

Spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives as candidate HDAC8 inhibitors: a molecular docking and ADMET analysis using the HDAC8–inhibitor complex (PDB 2V5X)

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Abstract

Histone deacetylase 8 (HDAC8) represents a promising therapeutic target for Cornelia de Lange syndrome and certain cancers. This study investigates novel spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives as HDAC8 inhibitors through molecular docking using the 2V5X HDAC8–inhibitor crystal structure, with preliminary cytotoxicity assessment of related compounds. Lead compounds KB-282 and KB-281 yielded docking scores of – 12.3 and – 11.0 kcal/mol, respectively, exceeding the docking scores obtained for the established HDAC8-selective reference inhibitors under identical conditions. Structure-activity relationship analysis identified critical pharmacophoric elements: carboxamide/carbothioamide groups at position 1 of the indoline ring and electron-withdrawing substituents on the phenyl ring. Preliminary cytotoxicity assessment of related compounds (KB-268, KB-270, KB-292) was consistent with the computational ranking, with KB-270 exhibiting moderate cytotoxic activity (IC_{50} 34.88–49.69 μ M) while lower-binding compounds showed minimal activity (IC_{50} >50 μ M). Unlike conventional hydroxamate inhibitors, these compounds employ π -interactions with aromatic residues (Phe152, Phe208, Tyr306) and electrostatic interactions with Asp101 rather than zinc chelation. This non-hydroxamate mechanism may confer advantages in isoform selectivity and could reduce metal-binding toxicity relative to conventional hydroxamate inhibitors. *In silico* ADMET analysis revealed favorable physicochemical properties and predicted toxicity profiles. These computational and experimental findings provide initial rationale for further development of spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives as selective HDAC8 inhibitors for treating HDAC8-associated disorders.

1. INTRODUCTION

Histone deacetylases (HDACs) are enzymes that remove acetyl groups from lysine residues in histones and non-histone proteins, thereby regulating gene expression and a broad range of cellular processes. Aberrant HDAC activity contributes to diverse pathological states, including cancer, neurodegenerative disorders, and autoimmune diseases, motivating the search for selective inhibitors [1]. Among the 18 human HDAC isoforms identified to date, HDAC8, a class I HDAC, has emerged as a particularly promising target due to its distinct structural features and association with specific pathologies, including Cornelia de Lange syndrome and certain cancers [2].

1.1. HDAC8 as a therapeutic target

HDAC8 exhibits several structural and functional characteristics that distinguish it from other class I HDACs, providing the molecular basis for selective inhibitor design. The enzyme possesses a characteristic L1 loop configuration that participates directly in substrate recognition through specific interactions with substrate residues [3, 4]. Unlike other class I HDACs, HDAC8 has a more open and conformationally flexible substrate-binding channel, which allows accommodation of a broader range of substrates [4]. Crystallographic and computational studies have revealed conformational flexibility in HDAC8 that mediates substrate recognition and catalytic function [5]. Mutations in HDAC8 are associated with Cornelia de Lange syndrome, highlighting its clinical relevance.

HDAC8 deacetylates several physiologically relevant substrates, including structural maintenance of chromosomes protein 3 (SMC3), where HDAC8-mediated deacetylation regulates cohesion dynamics during cell division [2]. The enzyme also deacetylates estrogen-related receptor alpha (ERR α), thereby modulating its transcriptional activity [6], and has been implicated in p53 regulation, though the precise mechanisms require further investigation [1].

1.2. Limitations of current HDAC inhibitors

The development of HDAC inhibitors (HDACi) has progressed over the past two decades, with several compounds receiving FDA approval for various malignancies. Current HDACi can be classified into several structural categories based on their zinc-binding groups, each exhibiting distinct potency and selectivity profiles [1, 7].

Hydroxamate-based inhibitors represent the most extensively studied class, characterized by potent zinc-chelating capacity and broad-spectrum inhibitory activity. Notable examples include trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA/Vorinostat), panobinostat (LBH-589), and belinostat [8–10]. While effective, these compounds often exhibit limited selectivity across HDAC isoforms due to the high conservation of the catalytic zinc site.

Benzamide-based inhibitors, including MS-275 (Entinostat) and mocetinostat, generally exhibit greater selectivity for class I HDACs compared to hydroxamates but still lack HDAC8 specificity [11]. Cyclic peptides such as romidepsin and apicidin offer distinct binding properties but face similar selectivity challenges [12, 13].

These limitations across multiple inhibitor classes have motivated efforts toward isoform-selective inhibitor design. PCI-34051 represents a highly selective HDAC8 inhibitor with minimal activity against other HDAC isoforms, demonstrating selective apoptosis induction in T-cell lymphomas [14]. NCC149 exploits unique structural features of the HDAC8 active site, achieving notable selectivity through a distinctive binding mode [15].

Despite these advances, current HDAC8-selective inhibitors face several limitations. Most rely on zinc chelation mechanisms, which can lead to metal-binding toxicity and off-target effects. Additionally, the achievable selectivity ratios may still be insufficient to avoid undesired side effects in clinical applications.

1.3. Rationale for the spiro[indoline-tetrazolo[1,5-c]quinazoline] scaffold

Structure-activity relationship (SAR) studies combined with molecular docking have proven valuable for evaluating biological activity in heterocyclic compound development, as demonstrated by prior investigations of spiro[1, 2, 4]triazolo[1,5-c]quinazoline derivatives using CB-Dock2 blind docking platform [16].

The 2V5X crystal structure, an HDAC8–hydroxamate inhibitor complex reported by Vannini et al. [17] alongside the companion HDAC8–substrate complex (2V5W), provides a well-resolved template for structure-based drug design. In that work, the substrate complex revealed the previously unrecognized role of Asp101 at the active-site rim in positioning the substrate, and a comparable Asp101 interaction was observed in the inhibitor complex. The inhibitor-bound 2V5X structure therefore captures the catalytic site in a ligand-occupied conformation while retaining the Asp101 contact relevant to inhibitor design.

The role of Asp101 in active-site recognition, characterized across this structural series [17], involves hydrogen bonding to the bound ligand and offers an opportunity to enhance inhibitor affinity and, potentially, isoform selectivity. Targeting this Asp101 contact, rather than relying solely on zinc chelation, provided the rationale for the present docking study.

Accordingly, in this study, we investigate the binding potential of novel spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives (Fig. 1, Table 1) to HDAC8 through molecular docking using the 2V5X HDAC8–inhibitor structure.

Table 1

Chemical nomenclature of reference compounds and novel spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives (KB series) evaluated for HDAC8 binding potential.

Compounds	Chemical name
Octanediamide	((2S)-N8-hydroxy-2-[[[(5-methoxy-2-methyl-1 <i>H</i> -indol-3-yl)acetyl]amino)-N ¹ -[2-(2-phenyl-1 <i>H</i> -indol-3-yl)ethyl]octanediamide)
NCC149	<i>N</i> -hydroxy-3-(1-((phenylthio)methyl)-1 <i>H</i> -1,2,3-triazol-4-yl)benzamide
Entinostat	pyridin-3-ylmethyl <i>N</i> -[[4-[(2-aminophenyl)carbamoyl]phenyl]methyl]carbamate
PCI-34051	<i>N</i> -hydroxy-1-(4-methoxybenzyl)-1 <i>H</i> -indole-6-carboxamide
p53-derived	<i>N</i> -Acetyl-L-arginyl-L-histidyl- <i>N</i> 6-acetyl-L-lysyl- <i>N</i> 6-acetyl-L-lysine
KB-268	6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazolin]-2-one
KB-269	<i>N</i> -(4-chlorophenyl)-2-(2-oxo-6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazolin]-1-yl)acetamide
KB-270	<i>N</i> -(3-chlorophenyl)-2-(2-oxo-6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazolin]-1-yl)acetamide
KB-271	2-(2-oxo-6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazolin]-1-yl)- <i>N</i> -(2-(trifluoromethyl)benzyl)acetamide
KB-272	6'-(2-chloroacetyl)-6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazolin]-2-one
KB-280	2-oxo- <i>N</i> -phenyl-6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazoline]-1-carbothioamide
KB-281	2-oxo- <i>N</i> -(3-(trifluoromethyl)phenyl)-6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazoline]-1-carboxamide
KB-282	<i>N</i> -(4-cyanophenyl)-2-oxo-6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazoline]-1-carboxamide
KB-283	2-oxo- <i>N</i> -propyl-6' <i>H</i> -spiro[indoline-3,5'-tetrazolo[1,5-c]quinazoline]-1-carboxamide
KB-292	5-methyl-5-(4-nitrophenyl)-5,6-dihydrotetrazolo[1,5-c]quinazoline

This approach was selected to identify compounds that might achieve selectivity by exploiting unique features of the substrate-enzyme complex rather than relying solely on zinc chelation. Such non-hydroxamate inhibitors could potentially avoid the metal-binding toxicity issues associated with traditional HDAC inhibitors [18] while maintaining high potency through optimized interactions with key residues such as Asp101 that are crucial for substrate recognition.

Our computational approach incorporates multiple reference compounds with experimentally verified binding data, representing diverse chemical scaffolds: a hydroxamate inhibitor (octanediamide) from the original 2V5X structure, a class I-selective benzamide (entinostat), established HDAC8-selective inhibitors (PCI-34051 and NCC149), and the co-crystallized p53-derived peptide substrate (Fig. 1, Table 1). This analysis aims to identify SAR and binding determinants that could guide the further development of selective HDAC8 inhibitors based on the spiro[indoline-tetrazolo[1,5-c]quinazoline] scaffold, with potential applications in treating HDAC8-associated disorders.

2. Materials and methods

2.1. Molecular docking

2.1.1. Protein template and preparation

The crystal structure of human HDAC8 in complex with a hydroxamate inhibitor (PDB: 2V5X, resolution 2.25 Å) served as the docking template [17]. Crystallographic water molecules were removed, missing hydrogen atoms were added, and side-chain conformations were optimized using standard preparation protocols. The co-crystallized inhibitor was retained during initial preparation to preserve the native binding-site architecture and was removed immediately prior to docking.

2.1.2. Ligand preparation

Nine novel spiro[indoline-3,5'-tetrazolo[1,5-c]quinazoline] derivatives (KB-268-272, KB-280-283) were docked alongside five reference compounds: the HDAC8-selective inhibitors PCI-34051 and NCC149, the class I HDAC inhibitor entinostat, the hydroxamate inhibitor octanediamide co-crystallized with 2V5X, and the p53-derived substrate peptide taken from the companion HDAC8–substrate complex (2V5W). Three-dimensional structures were generated and energy-minimized using RDKit as implemented in CB-Dock2; partial charges were assigned by the Gasteiger method and rotatable bonds were identified automatically.

2.1.3. Docking validation

Protocol reliability was verified by redocking co-crystallized ligands from multiple crystal structures of HDAC8 and related HDAC isoforms using the CB-Dock2 template-based docking protocol: 2V5X (octanediamide), 6HSG and 6HRG (NCC149), 6HSF and 6HQY (PCI-34051), and 3MAX and 6UOC (entinostat). Pose quality was assessed by root-mean-square deviation (RMSD) relative to the crystallographic conformation, with RMSD < 2.0 Å applied as the acceptance criterion [19]. For octanediamide redocked into its parent 2V5X structure, the template-based procedure in CB-Dock2 recovered the co-crystallized pose essentially exactly (RMSD 0.00 Å); this outcome is expected when the query ligand and its binding pocket derive from the same PDB entry used as the template, and is therefore reported here as an internal consistency check rather than an independent validation. Cross-validation is provided by the remaining six redocking experiments, in which the ligand–protein pairs differed from the template source. NCC149 redocked into 6HSG and 6HRG produced RMSD values of 0.49 Å and 0.32 Å, respectively. PCI-34051 redocked into 6HSF and 6HQY gave RMSD values of 0.30 Å and 0.36 Å. Redocking of entinostat into 3MAX yielded an RMSD of 1.25 Å, whereas redocking into 6UOC yielded 2.14 Å, marginally exceeding the acceptance threshold. Five of the six independent redocking experiments thus satisfied the RMSD < 2.0 Å criterion, supporting the reliability of the template-based docking protocol for subsequent virtual screening.

2.1.4. CB-Dock2 blind docking

Blind docking calculations were performed with CB-Dock2 [20, 24], which integrates structure-based and template-based pipelines in an automated workflow. Binding cavities were identified using CurPocket, a protein-surface-curvature algorithm approximately 4–5 times faster than its predecessor. Template matching searched the BioLip database (version 2021.09.15; 214,506 filtered protein–ligand interactions) using FP2 fingerprint similarity (threshold 0.4); when templates were found, the query protein was superposed to template structures for FitDock-based docking. Structure-based docking applied AutoDock Vina scoring. For each ligand, docking was performed simultaneously into the five highest-ranked cavities; pockets sharing more than 50% of binding residues were merged, otherwise treated independently. Binding scores (kcal/mol) and box dimensions for all five pockets are reported in Supplementary Material, S1; a compact score matrix across all 15 compounds and five pockets is provided in Supplementary Material, S2.

2.1.5. Binding site and interaction analysis

Pocket volumes, center coordinates, and amino acid compositions were extracted from CB-Dock2 CurPocket output files. The frequency of each amino acid residue type in Pocket 1 across all compounds is summarized in

Supplementary Material, S3. Protein–ligand interactions were characterized using Discovery Studio Visualizer 2017 R2 (Dassault Systèmes BIOVIA) with contact distances evaluated according to CASP criteria (sum of van der Waals radii plus 0.5 Å tolerance). Interaction types recorded included conventional and carbon–hydrogen bonds; parallel and T-shaped π – π stacking; π – σ , π –alkyl, π –anion, halogen, and π –sulfur contacts. The detailed residue-level interaction profile of KB-282 versus octanediarnide in both template and blind docking modes is presented in Supplementary Material, S4. Complete amino acid residue listings (Chain A and Chain B) for all five binding pockets across all 15 compounds are provided in Supplementary Material, S5; a summary of the top-5 most frequent residue types per pocket per compound appears in Supplementary Material, S6.

2.2. *In silico* ADMET prediction

Physicochemical descriptors and ADMET parameters were predicted with admetSAR 3.0 [25]. SlogP was calculated using the RDKit algorithm; logP was obtained from the CLMGraph deep-learning model. Predicted parameters are reported across seven supplementary tables. Absorption and distribution parameters—Caco-2 permeability (log units and probability class), human intestinal absorption (HIA), MDCK permeability, oral bioavailability at three thresholds (F20%, F30%, F50%), blood–brain barrier (BBB) permeability, plasma protein binding (PPB), and steady-state volume of distribution (VDss)—are presented in Supplementary Material, S7. Transporter interaction probabilities—inhibition and substrate classification for OATP1B1, OATP1B3, OATP2B1, OCT1, OCT2, BCRP, BSEP, MATE1, and P-glycoprotein—are provided in Supplementary Material, S8. Metabolism and excretion parameters—CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 inhibition and substrate probabilities, human liver microsomal (HLM) and rat liver microsomal (RLM) stability, UGT substrate probability, plasma clearance (CLp), renal clearance (CLr), elimination half-life ($T_{1/2}$), and mean residence time (MRT)—appear in Supplementary Material, S9. Toxicity parameters—hERG channel inhibition at IC^{50} thresholds of 1, 10, and 30 μ M; drug-induced liver injury (DILI); respiratory toxicity; nephrotoxicity; eye and skin irritation and corrosion; skin sensitization; Ames mutagenicity; rodent carcinogenicity; micronucleus genotoxicity; reproductive, mitochondrial, hemolytic, and repeated-dose toxicity; and acute oral toxicity—are compiled in Supplementary Material, S10. Endocrine disruption potential across the Tox21 nuclear receptor and stress-response panel (AR, ER, AR-LBD, ER-LBD, aromatase, AhR, ARE, ATAD5, HSE, p53, PPAR γ , MMP, TR, GR) is reported in Supplementary Material, S11. Ecotoxicity endpoints—aquatic toxicity to algae (*P. subcapitata*), crustaceans, *D. magna*, fish (fathead minnow, bluegill sunfish, rainbow trout, sheepshead minnow), and *T. pyriformis*; avian toxicity to *C. virginianus* and *A. platyrhynchos*; bee toxicity; bioconcentration factor (BCF); biodegradability; and photoinduced toxicity—are presented in Supplementary Material, S12. Full physicochemical descriptors—molecular weight, atom count, heteroatom count, ring count, rotatable bonds, HBA, HBD, TPSA, SlogP, logS, logP, and pKa values—are listed in Supplementary Material, S13 (admetSAR 3.0; SlogP from RDKit, logP from CLMGraph).

2.3. Cytotoxicity assessment

These data originate from a parallel investigation of the anticancer potential of tetrazolo[1,5-c]quinazoline derivatives and are included here as preliminary experimental validation. Cytotoxic activity of KB-268, KB-270, and KB-292 was evaluated at the Molecular Design Centre, Danylo Halytsky Lviv National Medical University, Ukraine, using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma-Aldrich, USA). The following cell lines were used: MCF-7 (RRID:CVCL_0031), HCT-116 (RRID:CVCL_0291), Jurkat (RRID:CVCL_0065), and J774.2 (RRID:CVCL_0357). All cell lines were verified as mycoplasma-free prior to experimental use. Adherent tumor cells (6,000 per well) were seeded in 96-well plates in 100 μ l DMEM or RPMI-1640 medium (Sigma-Aldrich) per ATCC recommendations and allowed to attach overnight. Compounds were added at concentrations of 1, 10, and 50 μ M in duplicate and incubated for 72 h at 37°C in 5% CO₂. The medium was removed, MTT reagent was added to a final concentration of 5 mg/ml, and cells were incubated for a further 4 h. Formazan crystals were dissolved in DMSO and

absorbance was measured at 570 nm (TECAN Infinite M Plex microplate reader). IC₅₀ values were determined by four-parameter logistic regression in GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

2.4. Statistical analysis

Structure–activity relationships were analyzed for the nine novel derivatives (KB-268-272, KB-280-283) using Pocket 1 AutoDock Vina binding scores as the dependent variable and admetSAR 3.0 descriptors as independent variables. Pearson correlation coefficients (*r*) between binding affinity and individual physicochemical properties (SlogP, logP, TPSA, MW, HBA, HBD, BBB permeability, HIA) were calculated with significance assessed at $\alpha = 0.05$ using the *t*-distribution (*df* = 7; Supplementary Material, S14). Quantitative SAR analysis by structural feature (substitution group means, standard deviations, ranges, and improvement relative to the unsubstituted baseline) is presented in Supplementary Material, S15. Physicochemical property distributions (mean $\pm$ SD, median, quartiles, interquartile range) for the nine novel compounds are summarized in Supplementary Material, S16. Amino acid residue frequency in Pocket 1 across all 15 compounds and a comparison of top- and bottom-performing compounds by residue counts are provided in Supplementary Material, S17 and S18, respectively.

A multiple linear regression model incorporating SlogP, TPSA, and HBA as predictors was constructed; model significance was evaluated by *F*-test ($R^2 = 0.797$, $F = 6.55$, *df* = 3,5, $p = 0.035$). Differences in binding affinity among substitution-pattern groups were evaluated with the Kruskal–Wallis test (*df* = 3), which is appropriate because two groups comprise single compounds; pairwise comparison of electron-withdrawing versus electron-donating substituents employed Welch's *t*-test with Satterthwaite degrees of freedom. Owing to the presence of singleton groups, Kruskal–Wallis results should be interpreted descriptively rather than inferentially. Drug-likeness was assessed according to Lipinski's Rule of Five and Veber criteria, with composite drug-like scores derived from weighted molecular descriptor combinations; results are tabulated in Supplementary Material, S19. Statistical test outcomes (Kruskal–Wallis, Welch *t*-test, Pearson correlations, and regression model summary) are reported in Supplementary Material, S20. Outlier identification used standardized residuals ($|z| > 2.0$) and Cook's distance ($D > 0.5$); results are presented in Supplementary Material, S21. All statistical computations were performed using SciPy (Python 3.12).

A comparative ADMET profile of the two lead spiro compounds (KB-281, KB-282) against reference HDAC8 inhibitors, including absorption, distribution, hERG, and DILI parameters, is presented in Supplementary Material, S22. Pairwise toxicity profile comparison and drug–drug interaction (DDI) assessment based on cumulative CYP inhibition probability scores are provided in Supplementary Material, S23 and S24, respectively.

2.5. Synthesis of substances analyzed for cytotoxicity

2.5.1. General

Melting points were determined in open capillary tubes in a «Mettler Toledo MP 50» apparatus (Mettler-Toledo, Greifensee, Switzerland), and were uncorrected. The elemental analyses (C, H, N) were performed using the vario EL Cube analyzer (Elementar Americas, Mount Laurel, NJ, USA). Analyses were indicated by symbols of the elements or functions within $\pm 0.3\%$ of the theoretical values. ¹H NMR spectra (400 MHz) and ¹³C NMR spectra (125 MHz) were recorded on a Varian-Mercury 400 (Varian Inc., Palo Alto, CA, USA) spectrometer with TMS as an internal standard in DMSO-*d*₆ solution. LC-MS was recorded using a chromatography/mass spectrometric system that consists of high-performance liquid chromatography «Agilent 1100 Series» (Agilent, Palo Alto, CA, USA) equipped with a diode-matrix and mass-selective detector «Agilent LC/MSD SL» (atmospheric pressure chemical ionization–APCI). Electron impact mass spectra (EI-MS) were recorded on a Varian 1200 L instrument at 70 eV (Varian, Palo Alto, CA, USA). The purity (> 95% pure) of obtained compounds was checked by ¹H, ¹³C-NMR, and LC-MS. Starting materials and solvents were obtained from Enamine Ltd. (Kyiv, Ukraine) and used without additional purification.

2.5.2. Procedure and spectral data

General procedure for the synthesis of KB-268 and KB-270.

A quantity of 1.0 g (6.2 mmol) of 2-(1*H*-tetrazol-5-yl)aniline, prepared by hydrolytic cleavage of the tetrazoloquinazoline precursor [26], was added to a suspension of (*N*-aryl)indoline-2,3-dione (6.2 mmol) in 15 mL of acetic acid. The resulting mixture was refluxed for 1 h. After cooling to r.t. the crystalline precipitate was filtered, dried, and recrystallized from propan-2-ol.

KB-268, (6'*H*-spiro[indoline-3,5'-tetrazolo[1,5-*c*]quinazolin]-2-one.

¹H NMR (400 MHz, DMSO-*d*₆): δ 10.77 (s, 1H, NH indol.), 8.01 (s, 1H, NH quinaz.), 7.88 (d, *J* = 7.7 Hz, 1H, H-4 indol.), 7.44 (m, 2H, H-10 quinaz., H-7 indol.), 7.35 (t, *J* = 7.9 Hz, 1H, H-5 indol.), 7.12 (t, *J* = 7.6 Hz, 1H, H-6 indol.), 7.04 (d, *J* = 7.8 Hz, 1H, H-7 quinaz.), 6.98–6.83 (m, 2H, H-8,9 quinaz.). IR (cm⁻¹): 1716, 1621, 1474, 749. LC-MS: *m/z* = 291 [M + H]⁺. Anal. Calcd. For C₁₅H₁₀N₆O: C, 62.06; H, 3.47; N, 28.95; O, 5.51. Found: C, 62.09; H, 3.45; N, 28.91; O, 5.55.

KB-270, (*N*-(3-chlorophenyl)-2-(2-oxo-6'*H*-spiro[indoline-3,5'-tetrazolo[1,5-*c*]quinazolin]-1-yl)acetamide.

¹H NMR (400 MHz, DMSO-*d*₆): δ 10.48 (s, 1H, NH indol.), 8.18 (s, 1H, NH quinaz.), 7.89 (d, *J* = 7.7 Hz, 1H, H-10 quinaz.), 7.79 (m, 1H, H-4 indol.), 7.55 (m, 2H, Ph-2,6), 7.44 (d, *J* = 8.3 Hz, 1H, H-7 quinaz.), 7.36 (t, *J* = 7.7 Hz, 1H, H-6 indol.), 7.23 (m, 2H, Ph-5, H-5 indol.), 7.03 (m, 2H, H-8 quinaz., H-7 indol.), 6.92 (m, 2H H-9 quinaz., Ph-4), 4.57 (q, *J* = 16.9 Hz, 2H, CH₂). IR (cm⁻¹): 1726, 1678, 1616, 1471, 748, 682. LC-MS: *m/z* = 458 [M + H]⁺. Anal. Calcd. For C₂₃H₁₆ClN₇O₂: C, 60.33; H, 3.52; N, 21.41; O, 6.99. Found: C, 60.35; H, 3.49; N, 21.44; O, 6.95.

General procedure for the synthesis of KB-292.

A quantity of 1.0 g (6.2 mmol) of 2-(1*H*-tetrazol-5-yl)aniline and 1.03 g (6.2 mmol) of 1-(4-nitrophenyl)ethan-1-one were dissolved in 15 mL of propan-2-ol with the addition of 1 drop of sulfuric acid. The resulting mixture was refluxed for 8 h. After cooling to r.t. the crystalline precipitate was filtered, dried, and recrystallized from methanol/water mixture.

KB-292 (5-methyl-5-(4-nitrophenyl)-5,6-dihydrotetrazolo[1,5-*c*]quinazoline).

¹H NMR (400 MHz, DMSO-*d*₆): δ 8.26 (s, 1H, NH), 8.13 (d, *J* = 8.5 Hz, 2H, Ph-3,5), 7.76 (d, *J* = 7.7 Hz, 1H, H-10 quinaz.), 7.41 (d, *J* = 8.5 Hz, 2H, Ph-2,6), 7.35 (t, *J* = 7.8 Hz, 1H, H-8 quinaz.), 7.06 (d, *J* = 8.2 Hz, 1H, H-7 quinaz.), 6.88 (t, *J* = 7.5 Hz, 1H, H-9 quinaz.), 2.35 (s, 3H, CH₃). IR (cm⁻¹): 1621, 1518, 1348, 1111, 853, 802, 776, 755, 698. LC-MS: *m/z* = 309 [M + H]⁺. Anal. Calcd. C₁₅H₁₂N₆O₂: C, 58.44; H, 3.92; N, 27.26; O, 10.38. Found: C, 58.48; H, 3.89; N, 27.29; O, 10.34.

Their IR, ¹H NMR, and LC-MS spectra are given in Supplementary Material, Tables S25-S27.

3. RESULTS AND DISCUSSION

3.1. Molecular docking studies

3.1.1. Computational method validation

The CB-Dock2 docking protocol was validated through comparing the redocked co-crystallized ligands in their respective HDAC8 structures. As depicted in the computational workflow (Fig. 1S, Supplementary Material), and

reported in Section 2.1.3, five of the six independent redocking experiments satisfied the $\text{RMSD} < 2.0 \text{ \AA}$ criterion; only entinostat in 6UOC marginally exceeded the threshold.

The success of this validation confirms that the CB-Dock2 algorithm accurately reproduces experimentally determined binding conformations and provides a reliable foundation for the evaluation of novel spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives. This approach is particularly important when studying substrate-binding conformations, as noted by Vannini et al. [17] in their structural characterization of the HDAC8-substrate complex.

3.1.2. Binding affinity analysis and pocket preferences

The blind docking approach revealed five potential binding pockets on the HDAC8 protein surface, with Pocket 1 (volume: $2020 \text{ \AA}^3$, coordinates: 16.465, - 0.699, 43.607) consistently exhibiting the highest binding affinities for most compounds. As illustrated in the heat map visualization (Fig. 2), this pocket corresponds to the catalytic site of HDAC8, which contains the catalytic zinc ion and key residues involved in substrate recognition and deacetylation, as previously characterized in crystallographic studies by Vannini et al. [17] and Somoza et al. [3].

Among the tested compounds, novel spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives exhibited substantially higher binding affinities to Pocket 1, with KB-282 demonstrating the highest binding affinity (-12.3 kcal/mol), exceeding that of the reference hydroxamate inhibitor octanediarnide (-10.8 kcal/mol), as shown in Table 2.

Table 2
Key binding affinities of compounds to HDAC8 Pocket 1 (catalytic site).

Compound	Binding score (kcal/mol)	Key interacting residues	Binding mechanism
KB-282	-12.3	ASP101, PHE152, PHE208, TYR306	π -anion, π - π stacking
KB-281	-11.0	ASP101, PHE152, PHE208, TYR306	π -anion, π - π stacking
Octanediamide	-10.8	TYR306, ASP101, ASP178, Zn ²⁺	Hydrogen bonding, Zn ²⁺ chelation
KB-269	-10.5	ASP101, PHE152, HIS143, TYR306	π -anion, π - π stacking
KB-271	-10.4	PHE152, ASP101, HIS143, TYR306	π -anion, π - π stacking
KB-280	-10.4	ASP101, PHE152, PHE208, TYR306	π -anion, π - π stacking
KB-270	-10.0	ASP101, PHE152, HIS143, TYR306	π -anion, π - π stacking
NCC149	-8.9	HIS143, GLY151, ASP101, Zn ²⁺	Hydrogen bonding, Zn ²⁺ chelation
PCI-34051	-8.8	TYR306, HIS143, ASP101, Zn ²⁺	Hydrogen bonding, Zn ²⁺ chelation
KB-268	-8.8	PHE152, ASP101, TYR306	π -anion, π - π stacking
KB-292	-8.7	ASP101, HIS143, GLY151, PHE152, TYR306	π - π stacking, van der Waals
KB-272	-8.5	PHE152, HIS143, ASP101, TYR306	π -anion, π - π stacking
KB-283	-8.5	ASP101, PHE152, HIS143, TYR306	π -anion, π - π stacking
Entinostat	-8.4	HIS143, ASP101, PHE152, TYR306	Hydrogen bonding, π - π stacking
p53-derived	-8.4	HIS143, ASP101, PHE152, Zn ²⁺	Hydrogen bonding, peptide-specific
<i>Note: Full blind docking results across all five binding pockets are provided in Supplementary Material, S1.</i>			

In contrast, established HDAC8-selective inhibitors exhibited more moderate binding affinities, with NCC149 (– 8.9 kcal/mol), PCI-34051 (– 8.8 kcal/mol), and entinostat (– 8.4 kcal/mol) showing similar binding energies to the p53-derived peptide substrate (– 8.4 kcal/mol), as detailed in Table 2. The control compound KB-292, representing the core tetrazolo[1,5-c]quinazoline scaffold without spiro-indoline modification, yielded a Pocket 1 binding score of – 8.7 kcal/mol, comparable to those of the reference HDAC8-selective inhibitors and consistent with the absence of the pharmacophoric elements identified as critical for high-affinity binding in the present series.

These values align with the relative potencies reported in experimental studies by Suzuki et al. [15] for NCC149 and Balasubramanian et al. [14] for PCI-34051. This suggests that the novel spiro compounds may interact with the HDAC8 binding site through additional favorable contacts not fully exploited by current inhibitors.

All compounds consistently bound more strongly to Pocket 1 than to alternative binding sites, with binding energies for secondary pockets typically 2–3 kcal/mol weaker than for the primary site, as demonstrated in Fig. 2 and detailed in Supplementary Material, S1. This preference for the catalytic pocket aligns with the mechanism of action of classical HDAC inhibitors, which primarily function through competition with natural substrates for the active site [7].

3.1.3. Structure-activity relationships

SAR studies combined with molecular docking have proven valuable for evaluating biological activity in heterocyclic compound development, as demonstrated by prior investigations of triazolo(triazino)quinazoline-derived acetamides and carboxylic acid derivatives [27, 28].

Examination of the current relationship between chemical structure and binding affinity also revealed several key structural features that enhance the interaction of spiro compounds with HDAC8. Based on the SAR visualization (Fig. 2S, Supplementary Material) and the data summarized in Table 3, the most potent compounds, KB-282 and KB-281, share a common structural motif: a carboxamide or carbothioamide group at position 1 of the indoline ring. This functional group appears to serve as a critical pharmacophore element, likely engaging in favorable interactions with key residues in the binding pocket, similar to the binding determinants identified by Whitehead et al. [21] in their analysis of HDAC inhibitor selectivity.

Table 3
Summary of SAR analysis for spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives.

Structural feature	Position	Effect on binding affinity	Compounds	Binding score (kcal/mol)
Carboxamide group	Position 1 of indoline ring	Strong enhancement	KB-282, KB-281	-12.3, - 11.0
Carbothioamide group	Position 1 of indoline ring	Moderate enhancement	KB-280	-10.4
Acetamide group	Position 1 of indoline ring	Moderate enhancement	KB-269, KB-270, KB-271	-10.5, - 10.0, -10.4
Cyano group	Phenyl ring of carboxamide	Strong enhancement	KB-282	-12.3
Trifluoromethyl group	Phenyl ring of carboxamide	Strong enhancement	KB-281	-11.0
Chloro group	Phenyl ring of acetamide	Moderate enhancement	KB-269 (4-position), KB-270 (3-position)	-10.5, - 10.0
No substitution	Position 1	Weak binding	KB-268	-8.8
2-chloroacetyl group	Position 6'	Decreased binding	KB-272	-8.5
Propyl group	Amide N-substituent	Decreased binding	KB-283	-8.5

Statistical analysis of the spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives revealed correlations between molecular properties and binding affinity (Supplementary Material, S14–S16). Binding scores showed strong negative correlations with topological polar surface area ($r = -0.698$, $p < 0.05$) and hydrogen bond acceptor count ($r = -0.830$, $p < 0.01$), indicating that excessive polarity diminishes HDAC8 binding affinity. The Kruskal–Wallis test was applied to evaluate differences in binding affinity among position 1 substitution groups; however, the test did not reach statistical significance ($H = 4.50$, $df = 3$, $p = 0.21$, Supplementary Material, S20), likely owing to the small group sizes (two groups comprise single compounds).

Given the non-significant Kruskal–Wallis result and the small sample sizes, the following group comparisons are interpreted descriptively. Compounds bearing carboxamide groups at position 1 (KB-281 and KB-282) achieved a mean binding score of -11.65 kcal/mol (range -11.0 to -12.3), compared with -10.4 kcal/mol for the carbothioamide derivative (KB-280), -9.58 ± 1.00 kcal/mol for acetamide derivatives (KB-269, KB-270, KB-271, KB-272, KB-283; $n = 5$), and -8.8 kcal/mol for the unsubstituted parent compound KB-268 (Supplementary Material, S15).

The presence of electron-withdrawing substituents on the phenyl ring of the carboxamide/carbothioamide moiety further enhances binding affinity, as exemplified by the cyano group in KB-282 and the trifluoromethyl group in KB-281 (Fig. 2S and Table 3). Electronic effects analysis demonstrated that electron-withdrawing substituents provided non-significant binding enhancement compared to electron-donating groups ($t = -0.94$, $p = 0.39$ (Welch), Supplementary Material, S20). These substituents may contribute to binding through electronic effects that strengthen electrostatic interactions or through conformational constraints that optimize the positioning of the molecule within the active site, consistent with SAR patterns observed for other HDAC inhibitor classes [23].

In contrast, compounds with acetamide functionalities at position 1 (KB-269, KB-270, KB-271, as shown in Fig. 1, and 2S) showed slightly lower binding affinities, although still comparable to or better than the reference hydroxamate inhibitor. This suggests that while the acetamide group provides favorable interactions, the direct attachment of the amide to the indoline ring as in KB-282 and KB-281 creates a more optimal binding geometry.

The unsubstituted parent compound KB-268 exhibited weaker binding (-8.8 kcal/mol), emphasizing the importance of appropriate functionalization for strong HDAC8 engagement. Similarly, KB-272, which features a 2-chloroacetyl group at position 6', showed reduced binding affinity (-8.5 kcal/mol), indicating that substitution at this position may create steric hindrance within the binding pocket. Outlier analysis (Supplementary Material, S21) identified three compounds deviating from the linear regression model: KB-280 (Cook's distance = 0.82), KB-282 (Cook's distance = 3.238), and KB-283 (Cook's distance = 0.522). The anomalous behavior of KB-282 is consistent with a non-linear structure–activity relationship at the high-affinity end of the series, while KB-280 and KB-283 likely reflect structural features not adequately captured by the three-descriptor model (SlogP, TPSA, HBA). KB-272 fell within expected model performance (Cook's distance = 0.010, standardized residual = -0.19). Given the small sample ($n = 9$), the three predictors, and the influence of these outliers, the regression is interpreted as exploratory rather than predictive.

3.1.4. Amino acid composition of the binding pocket

Analysis of the amino acid composition of Pocket 1 revealed a distinctive pattern of residues that contribute to ligand binding. As visualized in Fig. 3S and detailed in Supplementary Material, S3, aspartic acid (ASP), phenylalanine (PHE), histidine (HIS), lysine (LYS), and glycine (GLY) consistently appeared among the top five most frequent residues across all compounds, aligning with previous structural characterizations of the HDAC8 active site [4, 18].

A relationship was observed between binding score and the frequency of specific residues, as shown in Fig. 3S. Among the nine novel derivatives, stronger binders tended to contact more aspartic acid residues ($r = -0.82$, $p < 0.01$), with KB-282, the strongest binder, contacting eight ASP residues summed across the two protein chains present in the crystal lattice (Supplementary Material, S3); this association was negligible across the full 15-compound set ($r = -0.09$), in which high-ASP reference ligands such as the p53-derived peptide bound weakly, indicating multifactorial determinants of affinity. Similarly, phenylalanine residues were consistently present across all binding poses (six PHE residues for 13 of 15 compounds; KB-269 and KB-283 showed five and four PHE contacts, respectively), suggesting that aromatic interactions play a crucial role in stabilizing the protein–ligand complex, consistent with observations by Wu et al. [22].

3.1.5. Molecular interaction mechanisms

Detailed analysis of the molecular interactions revealed differences between the binding modes of the reference hydroxamate inhibitor (octanediamide) and the highest-scoring novel compound (KB-282). As illustrated in Fig. 3 and detailed in Supplementary Table S4, octanediamide primarily engages with HDAC8 through conventional hydrogen bonds with key residues such as Tyr306, Asp101, and Asp178, whereas KB-282 establishes a more diverse interaction network dominated by π -interactions.

The template-based docking of octanediamide showed three conventional hydrogen bonds with distances ranging from 2.40 to 2.53 Å, complemented by multiple hydrophobic interactions, particularly π - π stacking with Tyr306 (Table S4). This binding mode is consistent with the established mechanism of hydroxamate inhibitors, which chelate the catalytic zinc ion while forming hydrogen bonds with surrounding residues [4, 7].

In contrast, KB-282 exhibited a distinct binding pattern characterized by electrostatic π -anion interactions with Asp101 (distances of 4.22 and 4.06 Å) and extensive π - π stacking with aromatic residues including Phe152, Phe208, and Tyr306, as illustrated in Fig. 3 and detailed in Table S4. The absence of direct hydrogen bonding with Tyr306 in the KB-282 complex suggests an alternative mode of binding that does not strictly mimic the substrate recognition mechanism described by Vannini et al. [17].

These differences in interaction profiles may explain the enhanced binding affinity of KB-282 compared to conventional inhibitors. The combination of electrostatic interactions with Asp101 and multiple aromatic contacts creates a network of complementary interactions that stabilize the compound within the active site. Moreover, the interaction with Asp101 is notable given its established role in substrate recognition, as demonstrated by a substantial reduction in inhibitor binding affinity observed for the Asp101Ala mutant [17, 18], consistent with the critical role of this residue in substrate and inhibitor positioning.

3.2. Preliminary experimental comparison *via* cytotoxicity study and correlation analysis

To provide a preliminary experimental comparison, we incorporated cytotoxicity data from related tetrazolo[1,5-*c*]quinazoline compounds, namely KB-268, KB-270, and KB-292. KB-292 served as a structural control compound, representing the core tetrazolo[1,5-*c*]quinazoline scaffold without spiro-indoline modification. With a predicted binding score of -8.7 kcal/mol, it enabled assessment of computational accuracy within the moderate binding range while testing the necessity of specific structural features identified in lead compounds.

Cytotoxicity assessment using MTT assay revealed distinct activity patterns correlating with structural features (Table 4). KB-270 emerged as the most active compound, demonstrating measurable cytotoxicity against multiple cell lines (IC_{50} 34.88–49.69 μ M across the J774.2, Jurkat, and MCF-7 lines and > 50 μ M against HCT-116; Table 4). This activity is consistent with the computational ranking, which favoured compounds with stronger predicted binding. KB-268 and KB-292 both showed minimal activity (IC_{50} > 50 μ M), consistent with weaker predicted or observed binding affinities.

The moderate activity of KB-270 compared to the predicted high potency of KB-282 and KB-281 suggests that while computational binding scores provide valuable guidance, additional factors such as cellular permeability, metabolic stability, and off-target interactions influence biological outcomes. This discrepancy highlights the complexity of translating computational predictions to cellular responses and underscores the need for experimental evaluation of the most promising computational hits, particularly KB-282 and KB-281.

Table 4
Correlation between computational binding scores and experimental cytotoxic activity.

Compound	Docking score (kcal/mol)	J774.2 (μM)	MCF-7 (μM)	HCT-116 (μM)	Jurkat (μM)	Activity level (predicted / observed)
KB-270	-10.0	34.88	49.69	> 50	42.99	Predicted moderate / moderate
KB-268	-8.8	> 50	> 50	> 50	> 50	Predicted low / minimal
KB-292	-8.7	> 50	> 50	> 50	> 50	Predicted low / minimal

It is important to acknowledge that the correlation between general cytotoxicity and specific HDAC8 inhibitory activity requires further investigation. The cytotoxicity data, while providing initial validation of our computational approach, represents a broader measure of biological activity that may involve multiple cellular targets beyond HDAC8.

3.3. ADMET properties and drug-likeness analysis

In silico assessment of absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties for the novel spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives reveals several properties relevant to their drug development potential. As illustrated in Fig. 4S and detailed in Table 5, the lead compounds KB-282 and KB-281 demonstrate favorable ADMET profiles compared to reference inhibitors.

Table 5
Physicochemical properties and drug-likeness parameters of lead compounds.

Comp.	MW (Da)	LogP	TPSA (Å ²)	HBA	HBD	nRot	Caco-2 perm	HIA	BBB	hERG inhib	DILI prob	Lip. viol.
Lead spiro compounds												
KB-282	434.42	2.60	128.83	8	2	1	0.619	0.835	0.599	0.017	0.737	0
KB-281	477.41	3.85	105.04	7	2	1	0.681	0.972	0.735	0.026	0.826	0
KB-269	457.88	3.42	105.04	7	2	3	0.344	0.961	0.663	0.020	0.743	0
KB-280	425.48	3.41	87.97	7	2	1	0.515	0.961	0.673	0.024	0.786	0
Reference HDAC8 inhibitors												
NCC149	326.38	2.40	80.04	6	2	5	0.453	0.982	0.884	0.013	0.595	0
PCI-34051	296.33	1.67	63.49	4	2	4	0.122	0.912	0.661	0.026	0.478	0
Entino-stat	376.42	1.93	106.34	5	3	6	0.286	0.963	0.721	0.019	0.507	0
Octane-diamide	608.72	2.37	159.01	4	6	14	0.016	0.775	0.215	0.027	0.499	2
MW = Molecular Weight; LogP = Octanol-water partition coefficient; TPSA = Topological Polar Surface Area; HBA = Hydrogen Bond Acceptors; HBD = Hydrogen Bond Donors; nRot = Number of Rotatable Bonds; Caco-2 Perm = Predicted Caco-2 cell permeability; HIA = Human Intestinal Absorption; BBB = Blood-Brain Barrier permeability; hERG Inhib = hERG channel inhibition probability at 1μM; DILI Prob = Drug-Induced Liver Injury probability.												

The physicochemical properties of the spiro compounds reveal that KB-282 and KB-281 exhibit favorable molecular weights (434.42 Da and 477.41 Da, respectively) that comply with Lipinski's rule of five (MW < 500 Da), although KB-

281 approaches this threshold. Both compounds demonstrate moderate lipophilicity (logP values of 2.60 and 3.85, respectively), which balances membrane permeability with aqueous solubility. KB-271 represents an exception, with a molecular weight of 505.46 Da that marginally exceeds the 500 Da threshold, resulting in one Lipinski violation (Supplementary Material, S19).

KB-282 demonstrated high predicted Caco-2 permeability (0.619) and human intestinal absorption (HIA = 0.835), suggesting favorable oral absorption characteristics. KB-281 exhibited even higher intestinal absorption (HIA = 0.972) and Caco-2 permeability (0.681). For comparison, the hydroxamate inhibitor showed lower Caco-2 permeability (0.016) and reduced intestinal absorption (HIA = 0.775), likely due to its higher molecular weight, greater polarity, and increased hydrogen bond donors.

The metabolism parameters, detailed in Supplementary Material, S9, indicate that KB-282 exhibits lower inhibitory potential against most CYP450 enzymes compared to the hydroxamate inhibitor, suggesting a reduced risk of drug–drug interactions. KB-282 achieved the lowest composite CYP inhibition score (1.824) among all tested compounds, calculated as the sum of six CYP inhibition probabilities (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4, CYP2D6; Supplementary Material, S9), representing an approximately 53% reduction compared to the established HDAC8-selective inhibitors NCC149 and PCI-34051 (mean DDI score: 3.875 ± 0.663 , calculated by the same method).

The toxicity predictions reveal several characteristics of the lead spiro compounds relevant to their further development (Supplementary Material, S10). KB-282 exhibits a low predicted probability of hERG channel inhibition at 1 μ M (0.017), lower than KB-281 (0.026) and the reference compounds, suggesting reduced cardiotoxicity risk. Within the spiro series, KB-282 shows a lower predicted probability of drug-induced liver injury (DILI) (0.737) compared to KB-281 (0.826); however, it should be noted that this DILI probability exceeds that of the hydroxamate reference compound octanediamide (0.499), indicating a 47.7% higher predicted DILI risk relative to the hydroxamate scaffold (Supplementary Material, S23). In contrast, KB-282 demonstrates substantially lower predicted mutagenicity (Ames probability 0.136 vs. 0.576 for octanediamide; 76.4% lower) and lower hepatotoxicity (0.404 vs. 0.711; 43.2% lower), as detailed in Supplementary Material, S23. DILI and hepatotoxicity are distinct admetSAR endpoints; for KB-282 they diverge, with a low structural-alert hepatotoxicity probability (0.404) but a high DILI probability (0.737), and the liver-related signal should therefore be interpreted in light of this divergence. The lower predicted hERG and mutagenicity risk (the latter also 72.9% below that of KB-281) supports KB-282 as the lead compound for further development, while the elevated DILI signal warrants monitoring in subsequent experimental studies.

Comparative ADMET analysis using standardized prediction algorithms confirmed notable differences in the ADMET profile of the spiro scaffold relative to conventional hydroxamate inhibitors. Lead compounds KB-282 and KB-281 demonstrated much higher predicted Caco-2 cell permeability compared to the reference hydroxamate inhibitor (mean 0.650 ± 0.044 vs 0.016, Supplementary Material, S22), indicating a potentially enhanced oral absorption profile. Pairwise toxicity profile comparison and drug–drug interaction assessment based on cumulative CYP inhibition probability scores are provided in Supplementary Material, S23 and S24, respectively.

3.4. Comparative analysis with known HDAC8-selective inhibitors

The comparison of binding profiles between novel spiro compounds and established HDAC8-selective inhibitors provides context for evaluating the potential selectivity of the new derivatives. As illustrated in Table 6, reference compounds PCI-34051 and NCC149 exhibit notable selectivity for HDAC8 over other HDAC isoforms (> 200-fold and > 500-fold, respectively) despite their experimentally determined nanomolar binding affinities (IC_{50} values of 10 nM and 70 nM), as reported by Balasubramanian et al. [14] and Suzuki et al. [15], respectively.

The higher binding scores obtained for the lead spiro compounds (KB-282, KB-281) from our docking studies suggest that these molecules may achieve comparable or improved potency. However, it is important to note that binding scores do not directly translate to inhibitory activity or selectivity, as these properties are influenced by additional factors including pharmacokinetics and specific interaction patterns with residues that differ across HDAC isoforms, as demonstrated by Whitehead et al. [21].

The distinctive binding mode of KB-282, characterized by strong interactions with Asp101 and aromatic residues but without direct zinc chelation (Fig. 3, Table S4), represents a promising approach for achieving selectivity. This binding strategy differs from the hydroxamate-based inhibitors that typically bind promiscuously across HDAC isoforms due to the high conservation of the catalytic zinc site, as noted by Finnin et al. [7] in their pioneering structural studies.

Table 6
Comparison of selective HDAC8 inhibitors: Binding energies and key interaction profiles.

Compound	Binding score (kcal/mol)	Exp. determ. IC ₅₀ (nM)	Selectiv. ratio*	Zinc chel.	Key interaction types	Key selectivity determinants
Novel spiro compounds						
KB-282	-12.3	N/A†	N/A†	No	π-anion interactions with Asp101; π-π stacking with Phe152, Phe208, Tyr306	Unique interaction with Asp101; distinctive binding mode without zinc chelation
KB-281	-11.0	N/A†	N/A†	No	π-anion interactions with Asp101; π-π stacking with Phe152, Phe208, Tyr306	Unique interaction with Asp101; distinctive binding mode without zinc chelation
Reference HDAC8-selective inhibitors						
PCI-34051	-8.8	10	> 200	Yes	Hydrogen bonding with Tyr306, His143; zinc coordination	Exploits unique pocket near catalytic site; narrow zinc-binding group
NCC149	-8.9	70	> 500	Yes	Hydrogen bonding with His143, Gly151; zinc coordination	Exploits unique foot pocket of HDAC8; optimized zinc-binding geometry
Other HDAC inhibitors						
Entinostat	-8.4	1,200	0.3	No	Hydrogen bonding with His143, Asp101; π-π stacking	Non-selective, primarily Class I HDAC inhibitor
Octane-diamide	-10.8	350	5	Yes	Hydrogen bonding with Tyr306, Asp101, Asp178; zinc coordination	Moderate selectivity; primarily relies on zinc chelation
*Selectivity ratio indicates fold-selectivity for HDAC8 over other HDAC isoforms (primarily HDAC1). †Experimental determination pending; computational analysis suggests potential for high selectivity based on unique binding mode.						

The successful development of selective inhibitors such as PCI-34051 and NCC149 demonstrates that HDAC8 selectivity is achievable despite the high conservation of the catalytic domain across HDAC isoforms, as previously documented by Somoza et al. [3] and Vannini et al. [4]. The novel spiro compounds, with their unique interaction profiles detailed in Table 3, contribute to this growing body of evidence that selective targeting of HDAC8 is feasible through exploitation of subtle structural differences in the active site.

3.5. Mechanistic insights and clinical implications

The role of Asp101 as a critical interaction partner, discussed earlier, further supports the rationale for targeting this residue to achieve enhanced potency and selectivity. The higher binding affinities observed for non-hydroxamate spiro derivatives (Table 2, Fig. 2) indicate that zinc chelation, while effective for inhibition, is not the only strategy for achieving potent HDAC8 engagement. A notable advantage of the spiro[indoline-tetrazolo[1,5-c]quinazoline] scaffold is the absence of the hydroxamate group present in many HDAC inhibitors. Hydroxamate-based compounds often exhibit metal-binding toxicity due to their ability to chelate various metal ions besides the catalytic zinc in HDACs. Our lead compounds, KB-282 and KB-281, achieve high binding affinity through alternative interaction mechanisms, as demonstrated in the molecular docking studies and visualized in Fig. 3, potentially reducing metal-binding-related toxicities.

Analysis of binding pocket compositions (Fig. 3S, Supplementary Material, S3) and interaction patterns (Table S4) provides a basis for understanding the molecular determinants of HDAC8 selectivity. The distinctive constellation of residues in Pocket 1, particularly the arrangement of acidic and aromatic amino acids, creates an environment that may be selectively targeted through appropriate molecular design, building upon the selectivity principles established by Suzuki et al. [15] and Balasubramanian et al. [14] in their development of HDAC8-selective inhibitors.

3.6. Limitations and future directions

This computational study has several limitations. Molecular docking scores represent relative binding affinities under idealized conditions and do not directly translate to experimental IC_{50} values or biological activity. The moderate correlation between computational predictions and cellular activity underscores the necessity of direct HDAC8 enzyme inhibition assays to validate the predicted binding affinities.

The docking protocol, while validated through successful reproduction of crystallographic poses ($RMSD < 2.0 \text{ \AA}$), assumes rigid protein conformations and may not capture the dynamic nature of protein-ligand interactions or allosteric effects that influence binding in physiological conditions. The extrapolation from the static 2V5X structure to dynamic binding behavior introduces uncertainty in our predictions. In addition, the top blind-docking cavity (Pocket 1, approximately $2020 \text{ \AA}^3$) and the residue-contact counts were derived from the crystallographic arrangement, which contains a second protein chain; contacts attributed to that chain may reflect lattice packing rather than the biological monomer and would benefit from confirmation against the biological assembly or by molecular dynamics. The present poses also do not include explicit coordination to the catalytic Zn^{2+} ion, so the proposed non-chelating binding mode requires confirmation by approaches that explicitly model the metal center.

The experimental validation relies on cytotoxicity data rather than direct HDAC8 inhibition assays. The tested compounds (KB-268, KB-270, KB-292) were not selected based on computational rankings but were available from parallel research efforts, limiting the direct validation of our highest-scoring computational predictions. This gap between computational predictions and experimental validation represents a limitation that requires addressing in future studies. Moreover, the redocking protocol was assessed using co-crystal structures that include HDAC8 orthologues and related HDAC isoforms in addition to human HDAC8; validation restricted to human HDAC8 co-crystal complexes would more directly support the present application.

Beyond HDAC8 inhibition, the broader pharmacological potential of nitrogen-rich heterocyclic scaffolds related to the tetrazolo[1,5-c]quinazoline core – including antitumor, antimicrobial, anti-inflammatory, and anticonvulsant activities documented for 1,2,4-triazole-containing compounds – warrants systematic exploration of this compound class against additional biological targets [29].

4. CONCLUSIONS

This computational and experimental investigation of spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives provides initial evidence supporting their further evaluation as selective HDAC8 inhibitors.

Molecular docking studies using an inhibitor-bound HDAC8 crystal structure (PDB ID: 2V5X) demonstrated that spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives exhibit notable binding affinities for the HDAC8 catalytic site. Lead compounds KB-282 and KB-281 achieved binding energies (– 12.3 and – 11.0 kcal/mol, respectively) that exceeded the docking scores of the established HDAC8-selective reference inhibitors, suggesting their potential as inhibitors warranting further experimental evaluation. Critical pharmacophoric elements were identified that may enhance binding affinity: carboxamide or carbothioamide groups at position 1 of the indoline ring and electron-withdrawing substituents on the phenyl ring of the amide moiety. These structural determinants suggest directions for rational optimization of this scaffold.

The lead compounds employ a distinctive binding mode that differs fundamentally from conventional hydroxamate-based inhibitors. Instead of zinc chelation, these compounds establish extensive π -interactions with aromatic residues (Phe152, Phe208, Tyr306) and critical electrostatic interactions with Asp101. This non-hydroxamate mechanism may confer selectivity advantages and reduced metal-binding toxicity. *In silico* ADMET assessment revealed favorable physicochemical properties, including predicted oral bioavailability improvements compared to hydroxamate inhibitors and predicted acceptable toxicity profiles.

Cytotoxicity assessment of related compounds (KB-268, KB-270, KB-292) provided a preliminary experimental comparison. The qualitative consistency between predicted binding and observed activity, exemplified by KB-270's moderate cytotoxic activity (IC_{50} 34.88–49.69 μ M) versus minimal activity for lower-binding compounds (IC_{50} >50 μ M), supports the predictive value of the docking methodology while highlighting the complexity of translating computational predictions to cellular responses.

The identification of compounds with predicted binding affinities exceeding established HDAC8-selective inhibitors, coupled with preliminary experimental validation and favorable predicted ADMET profiles, supports further investigation of spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives as candidates for treating HDAC8-associated disorders, including Cornelia de Lange syndrome and specific malignancies. However, the moderate correlation between computational predictions and cellular activity underscores the necessity of direct experimental validation to realize this therapeutic potential.

Abbreviations

ADMET

absorption,distribution,metabolism,excretion,and toxicity

AhR

aryl hydrocarbon receptor

APCI

atmospheric pressure chemical ionization

AR
androgen receptor
AR-LBD
androgen receptor ligand-binding domain
ARE
antioxidant response element
ATAD5
ATPase family AAA domain-containing protein 5
ATCC
American Type Culture Collection
BBB
blood–brain barrier
BCF
bioconcentration factor
BCRP
breast cancer resistance protein
BioLip
biological ligand–protein interaction database
BSEP
bile salt export pump
Caco-2 perm
predicted Caco-2 cell permeability
CB-Dock2
cavity detection-guided blind docking server version 2
CLMGraph
deep-learning logP prediction model
CLp
plasma clearance
CLr
renal clearance
CO₂
carbon dioxide
CurPocket
protein-surface-curvature-based pocket detection algorithm
CYP
cytochrome P450
DDI
drug–drug interaction
df
degrees of freedom
DILI
drug-induced liver injury
DMEM
Dulbecco's Modified Eagle Medium
DMSO

dimethyl sulfoxide
EI-MS
electron impact mass spectrometry
ER
estrogen receptor
ER-LBD
estrogen receptor ligand-binding domain
ERR α
estrogen-related receptor alpha
F
F-statistic
FDA
Food and Drug Administration
FitDock
template-based fitting docking algorithm
FP2
fingerprint type 2
GR
glucocorticoid receptor
H
Kruskal–Wallis test statistic
HBA
hydrogen bond acceptors
HBD
hydrogen bond donors
HDAC
histone deacetylase
HDAC8
histone deacetylase isoform 8
HDACi
HDAC inhibitor(s)
hERG
human ether-à-go-go-related gene channel
HIA
human intestinal absorption
HLM
human liver microsomal stability
IC₅₀
half-maximal inhibitory concentration
IR
infrared spectroscopy
L1 loop
loop 1 of HDAC8
LC-MS
liquid chromatography–mass spectrometry

LogP
logarithm of octanol–water partition coefficient
logS
logarithm of aqueous solubility
MATE1
multidrug and toxin extrusion protein 1
MMP
mitochondrial membrane potential
MRT
mean residence time
MTT
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW
molecular weight
N/A
not available
NMR
nuclear magnetic resonance
nRot
number of rotatable bonds
OATP1B1
OATP1B3,OATP2B1,organic anion transporting polypeptides
OCT1
OCT2,organic cation transporters
p53
tumor protein p53
PDB
Protein Data Bank
P-glycoprotein
P-gp efflux transporter
pKa
acid dissociation constant (logarithmic form)
PPAR γ
peroxisome proliferator-activated receptor gamma
PPB
plasma protein binding
r
Pearson correlation coefficient
R²
coefficient of determination
RDKit
open-source cheminformatics toolkit
RLM
rat liver microsomal stability
RMSD

root-mean-square deviation
RPMI-1640
Roswell Park Memorial Institute medium 1640
r.t.
room temperature
SAR
structure–activity relationship
SD
standard deviation
SlogP
logP calculated by the RDKit algorithm
SMC3
structural maintenance of chromosomes protein 3
 $T_{1/2}$
elimination half-life
TMS
tetramethylsilane
Tox21
Toxicology in the 21st Century panel
TPSA
topological polar surface area
TR
thyroid receptor
TSA
trichostatin A
UGT
UDP-glucuronosyltransferase
VDss
steady-state volume of distribution
 ^1H NMR
proton nuclear magnetic resonance spectroscopy
 ^{13}C NMR
carbon-13 nuclear magnetic resonance spectroscopy.

Declarations

Ethics approval

No primary human tissue, animal experiments, or patient data were involved in this study. All computational analyses were performed on publicly available structural data (PDB ID: 2V5X). Accordingly, ethical committee approval was not required for this study.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Conceptualization and design of the study, methodology, project administration: L.A.; software, L.A. and O.A.; investigation: L.A., O.A., Y.K.; resources: S.K.; original draft preparation: L.A.; data curation, formal analysis, visualization, writing—review and editing: L.A., O.A., V.S., M.M., H.L.; supervision: S.K. All authors reviewed the manuscript.

Data availability

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

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Figures

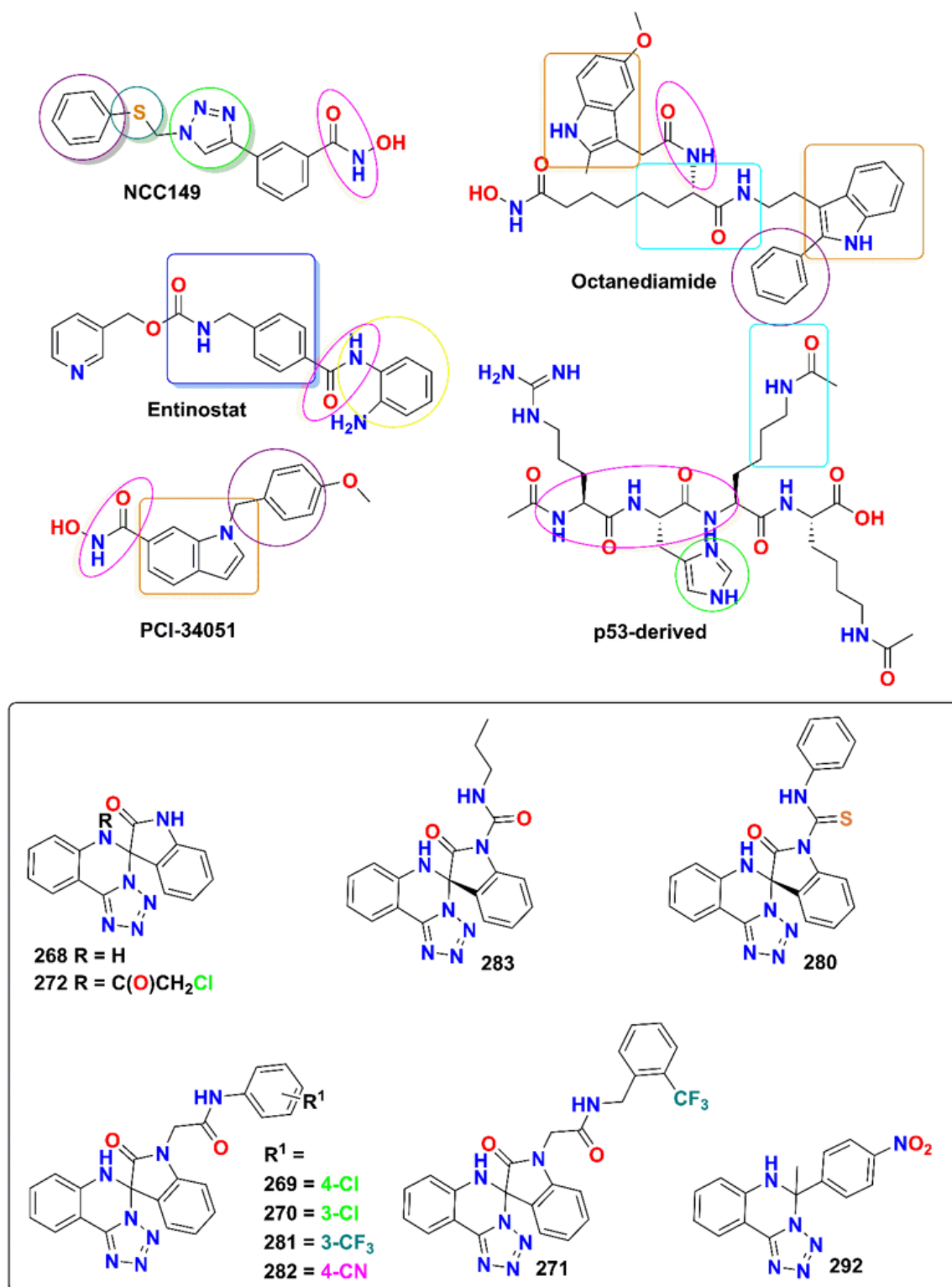


Figure 1

Molecular structures of HDAC8 inhibitors. Chemical structures of reference HDAC8 inhibitors including octanediamide (hydroxamate inhibitor), NCC149 (HDAC8-selective inhibitor), entinostat (benzamide inhibitor), PCI-34051 (HDAC8-selective inhibitor), and p53-derived peptide substrate. Novel spiro[indoline-tetrazolo[1,5-c]quinazoline] derivatives (KB-268-272, 280-283, and 292) evaluated for HDAC8 binding.

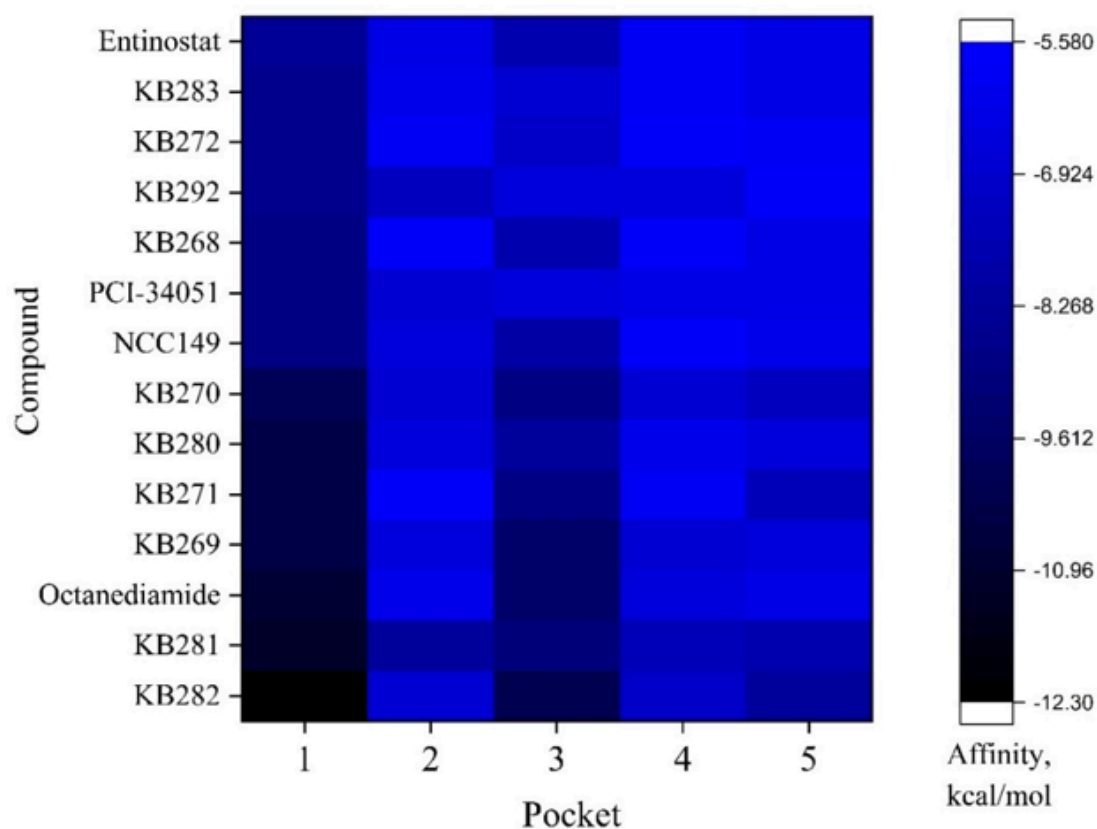


Figure 2

Heat map of binding affinities for HDAC8 pockets. Heat map visualization of binding affinities (kcal/mol) for all compounds across the five binding pockets identified in HDAC8 (PDB ID: 2V5X). Color intensity represents binding strength with darker colors indicating stronger binding (more negative values). Compounds are arranged in order of decreasing binding affinity to Pocket 1 (catalytic site).

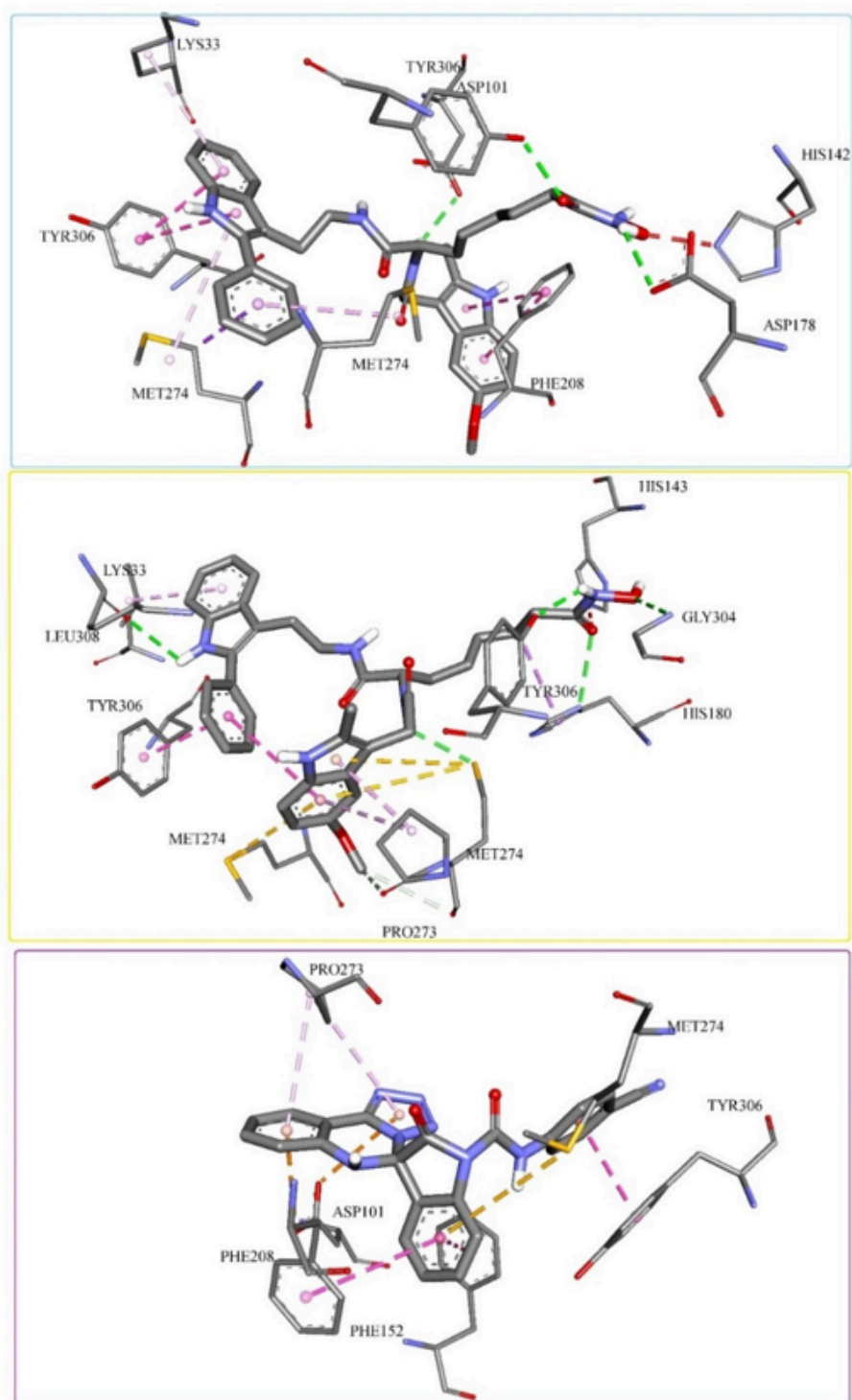


Figure 3

Comparative binding pose analysis in the HDAC8 active site (PDB ID: 2V5X). The figure illustrates the superimposed binding conformations of three ligands in the catalytic pocket 1 (volume: 2020 Å³) of HDAC8: co-crystallized (blue) octanediamide inhibitor demonstrating the experimentally validated binding mode, octanediamide from blind docking simulation (yellow) showing the computational prediction that closely matches the crystallographic pose, and the novel compound KB-282 (violet) which exhibited the highest binding affinity among all tested compounds. Key interacting residues are labeled and color-coded according to interaction type: hydrogen bonds (green), highlighting

the conventional hydrogen bonding network involving Tyr306, Asp101, and Asp178 for octanediamide; hydrophobic interactions (pink), including critical π - π stacking interactions with aromatic residues such as Phe152, Phe208, and Tyr306; and electrostatic interactions (orange), notably the π -anion interactions between KB-282 and Asp101 (distances of 4.22 Å and 4.06 Å), that appear critical for its enhanced binding affinity.

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