscience, Catalog No. 50030) was then added to each well and the plate was incubated for another 15 minutes. The fluorescence was measured at excitation and emission wavelengths of 360 nm and 460 nm, respectively. We had the negative control containing protein without inhibitors and the positive control with TSA inhibitor. In addition, the readout of blank well containing no inhibitor and protein was subtracted from each well in measurement. All the assays were performed in triplicate.

RESULTS AND DISCUSSION

Scaffold-Merging Hybrid Query Generation and Validation

Detailed analysis of the binding orientation of N-(2-aminophenyl) benzamide (LLX) in the co-crystal complex reveals important interactions formed within the HDAC2 active site, which assisted greatly in defining the pharmacophoric features during our hybrid query preparation. As being a metalloenzyme, the most relevant type of interaction for any HDACs inhibitor is the chelating interaction with the zinc ion (Zn^{2+}) in the active site. The carbonyl oxygen and the *ortho*- NH_2 functional groups on LLX constitute the bidentate chelating interactions with the zinc ion as indicated in Fig. (2A). Additionally, both the carbonyl oxygen and the *ortho*- NH_2 groups along with the amide group are involved in a network of hydrogen bond interactions with Tyr308, His145 and His146, respectively as shown in Fig. (2B). Also there are π - π stacking interactions between the benzamide ring and two phenylalanines (Phe155 and Phe210) as well as a hydrophobic interaction with the diphenyl ring.

The foremost step while carrying out shape-based virtual screening is the preparation of the reference or the query molecule. The ROCS is a shape-based superposition method wherein employs smooth Gaussian function to represent the molecular volume.^{22b} Since this is a rigid alignment, no conformers are generated thus it is important to start with a 'reasonable' structure for each compound that represents a putative binding mode, e.g. a set of docked ligands or a set of X-ray crystal structures. Notably, our hybrid query generated by an alignment of multiple active ligands of different structural classes may not only avoid losing potentially important information from single class but also enhance the chance for screening hits of structural diversity. In this study, the structure of LLX was extracted from the co-crystal complex and re-docked into the receptor to evaluate the effectiveness of our docking protocol (cf. Fig. (S2) in Supportive Material). After that, for enhancing the structural diversity, four known HDAC2 inhibitors of 5-[5-[[4-(3-aminophenyl)-1,3-thiazol-2-yl]amino]-5-oxopentyl]-N-hydroxy-1,2-oxazole-3-carboxamide, 6-[(2-fluoren-9-ylideneacetyl)amino]-N-hydroxyhexanamide, (2*E*,4*E*,6*R*)-7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxohepta-2,4-dienamide and 5-[5-[[4-(3-aminophenyl)-1,3-thiazol-2-yl]amino]-5-oxopentyl]-N-hydroxy-1,2-oxazole-3-carboxamide of phenylisoxazole, triazole, dihydroquinoxalin, and isoxazole scaffolds respectively were docked into the 3MAX due to the lack of their cocrystal complexes. Their top-ranked docking conformations that fit to the aforementioned binding mode of LLX were superimposed to build the hybrid query. For comparison purpose, the query of LLX itself was also built. As mentioned previously, important functional groups common to individual inhibitor class such as carbonyl, amino, hydroxyl, and aromatic/heteroaromatic rings on the query molecules with chelating interaction, hydrogen bond interaction, π - π stacking interaction as well as hydrophobic interaction were defined to be pharmacophoric features to prepare the query (cf. Fig. (3)). Although each of them could contribute to the binding affinities/functional activities of individual class at the active site, their weights should be different. Basically, the weight number was assigned to individual pharmacophoric feature according to the relevance of the interactions they form in the HDAC2 active site. As chelation of the metal ion being the most important interaction for inhibitors targeting

metalloenzymes, the chelating interaction by carbonyl oxygen and *ortho*-NH₂ groups were assigned with the highest weight, followed by hydrogen bond interaction and π - π stacking interaction, while the least weight being added to the hydrophobic interactions of aromatic/heteroaromatic rings. Likewise, the query of LLX was prepared using similar procedures (cf. Fig. (3)).

After the preparation of queries, their validations were carried out by screening against multiple benchmarking sets comprised of known actives (molecules with reported binding affinity values against human HDAC2) and decoys (drug-like small molecules but non-binders). Validation step helps in ascertaining the ability of the query to screen out and score the true actives higher than the decoys at the ranking list, ensuring a robust and rigorous screening process. For this purpose, we searched ChEMBL database thoroughly for known HDAC2 inhibitors as actives, with the emphasis on spanning available structural scaffolds. A generic dataset of 1000 drug-like molecules from Schrodinger LLC constitutes as decoys. Specifically, three different validation sets were prepared depending upon the selection of structural scaffolds of active molecules found in the database (cf. Fig. (S3) in Supportive Material). Validation Set A consisted solely of 29 actives of benzamide scaffolds, with their binding affinities ranging from 0.007 μ M to 5.8 μ M. Validation Set B consisted of 28 actives of non-benzamide scaffolds, like isoxazoles, triazoles, dihydroquinoxalins and ketones, whereas Validation Set C consisted of a combination of 50 actives of both benzamides and non-benzamide scaffolds. The prepared three validation sets were then screened against by our hybrid and single queries. The comparison of results was performed using classical statistical metrics such as the Receiver Operating Characteristic (ROC) curves, the corresponding Area Under the Curve (AUC) values and initial enrichment at 0.5%, 1% and 2%.²⁵ For Set A, both the hybrid- and single-molecule queries showed an AUC value greater than 0.994 and high enrichment factor values, which indicated that both queries can distinguish the active benzamides from drug-like decoys (cf. Fig. (S4) in Supportive Material). However, for Sets B and C that involve diverse scaffold actives, the LLX query only showed an AUC value of 0.666 and low enrichment factor values, while the hybrid query performed so well that it ranked the same analogues higher than the decoys with an AUC value 0.800 as well as high enrichment factors (cf. Fig. (4)). These validation results confirmed that, in comparison to single-molecule query, our hybrid query showed marked improvement in “scaffold hopping” toward novel HDACs inhibitors of diverse core structures, which made it ready to the practical screening effort for novel HDAC2 inhibitors.

Database Screening and Experimental Validation

Once the hybrid query was validated, it was employed to screen against our in-house chemical databases collection including the TimTec Diversity Screening Set 10K. The screening hits were ranked by the combo scores. The scored hit list was visually inspected, and the hits did show sufficient structural diversity to each other. The top-ranked ten hits (cf. Fig. (S5) in Supportive Material) were subsequently validated by running the primary and secondary fluorogenic HDAC2 based assays,²⁶ where their inhibition curves and IC₅₀ values were determined. Among them, ST088357 showed moderate inhibitory activity against HDAC2 with an IC₅₀ value of 16.87 μ M (cf. Fig. (5)). Interestingly, a series of analogues of ST088357 have been reported in 2010 to show potent neuroprotective activity

through blocking the interaction of neuronal nitric oxide synthase (nNOS) with postsynaptic density protein-95 (PSD-95) specifically.²⁷ This information indicated that the neuroprotective mechanism of ST088357 and its analogues may include not only blocking the interaction of nNOS-PSD-95 but also inhibiting the HDAC2 activity.

From the perspective of structural novelty, the chemical scaffold of ST088357 is fairly different from most known HDAC2 inhibitors, which was confirmed by similarity calculations using Functional Class Fingerprints 6 (FCFP_6) molecular fingerprint²⁸ and MACCS 166-bit structural key²⁹, respectively. As shown in Fig. (5) and Table S1, on the top of all 309 known HDAC2 inhibitors we collected from ChEMBL database CHEMBL211625 showed the maximum similarities of 0.307692 to ST088357 by the FCFP_6 molecular fingerprint while CHEMBL180911 was 0.5 by the MACCS structural key. Although some of the top-ten similar compounds from the FCFP_6 and MACCS calculations also embed a N-benzylaniline subunit, they belong to benzamide derivatives. Clearly, their structures are fairly different from that of ST088357 in both 1D fingerprint and 3D conformations (cf. Figs. (S5) and (S6) in Supportive Material). In comparison, we conducted the FCFP_6 and MACCS fingerprint based searching by LLX as well as the ROCS screening using single-molecule query of LLX, ST088357 could not be located from the hit list, which further confirmed that our hybrid query exhibits greater advantage in scaffold hopping.

We also measured the inhibition rate of ST088357 against human HDAC1, HDAC2 and HDAC6, in an effort to explore the selectivity profile of this hit compound. As mentioned in the Introduction, HDACs 1, 2 belong to the Zn²⁺-dependent Class I HDACs while HDACs 6 and 10 are in Class IIb. We chose HDAC6 as the representative isoform from Class II, to distinguish first whether or not ST088357 is class-selective. It has also been heavily pursued in recent years among all isoforms in this class as a promising target for a variety of diseases³⁰. Notably, at the concentration of 100 μ M ST088357 showed the inhibition percentage of 34.28% to HDAC6 thus is regarded to be inactive to this particular isoform, and likely to the Class II as well. This is normally true for the primary bioassay, in which the inhibition rate is measured at the single concentration in multiple times. If the rate is smaller than 50%, the compound is deemed to be inactive and there is no need to move further for the IC₅₀ measurement. To HDAC1, the inhibition rate is 86.90%, the similar range of the rate for HDAC2 (80.55%). It indicates that ST088357 is non-selective to HDACs 1 and 2, which is not surprised considering the high homolog between these two isoforms in whole sequences, and particularly the active sites. Nevertheless, it provides us a valuable hit compounds for hit-to-lead and lead optimization, to further improve its potency and selectivity.

The binding pattern of ST088357 toward HDAC2 was analyzed by molecular docking (cf. Fig. (6)). The compound posits well into the active site, with three hydrogen bonds detected: one between the -NH group and Gly154, a residue in the vicinity of the entrance; the other two were from one of *meta*-substituted chlorine atoms on the phenyl ring to His145 and Gln 265, two residues on the hydrophobic pocket near the Zn²⁺ ion. It is noteworthy that Gly154 and His145 are the same residues that has been identified interacting with the -NH and -NH₂ groups of LLX in 3MAX, respectively. Additionally, the π - π stacking interaction

between the phenyl ring substituted with a dimethylamino group and two phenylalanine residues (Phe155 and Phe210) as well as their hydrophobic interaction may also impart the inhibitory activity of ST088357. However, one disadvantage of this compound is its weak Zn^{2+} chelating functionality, which is thought to be an important interaction for HDACIs, and this could explain why ST088357 alone showed moderate inhibitory activity against HDAC2 and can be utilized for lead optimization. Moreover, both the foot pocket described above and the large pocket on the entrance (cf. Fig. (S7) in Supportive Material) can provide space for the structural modification of ST088357.

CONCLUSION

In conclusion, our scaffold-merging hybrid query was built based upon the ROCS method, and it was found to be more suitable to identify novel HDAC2 inhibitors compared to single-molecule query by our designed benchmarking actives/decoys sets. Searching our in-house database collection resulted in the discovery of lead compound ST088357 with a novel N-benzylaniline scaffold. It showed moderate inhibitory activity against HDAC2, and its scaffold is quite diverse compared to known HDAC2 inhibitors, which can also be confirmed by similarity calculations based on FCFP_6 and MACCS molecular fingerprints. Finally, the molecular docking was carried out for the binding pattern analysis of ST088357, which not only helped in better understanding of the relevance of function groups of ST088357 but also suggests the potential space involving the zinc ion and remaining pockets for further lead optimization. Our present work here supplies an efficient approach in the identification of novel HDAC2 inhibitors, and the ST088357 can be regarded as an ideal lead for further modifications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

HDACs	histone deacetylases
HDACIs	HDACs inhibitors
HATs	histone acetyltransferases
CNS	central nervous system
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand

TSA	trichostatin A
LLX	N-(2-aminophenyl) benzamide
ROCS	Rapid Overlay of Chemical Structures
ROC	Receiver Operating Characteristic
AUC	Area Under the Curve
Tc	Tanimoto coefficient
FCFP_6	Functional Class Fingerprints 6
RMSD	root-mean-square distance

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Fig. (1).

Overview of our hybrid query based screening workflow employed in the present study.

Fig. (2).

(A) Chelating interaction formed between benzamide inhibitor (LLX) and the zinc ion (red) present in the human HDAC2 active site. Both carbonyl oxygen and *ortho*-NH₂ groups form bi-dentate covalent interactions with the zinc ion. (B) Hydrogen bond interactions between *ortho*-NH₂ group and His145/His146; carbonyl oxygen and Tyr308; π - π stacking interactions between the benzamide ring and Phe155/Phe210.

Fig. (3).

(Top) The hybrid query derived from multiple known HDAC2 inhibitor classes, which includes Hydroxamate phenylisoxazole, Triazole, Hydroxamate and Isoxazole; **(Bottom)** The single molecule query generated from LLX in 3MAX, comprised of carbonyl oxygen, *ortho*-N act as anions, H-bond acceptor and donor respectively.

Fig. (4).

The screening performance of the hybrid query (**Left**, AUC=0.813) in comparison to single-molecule query (**Right**, AUC=0.666) against the designed actives and decoys set C.

Fig. (5).
(Top) The inhibitory curves of ST088357 against human HDAC2 and its IC_{50} value;
(Middle) The most similar compounds to ST088357 from known HDAC2 inhibitors measured by the highest Tanimoto coefficient (Tc) calculated by MACCS and FCFP_6 fingerprints; **(Bottom)** The inhibition rate (%) of ST088357 against human HDAC1, HDAC2, and HDAC6 at 100 μM.

Fig. (6).

The complexes of ST088357 bound to human HDAC2. The residues close to the ligand with the cutoff distance of 8 Å were rendered by PyMOL software.