Human Histone Deacetylase 6 (HDAC6)

A Target Enabling Package (TEP)

Gene ID / UniProt ID / EC	10013 / Q9UBN7 / 3.5.1.98
Target Nominator	SGC Internal Nomination
SGC Authors	Harding R, Mader P, Ferreira de Freitas R, Kennedy S, Walker JR, Vedadi M, Von Delft F, Min J, Schapira M, Santhakumar V, Arrowsmith CH
Collaborating Authors	N/A
Target PI	Cheryl Arrowsmith
Therapeutic Area(s)	Oncology
Disease Relevance	Targeting the ubiquitin binding domain of HDAC6 should synergize with proteasome inhibitors in myeloma and lymphoma
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Affiliations	N/A

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SUMMARY OF PROJECT

HDAC6 is a cytosolic deacetylase with diverse roles in cellular trafficking, autophagy and cell signalling. Specifically targeting the unique zinc-finger ubiquitin-binding domain (ZnF-UBD) of HDAC6 may be an attractive strategy in myeloma and lymphoma but no inhibitor has been reported to date. Presented here are 4 co-crystal structures of HDAC6 ZnF-UBD in complex with different compounds, and associated SPR, ITC and FP assays.

SCIENTIFIC BACKGROUND

HDAC6 is a cytosolic deacetylase which functions in a diverse range of cellular processes including cell trafficking, autophagy and cell signalling. It is composed of two catalytic domains as well as a unique zinc-finger ubiquitin-binding domain (ZnF-UBD). HDAC6 gathers scattered polyubiquitinated protein aggregates via the ZnF-UBD and loads them on dynein/microtubules for aggresome degradation (1). Catalytic domain inhibitors antagonize the interaction of HDAC6 with dynein (2) and disrupt transport to the aggresome through acetylation of α-tubulin (3). Synergistic inhibition of HDAC6 and the proteasome is a promising strategy against myeloma and lymphoma (3,4). Current selective catalytic HDAC6 inhibitors are hydroxamic acids, and toxicity may be an issue. Targeting the recognition of protein aggregates with ZnF-UBD inhibitors rather than their loading on microtubules (as catalytic inhibitors do) could be a more direct way to antagonize aggresome degradation. SGC Toronto previously solved the apo structure of the ZnF-UBD (PDB code 3C5K) as well as in complex with the C-terminal ubiquitin peptide RLRGG, the native substrate for this domain (PDB code 3GV4). No inhibitor of HDAC6 ZnF-UBD has been reported to date. The goal of this TEP is to enable discovery of potent and selective compounds to target the ZnF-UBD of HDAC6.

RESULTS – THE TEP

Proteins purified

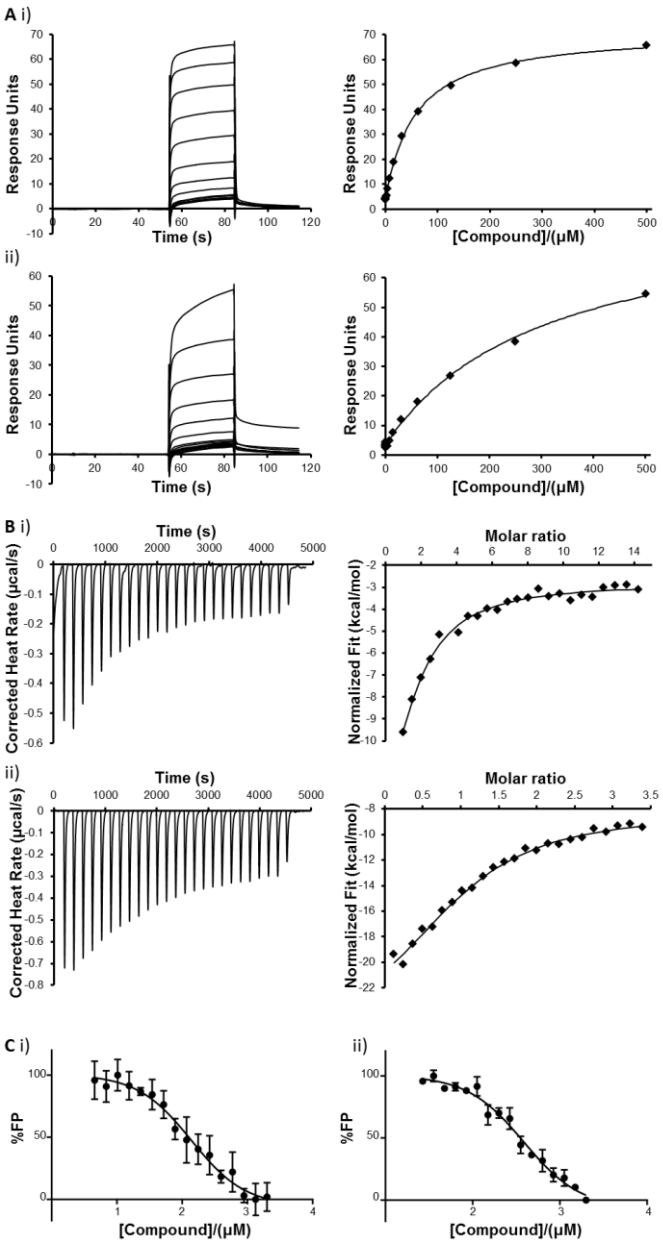
HDAC6 ZnF-UBD$^{1109\text{-}1215}$ pET28-LIC – expression construct of N-terminally His-tagged construct transformed into BL21(DE3)-RIL *E. coli* cells for protein expression. Used in ITC and FP assays. Construct also used to grow crystals with which to generate a seed-mix.

HDAC6 ZnF-UBD$^{1109\text{-}1213}$ pET28-LIC – expression construct of N-terminally His-tagged construct transformed into BL21(DE3)-RIL *E .coli* cells for protein expression. Used to grow crystals, in combination with the HDAC6 ZnF-UBD$^{1109\text{-}1215}$ seed mix, with a solvent accessible ubiquitin binding pocket in the crystal lattice, permissible to compound soaking.

HDAC6 ZnF-UBD$^{1109\text{-}1215}$ pET28BIOH-LIC – expression construct of N-terminally biotin-tagged and C-terminally his-tagged construct transformed into BL21(DE3)-BirA *E. coli* cells for protein expression. Used in SPR assays.

All cultures were grown in M9 minimal media supplemented with 50 µM $ZnSO_4$ and 10 µg/ml biotin in the case of HDAC6 ZnF-UBD$^{1109\text{-}1215}$ pET28BIOH-LICProteins were purified using Ni-affinity chromatography and gel filtration. For crystallisation, His-tags were cleaved using thrombin.

Full-length HDAC6 protein spanning 1-1215 was successfully expressed and purified from sf9 cells for use in FP assays.



Compounds i) SGC-T094 and ii) SGC-T164 were characterized by A) SPR, B) ITC and C) FP.

In vitro assays

The binding of HDAC6 ZnF-UBD$^{1109-1215}$ to ubiquitin and the ubiquitin C-terminal peptide (RLRGG) has been characterised thoroughly by ITC, SPR and FP. For compound screening the following assays have been established:

- FP peptide (FITC-labelled RLRGG) displacement assay
- SPR assay using streptavidin-biotin coupled HDAC6 ZnF-UBD$^{1109-1215}$
- ITC assay

FP peptide (FITC-labelled RLRGG) displacement assay was also optimised for full-length HDAC6 spanning residues 1-1215.

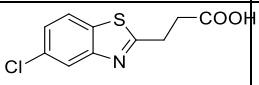
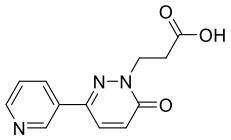
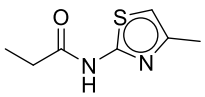
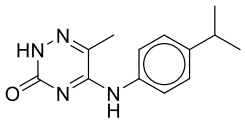
Structural data

Structures of apo and C-terminal ubiquitin peptide (RLRGG) bound HDAC6-ZnF were previously solved (PDB codes 3C5K and 3GV4). Soaked crystals of HDAC6 Znf-UBD$^{1109-1213}$ diffracted to 1.6 Å at the home source and 1.1 Å at the i04-1 Diamond Light Source beamline. Co-crystal structures have been solved with ligands which bind in either the ubiquitin pocket or the adjacent pocket as well as ligands which bind both pockets.

Chemical starting points

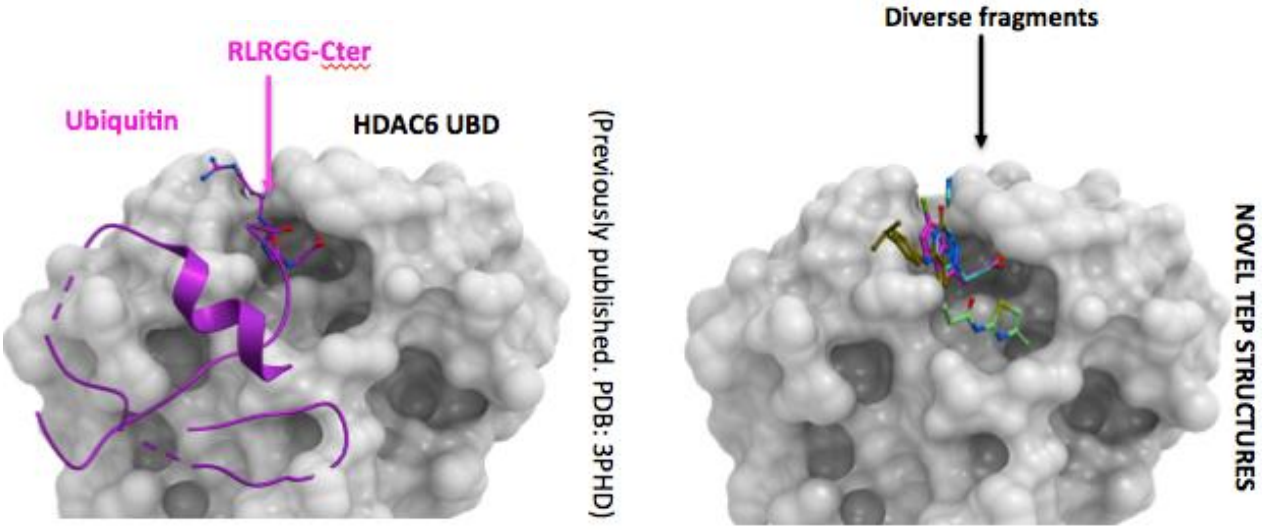
Initial fragment hits:

Fragments binding in the ubiquitin binding pocket and the adjacent pocket:

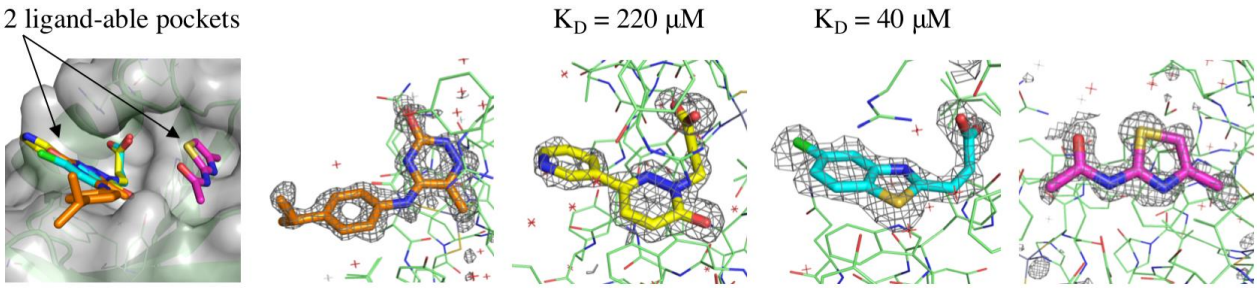
PDB	5KH3	5KH7	5B8D	5KH9
Structure				
Identification Method	Virtual screen	Virtual screen	High throughput X-ray crystallography	High throughput X-ray crystallography
Pocket bound	Ubiquitin	Ubiquitin	Adjacent	Ubiquitin
SPR (K_D, µM)	60 ± 3	220 ± 20	NB[a]	NB
ITC (K_D, µM)	40 ± 4	210 ± 30	NB	NB
FP (IC_{50}, µM)	55 ± 11	350 ± 50	NI[b]	NI
LE[c] (ITC)	0.39	0.28	-	-

Values calculated as average of 3 independent measurements; [a]No Binding; [b]No Inhibition [c]Ligand efficiency was calculated using the equation LE = (1.37 × pK_D)/HA; LE is expressed as kcal mol^{-1} atom^{-1}

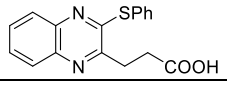
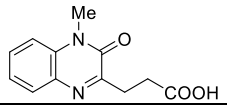
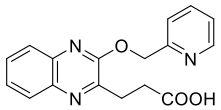
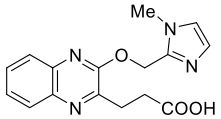
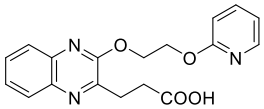
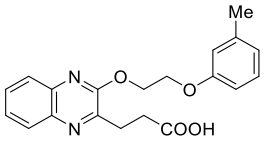
Structure of HDAC6 ZnF-UBD in complex with ubiquitin (left) and initial fragments (right):



Pockets found to bind the fragments (left) and the density for each fragment (right four images) along with KD where known:

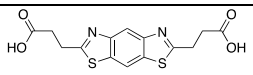
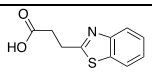
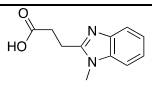
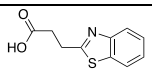
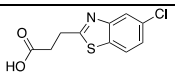
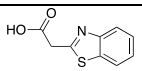
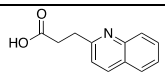
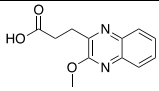
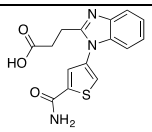
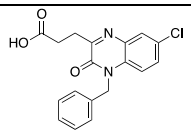
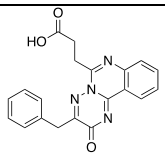
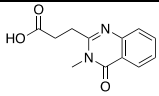
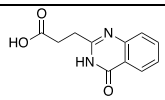
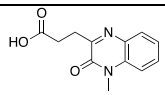


Initial SAR studies:

Structure	FP calculated IC_{50} (µM)[a]	PDB
	60 ± 9	
	70 ± 10	
	270 ± 80	5WPB
	140 ± 60	
	110 ± 40	
	180 ± 40	

[a]Average of three independent measurements.

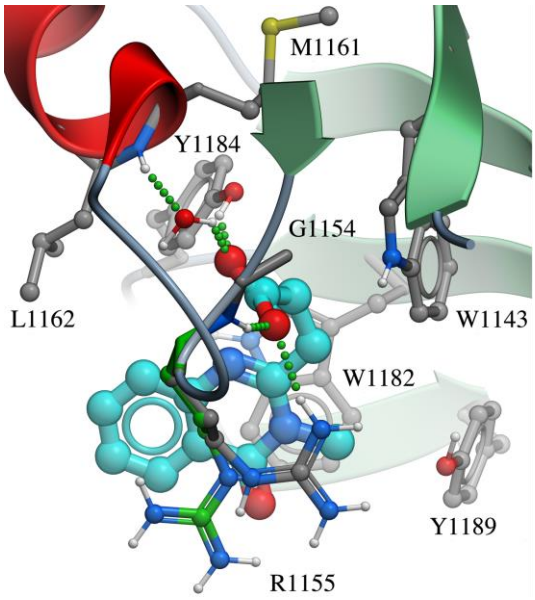
SAR development of lead ligands:

Structure	IC_{50} (□M)	LE[a]	logD[b]	PDB
	5.1 ± 0.61	0.34	-4.14	6CE6
	180 ± 52	0.37	-0.79	6CEF
	100 ± 39	0.37	-1.45	
	180 ± 52	0.37	-0.79	
	55 ± 11	0.39	-0.41	
	390 ± 160	0.36	-0.90	6CE8
	> 720	< 0.29	-0.81	
	45 ± 8.0	0.35	-1.55	6CEC
	640 ± 370	0.20	-1.00	
	150 ± 31	0.22	-0.04	
	25 ± 3.2	0.23	-0.78	5WBN
	2.3 ± 0.60	0.45	-1.83	6CED
	230 ± 70	0.31	-2.15	
	65 ± 9.7	0.34	-2.34	6CEE

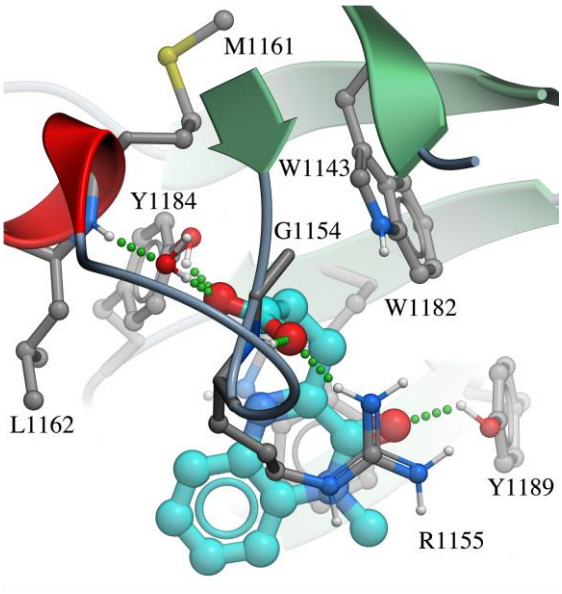
[a] Ligand efficiency (in kcal mol^{-1} $atom^{-1}$) was calculated as LE = (1.37 × pIC_{50})/HA;

[b]The log D was calculated using ChemAxon's JChem for Excel, version 16.11.2800.3575; IC_{50} determination experiments were performed in triplicate, and the values are presented as mean ± SD.

Below, co-crystal structures of two lead ligands along with PDB ID:



6CED



6CEE

IMPORTANT: Please note that the existence of small molecules within this TEP may indicate that chemical matter can bind to the protein in a functionally relevant pocket. As such these molecules should not be used as tools for functional studies of the protein unless otherwise stated as they are not sufficiently potent or well-characterised to be used in cellular studies. The small molecule ligands are intended to be used as the basis for future chemistry optimisation to increase potency and selectivity and yield a chemical probe or lead series.

Future work

Using structure guided compound design and validation with the mentioned assays, work is ongoing to grow or link compounds to increase potency and facilitate SAR understanding.

A collaboration with Mark Lauten's group (University of Toronto) is established to allow next stage chemistry to be followed up with rapid compound synthesis.

Twelve ubiquitin specific proteases (USPs) contain a ZnF-UBD with structural similarity to that of HDAC6. In particular, the ZnF-UBD of USP3, USP5 and USP16 have been shown to bind ubiquitin with physiologically relevant affinity. We have characterized the ubiquitin binding properties of these domains using ITC, SPR and NMR. Assays will be established to enable profiling of compound selectivity.

To probe the biology of HDAC6 ZnF-UBD in a disease environment, cell assays will be conducted in collaboration with Grace Zhai (University of Miami).

Following this strategy, we hope to develop more potent and selective compounds with comprehensive SAR analysis, potentially resulting in the development of a chemical probe.

CONCLUSION

A variety of compound screening assays, a robust crystal system, and numerous structures in complex with chemical fragments and ligands are presented for HDAC6 ZnF-UBD. Our future plan is to develop a chemical probe and test whether it synergizes with proteasome inhibitors to kill myeloma and lymphoma cells. A chemical probe would also help better characterize the functional role of HDAC6 ZnF-UBD.

FUNDING INFORMATION

The SGC is a registered charity (number 1097737) that receives funds from AbbVie, Bayer Pharma AG, Boehringer Ingelheim, Canada Foundation for Innovation, Eshelman Institute for Innovation, Genome Canada through Ontario Genomics Institute [OGI-055], Innovative Medicines Initiative (EU/EFPIA) [ULTRA-DD grant no. 115766], Janssen, Merck KGaA, Darmstadt, Germany, MSD, Novartis Pharma AG, Ontario Ministry of Research, Innovation and Science (MRIS), Pfizer, São Paulo Research Foundation-FAPESP, Takeda, and Wellcome.

ADDITIONAL INFORMATION

Structure Files

PDB ID	Structure Details
6CE6	Crystal structure of HDAC6
5KH3	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
5KH7	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
5KH9	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
5B8D	Fragment bound adjacent to ubiquitin binding pocket of HDAC6 zinc finger domain
5WPB	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
5WBN	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
6CE8	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
6CEC	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
6CED	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
6CEE	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain
6CEF	Fragment bound in ubiquitin binding pocket of HDAC6 zinc finger domain

Materials and Methods

Cloning, expression and purification of HDAC6 protein samples

DNAs encoding HDAC6$^{1109\text{-}1213}$ and HDAC6$^{1109\text{-}1215}$ were both subcloned into a modified pET28 vector encoding a thrombin cleavable (GenBank EF442785) N-terminal His6-tag (pET28-LIC) and HDAC6$^{1109\text{-}1215}$ was also subcloned into a modified pET28 vector encoding an N-terminal AviTag for *in vivo* biotinylation and a C-terminal His6-tag (p28BIOH-LIC). Subcloning was performed using ligation-independent InFusion™ cloning kit (ClonTech) and verified by DNA sequencing. All proteins were over-expressed in a BL21 (DE3) Codon Plus RIL E. coli strain (Agilent). Cultures were grown in M9 minimal media supplemented with 50 µM $ZnSO_4$ and 10 µg/mL biotin for HDAC6$^{1109\text{-}1215}$ p28BIOH-LIC expression. Expression cultures were induced using 0.5 mM IPTG overnight at 15 °C. Proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen) and the His6-tag was removed by thrombin protease for HDAC6$^{1109\text{-}1213}$ pET28-LIC. Uncleaved proteins and thrombin were removed by another pass with Ni-NTA resin. Proteins were further purified using gel filtration (Superdex 75, GE Healthcare). The final concentrations of purified proteins were 5-10 mg/mL as measured by UV absorbance at 280 nm.

DNA encoding HDAC6$^{1\text{-}1215}$ was cloned into a modified derivative pFastBac Dual vector (Invitrogen) encoding an N-terminal AviTag and a C-terminal His6 tag (pFBD-BirA). Cloning was completed using a ligation-independent InFusionTM cloning kit (ClonTech) and verified by DNA sequencing. HDAC6$^{1\text{-}1215}$ was over-expressed in sf9 insect cells. Cultures were grown in HyQ SFX Insect Serum Free Medium (Fisher Scientific) to a density of 4x10^6 cells/mL and infected with 10 mL of P3 viral stock media per 1 L of cell culture. Cell culture medium was collected after 4 days of incubation in a shaker. Protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen). Following overnight dialysis, protein was further purified using anion exchange (HiTrap Q HP, GE Healthcare) and gel filtration (Superdex 75, GE Healthcare). The final concentration of purified HDAC6$^{1\text{-}1215}$ was 3.5 mg/mL as measured by UV absorbance at 280 nm.

Crystallisation and structure determination of HDAC6-ligand cocrystals

The apo crystal form of HDAC6$^{1109\text{-}1215}$ (PDB ID: 3C5K) was previously reported and can be obtained by mixing the protein solution at 3.5 mg/mL 1:1 with 3.5 M sodium formate, 0.1 M bis tris propane, 5 % (v/v) ethylene glycol, pH 7 using the vapor diffusion method at room temperature. These crystals can be used to seed for the soaking amenable crystal form for fragment screening. Diluting a 1 µL drop of these crystals 1:10,000 with mother liquor and vortexing the sample vigorously yields a seed mix. HDAC6$^{1109\text{-}1213}$ can then be crystallized in a high salt condition containing 2 M Na formate, 0.1 M Na acetate pH 4.6, 5 % ethylene glycol, again by the vapor diffusion method. 500 nL of protein are added to 400 nL mother liquor and then 100 nL seed mix per drop, typically plates are set up using a Mosquito (TTP LabTech). These crystals have a solvent exposed ubiquitin binding pocket, amenable to soaking. For all compounds, HDAC6$^{1109\text{-}1213}$ crystals were soaked by

adding 5 % (v/v) of a 200 mM or 400 mM DMSO-solubilized stock of each compound to the drop for 2 hours prior to mounting and cryo-cooling.

X-ray diffraction data for HDAC6$^{1109-1213}$ crystals soaked with SGC-T094 or SGC-T164 were collected at 100K at Rigaku FR-E Superbright home source at a wavelength of 1.54178 Å. HDAC6$^{1109-1213}$ crystals soaked during LabXChem pipeline were collected at 100K at i04-1, Diamond Light Source (Harwell, U.K.) at a wavelength of 0.92819 Å, hit fragment datasets were identified using a DIMPLE-PANDDA pipeline. All datasets were processed with XDS and Aimless. Cycles of COOT, for model building and visualization, with REFMAC, for restrained refinement, were used to refine the models. Final models were validated with MOLPROBITY.

In vitro assays

Surface Plasmon Resonance (SPR): SPR studies were performed using a Biacore T200 (GE Health Sciences). Approximately 5000 response units (RU) of biotinylated HDAC6$^{1109-1215}$ were coupled onto one flow cell of a SA chip as per manufacturer's protocol, while an empty flow cell was used for reference subtraction. 2-fold serial dilutions for all compounds were prepared in 10 mM HEPES pH 7.4, 150 mM NaCl, 0.005 % (v/v) Tween-20 and 1 % (v/v) DMSO. K_D determination experiments were performed using single-cycle kinetics with a contact time of 30 s and a flow rate of 30 µL/min at 20 °C. K_D values were calculated using steady state affinity fitting and the Biacore T200 Evaluation software (GE Health Sciences).

Fluorescence Polarisation (FP) RLRGG Peptide Displacement: All experiments were performed in a total volume of 10 µL in 384-well black polypropylene PCR plates (Axygen). Fluorescence polarization (FP) was measured using a BioTek Synergy 4 (BioTek) after 10 min of incubation. The excitation and emission wavelengths were 485 nm and 528 nm, respectively. In each well, 9 µL 2 mM compound solutions in buffer containing 10 mM HEPES pH 7.4, 150 mM NaCl and 1 % (v/v) DMSO were diluted in the same buffer. 1 µL 30 µM HDAC6$^{1109-1215}$ or 10 µM HDAC6$^{1-1215}$ and 500 nM N-terminally FITC-labelled RLRGG were then added to each well. Following 1 in centrifugation at 250 g, the assay was incubated for 10 min before FP analysis.

Isothermal Titration Calorimetry (ITC): HDAC6$^{1109-1215}$ was diluted to 50 µM in 10 mM HEPES pH 7.4, 150 mM NaCl and 1 % (v/v) DMSO and all compounds were diluted to 1 mM in the same buffer. All ITC measurements were performed at 25 °C on a Nano ITC (TA Instruments). A total of 25 injections, each of 2 µL, were delivered into a 0.167 mL sample cell at a 180-second interval. The data were analyzed using Nano Analyze software and fitted to a one-site binding model.

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Publications arising from this work

- Harding RJ, Ferreira de Freitas R, Collins P, Franzoni I, Ravichandran M, Ouyang H, Juarez-Ornelas KA, Lautens M, Schapira M, von Delft F, Santhakumar V, Arrowsmith CH (2017) "Small Moleculte Antagonists of the Interaction between the Histone Deacetylase 6 Zinc-Finger Domain and Ubiquitin." J Med Chem 60(21):9090-9096.
- Ferreira de Freitas R, Harding R, Franzoni I, Ravichandran M, Mann MK, Outang H, Lautens M, Santhakumar V, Arrowsmith CH, Schapira M (2018). "Identification and Structure-Activity Relationship of HDAC6 Zinc-finger Ubiquitin Binding Domain Inhibitors" bioRxiv 268557; doi: https://doi.org/10.1101/268557

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