Supplementary Information

Chemical Phylogenetics of Histone Deacetylases

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I. Supplementary Results

Supplementary Figure 1

Supplementary Figure 1 Comparative profiling of HDAC substrates identifies preferences distinct from molecular phylogenetic class assignments. (a) Chemical structure of substrates 3 and 5. (b) Comparative activity of HDAC1, 2, 3 and 6 for Boc-protected acetyl-lysine substrate 5 and tripeptide acetyl-lysine substrate 3, studied at equivalent substrate concentrations (10 μM). Substrate 3 is the preferred substrate for these Class I and IIb enzymes. (c) Chemical structure of substrates 4 and 6. (d) Comparative activity of HDAC4, 5, 7, 8 and 9 for Boc-protected trifluoro acetyl-lysine substrate 4 and tripeptide trifluoro acetyl-lysine substrate 6, studied at equivalent substrate concentrations (10 μM). Substrate 6 is the preferred substrate for these Class I and IIa enzymes. The robust activity of HDAC8 for trifluoro acetyl lysine-based substrates resonates with published observations from the Schwienhorst laboratory, who have innovated HDAC assay design and substrate preference determination. See Supplementary Methods for complete assay details.
Supplementary Figure 2  Determination of $K_M$ for substrate 6. (a-e) Michaelis-Menten Plots for substrate 6 and human, recombinant HDACs (as labeled) in a miniaturized, kinetic trypsin-coupled assay. (f) Table of $K_M$ values derived. Also provided are concentrations of enzymes required for the miniaturized HDAC assay, afforded by substrate 6. The reduction in enzyme used per well enables reagent-efficient compound annotation as well as high-throughput screening. See Supplementary Methods for complete assay details.
Supplementary Figure 3

Supplementary Figure 3  Chemical structures of tool and pharmaceutical HDAC inhibitors used for biochemical profiling.
Phylogenetic analysis of human HDAC1-11. Amino acid sequences for each human histone deacetylase were retrieved from the National Centers for Biotechnology Information, and aligned using MAFFT as described below. (a) Maximum parsimony (MP) tree for full-length HDAC1-11. (b) Maximum likelihood (ML) tree for full-length HDAC1-11. Bootstrap support values are provided on the ML tree. The MP and ML trees have identical topologies, consistent with high observed bootstrap support. See Supplementary Methods for a complete description of computational methods.
Supplementary Figure 5 Pairwise comparative biochemical profiling of meta- (red) and para-substituted (blue) sub-libraries for relative inhibition of Class IIa HDACs. The complete library was studied and is displayed at a range of concentrations (0.03, 0.3, 3.0 and 30.0 μM). a) HDAC4 and HDAC5, b) HDAC4 and HDAC7, c) HDAC4 and HDAC9, d) HDAC5 and HDAC7, e) HDAC5 and HDAC9, f) HDAC7 and HDAC9,
Supplementary Figure 6

Comparative biochemical profiling of (a) trichostatin A (TSA), (b) SAHA and (c) pandacostat, for inhibition of HDAC1-9. Compounds were arrayed in 384-well plate format as library stock solutions at 10 mM top concentration. Dilution series (3-fold) were created by hand micropipette. Compounds were studied for inhibition of HDACs following robotic pin transfer and a brief pre-incubation period as described in Supplementary Methods. Dose-response data are presented for each compound. Data comprise the mean of three replicates. Curves were fit by logistic regression using Graph Pad Prism. These data confirm the unexpected selectivity of TSA and SAHA; they also confirm the markedly improved selectivity of pandacostat.
Supplementary Figure 7

(a) Visualization of biochemical inhibition of individual HDAC isoforms by pandacostat. (b) Summary of pandacostat Ki values for HDAC1-9 presented with standard deviation (Spotfire DecisionSite).
Supplementary Figure 8

Examination of Zinc chelation by HDAC inhibitors in published, crystallographic data. (a) Trichostatin A bound to HDAC8 (1T64)\(^3\). (b) SAHA bound to a bacterial Class II histone deacetylase homologue (1ZZ1)\(^4\). (c) HDAC4 in complex with hydroxamate based inhibitors (2VQM)\(^5\). (d) HDAC7 in complex with TSA (3C10)\(^6\). All data were obtained from the Protein Data Bank (Research Collaboratory for Structural Bioinformatics) and images were created in PyMOL Molecular Viewer (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, Palo Alto, CA, USA.).
**Supplementary Table 1**  Biochemical inhibition of HDAC1-9 by tool and pharmaceutical HDAC inhibitors (Ki [μM]).

| Compound   | HDAC1  | HDAC2  | HDAC3  | HDAC4  | HDAC5  | HDAC6  | HDAC7  | HDAC8  | HDAC9  |
|------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| APHA       | 0.055  | 0.125  | 0.25   | 17.5   | 11.5   | 0.03   | 7      | 0.6    | 10     |
| StdDev     | 0.004  | 0.01   | 0.02   | 1.2    | 0.6    | 0.004  | 0.9    | 0.04   | 1      |
| Apicidin   | 0.000004 | 0.0000012 | 0.000026 | -      | -      | -      | -      | 0.049  | -      |
| StdDev     | 0.000004 | 0.00000003 | 0.000005 | -      | -      | -      | -      | 0.02   | -      |
| CI-994     | 0.05   | 0.19   | 0.55   | -      | -      | -      | -      | -      | -      |
| StdDev     | 0.0045 | 0.015  | 0.035  | -      | -      | -      | -      | -      | -      |
| Depudecin  | 5.5    | 12.5   | 14.5   | -      | -      | -      | -      | -      | -      |
| StdDev     | 0.4    | 0.65   | 0.95   | -      | -      | -      | -      | -      | -      |
| FK-228     | 0.0000015 | 0.0000038 | 0.00015 | 0.0205 | 0.55   | 0.0095 | 1.25   | 0.00015 | 1.1  |
| StdDev     | 0.000001 | 0.000003 | 0.000025 | 0.0035 | 0.06   | 0.004  | 0.2    | 0.00033 | 0.22 |
| HCToxin    | 0.19   | 0.47   | 1.35   | -      | -      | -      | -      | -      | 10.5   |
| StdDev     | 0.02   | 0.06   | 0.115  | -      | -      | -      | -      | -      | 1.4    |
| ITF-2357   | 0.002  | 0.003  | 0.003  | 1.05   | 0.6    | 0.0042 | 0.24   | 0.0039 | 0.39   |
| StdDev     | 0.0001 | 0.0001 | 0.0001 | 0.15   | 0.085  | 0.0002 | 0.025  | 0.001  | 0.05   |
| LAO-824    | 0.00055 | 0.00014 | 0.0042 | 2.25   | 0.42   | 0.0095 | 9.5    | 0.34   | 9      |
| StdDev     | 0.0005 | 0.00003 | 0.0001 | 0.3    | 0.04   | 0.00036 | 4.55  | 0.035  | 7.5    |
| LBH-589    | 0.001  | 0.00065 | 0.0011 | 0.55   | 0.08   | 0.0015 | 4.55   | 0.105  | 3.2    |
| StdDev     | 0.0001 | 0.0001 | 0.00015 | 0.05   | 0.01   | 0.0005 | 0.315  | 0.02   | 0.2    |
| MGCD-0103  | 0.009  | 0.034  | 0.265  | -      | -      | -      | -      | -      | -      |
| StdDev     | 0.001  | 0.002  | 0.015  | -      | -      | -      | -      | -      | -      |
| MS-275     | 0.022  | 0.065  | 0.36   | -      | -      | -      | -      | -      | -      |
| StdDev     | 0.002  | 0.005  | 0.015  | -      | -      | -      | -      | -      | -      |
| Nilubacin  | -      | -      | -      | -      | 2.2    | -      | 0.75   | -      | -      |
| StdDev     | -      | -      | -      | -      | 0.38   | -      | 0.07   | -      | -      |
| 4-PBHA     | 0.295  | 0.43   | 1.85   | 16     | 1     | 0.15   | 1.86   | -      | -      |
| StdDev     | 0.04   | 0.03   | 0.1    | 1.26   | 0.01   | -      | 0.1    | -      | -      |
| PXD-101    | 0.00065 | 0.00085 | 0.0015 | 0.38   | 0.175  | 0.0016 | 0.075  | 0.025  | 0.25   |
| StdDev     | 0.0005 | 0.00005 | 0.0005 | 0.06   | 0.02   | 0.00015 | 0.01  | 0.002  | 0.05   |
| Pyroxamide | 0.0027 | 0.0036 | 0.008  | -      | 4.75   | 0.0048 | -      | 1      | -      |
| StdDev     | 0.00015 | 0.0002 | 0.0015 | 1.1    | 0.0003 | -      | 0.11   | -      | -      |
| SAHA       | 0.0013 | 0.0016 | 0.005  | -      | 3.6    | 0.0016 | -      | 0.48   | -      |
| StdDev     | 0.00005 | 0.00005 | 0.0002 | -      | 0.38   | 0.00005 | -     | 0.02   | -      |
| Scriptaid  | 0.0015 | 0.0022 | 0.0041 | 7.5    | 1      | 0.00025 | 2.25  | 0.105  | 8      |
| StdDev     | 0.00005 | 0.00005 | 0.0005 | 0.75   | 0.1    | 0.0001 | 0.35   | 0.01   | 1      |
| Suberoha   | 0.19   | 0.029  | 0.125  | 9.5    | 0.0145 | -      | 0.95   | -      | -      |
| StdDev     | 0.0035 | 0.0045 | 0.01   | 0.5    | 0.0015 | -      | 0.1    | -      | -      |
| Trichostatin | 0.0002 | 0.00065 | 0.0005 | 1.4    | 0.26   | 0.001  | 0.185  | 0.045  | 0.8    |
| StdDev     | 0.00045 | 0.00005 | 0.001  | 0.035  | 0.0001 | 0.02   | 0.015  | 0.1    | -      |
| Tubacin    | 0.028  | 0.042  | 0.275  | 17.5   | 1.5    | 0.016  | 8.5    | 0.17   | -      |
| StdDev     | 0.004  | 0.0035 | 0.02   | 2.5    | 0.25   | 0.002  | 1.5    | 0.01   | -      |
### MAFFT Alignment of HDAC1-11

|    | HDAC1          | HDAC2          | HDAC3          | HDAC4          | HDAC5          | HDAC6          | HDAC7          | HDAC8          | HDAC9          | HDAC10         | HDAC11         |
|----|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|    | M---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
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| HDAC4 | MSSQSHDGL       | SGRDQPVELL       | NPRAVNHMPS       | TVDVATALPL       | QVAPSAV---       |               |               |               |               |               |               |
| HDAC5 | MNPSNESDOM       | SGRESFEILEL       | PRTSLHISIPV       | TVEVKPVLP-       | RAMPSSMGGG       |               |               |               |               |               |               |
| HDAC9 | M---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| HDAC7 | M---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| HDAC6 | M---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| HDAC10| M---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| HDAC11| M---------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|

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|    | -----PMDL      | RLDHQFSLPV       | AEPALREQQL       | QDELALQKQ       |               |               |               |               |               |               |               |
|    | GGSPSPVEL       | RGalVGSV---       | DPTLREQQL       | QDELLALKQQ       |               |               |               |               |               |               |               |
|    | PLDL          | RTDLRMMMPFV       | VDFVREKQQL       | QDELLLIQQQ       |               |               |               |               |               |               |               |
|    | DL           | RVGQR---PP       | VEP-----PP       | EPTLLALQRP       |               |               |               |               |               |               |               |
| IPNLAEVKKK | GKMKKLCQAM       | EEDLIVGLQG       | MDLNLEAEAL       | AGTGLVLDEEQ       |               |               |               |               |               |               |               |
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|    | QQIQRQILIA       | EFRQRHEQLS       | RQHEAQLHEH       | IKQQQEMLAM       | KHHQEL----       |               |               |               |               |               |               |
|    | QQLQKOLLFA       | EFKQVHDHITL       | RQHEVQQLKH       | LKQQQEMLAA       | KQQQEMLAAK       |               |               |               |               |               |               |
|    | QIQIQtitLLIA       | EFKQHENLIT       | RQHQAQLQEH       | IK---EILAI       | KQQQEL---       |               |               |               |               |               |               |
|    | QRLIHHLFLA       | GLQQQRS---       |               |               |               |               |               |               |               |               |               |
|    | LNEFHLWDWDD       | SFPEGPERLH       | AIKQLIQEG       | LLDRCVSFQA       | RFAEKEELML       |               |               |               |               |               |               |
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|    | LEHQQK          | LERHRQEQEL       | EKQHREQKLQ       | QLKNKEKGE       | SAVASTEVKM       |               |               |               |               |               |               |
|    | RQQLEQQRQ       | REQQRQ---EL       | EKQRLEQQLL       | IIRNKEKSE       | SAVASTEVKL       |               |               |               |               |               |               |
|    | LEKEQK          | LEQQRQEQEV       | EHRHRQQQLP       | PLRGKDRG'RE       | RAVASTEVQ       |               |               |               |               |               |               |
|    | VEPMR-         | LSMDDTPMPEL       | QVGQPQQELR       | QLLHKDKSKR       | SAVASSVVKQ       |               |               |               |               |               |               |
|    | VHS-LEYIDL      | METTQYMNNEG       | ELRVLADTYD       | SYLHPNSYS       | CAACLASGVS       |               |               |               |               |               |               |
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|    | KLQEFVINNKK      | --KALA---       |               |               |               |               |               |               |               |               |               |
| Sequence | Description |
|----------|-------------|
| LSVELQ   | P LDEAVLQKQP | ---NINAVAT LEKVIIEQSK |
| LGNELE   | P LAEDEILQSP | ---NMNAVIS LQKIIIEQSP |
| LGNRTD   | P LSEEGWQKQP | ---NLNAIRS LEAVIRVHSDK |
| LGD      | P PPLTLPPP  | ---LSGALAS ITETIQVHRR |
| LGD      | P APPLSGMAP  | ---CSALES IQSARAAQP |
| FGLGL    | I-GPESP      | ---SVSAQNS DTPPLL  |
| LFENLRLM | PHAPG        | VMQQAIPEDA IPEESGDIE DEDD--PDKRI |
| LFENLRLM | PHAPG        | VMQQAIPEDA VHEDSGDIE GEDD--PDKRI |
| IFENLKLML| NHAPSA       | VQIHDPAPDL LTYDRTDEAE AERGEPENY |
| IKNLKHV  |             | ---TYDRT---T CENNEAETS |
| YWRCLQRT | TSTAG        | RSLREAQ---A GETEEAETVS |
| HSVCQKFP |             | ---MSLKFSS---G ADKIVEAIVT |
| YWGCMQRL | ASCPD        | SWVPKVP---A GETEAAETVS |
| HWKSLQQO |             | ---DVTA VM---P SPSHSPERMP |
| SICSSD    | CEEFSDSEE   | EGEEGRKNSS NFKK-AKRVK TEDEKEKDE |
| SIRASSDKRIA | CDEEFSDEDE | EGEEGRNNVA DHKGTKNKAAR IEEDKKEETED |
| S-------RPE | APNEFYDGDH  | DN---------D |
| AMASLSVGK | PAEK        | ---A QAAEAAREH |
| AMALLSVGAE| QAAAAAAREH  | ---SP---SP--- |
| ALASLSVGIL| AED         | ---A QAAAAAAREH |
| GKVTSASFG | ESTPQTNSE   | TAVVATQQDQ PSEAATGQAT LAQTISEAAL |
| PPLLPGGPVC | KAAASCPLP  | LDQPCLCPAP SVRTAVALT PDITLVLPDP |
| SICSSDKRIA | CEEFSDSEE   | EGEEGRKNSS NFKK-AKRVK TEDEKEKDE |
| SIRASSDKRIA | CDEEFSDEDE | EGEEGRNNVA DHKGTKNKAAR IEEDKKEETED |
| S-------RPE | APNEFYDGDH  | DN---------D |
| AMASLSVGK | PAEK        | ---A QAAEAAREH |
| AMALLSVGAE| QAAAAAAREH  | ---SP---SP--- |
| ALASLSVGIL| AED         | ---A QAAAAAAREH |
| GKVTSASFG | ESTPQTNSE   | TAVVATQQDQ PSEAATGQAT LAQTISEAAL |
| PPLLPGGPVC | KAAASCPLP  | LDQPCLCPAP SVRTAVALT PDITLVLPDP |
| EKKEVTEEREK | TKE---EKPE  | AKGVKEE--- --- --- --- --- --- |
| KKTVKEEDEK | SDKNSGEKTD  | TGKTKE--- --- --- --- --- --- |
| KESDVE--- | ---RPF EEPEEDEE--- --- --- --- --- --- |
| EKKEVTEEREK | TKE---EKPE  | AKGVKEE--- --- --- --- --- --- |
| KKTVKEEDEK | SDKNSGEKTD  | TGKTKE--- --- --- --- --- --- |
| KESDVE--- | ---RPF EEPEEDEE--- --- --- --- --- --- |
| SICSSDKRIA | CEEFSDSEE   | EGEEGRKNSS NFKK-AKRVK TEDEKEKDE |
| SIRASSDKRIA | CDEEFSDEDE | EGEEGRNNVA DHKGTKNKAAR IEEDKKEETED |
| S-------RPE | APNEFYDGDH  | DN---------D |
| AMASLSVGK | PAEK        | ---A QAAEAAREH |
| AMALLSVGAE| QAAAAAAREH  | ---SP---SP--- |
| ALASLSVGIL| AED         | ---A QAAAAAAREH |
| GKVTSASFG | ESTPQTNSE   | TAVVATQQDQ PSEAATGQAT LAQTISEAAL |
| PPLLPGGPVC | KAAASCPLP  | LDQPCLCPAP SVRTAVALT PDITLVLPDP |
| EKKEVTEEREK | TKE---EKPE  | AKGVKEE--- --- --- --- --- --- |
| KKTVKEEDEK | SDKNSGEKTD  | TGKTKE--- --- --- --- --- --- |
| KESDVE--- | ---RPF EEPEEDEE--- --- --- --- --- --- |
| EKKEVTEEREK | TKE---EKPE  | AKGVKEE--- --- --- --- --- --- |
| KKTVKEEDEK | SDKNSGEKTD  | TGKTKE--- --- --- --- --- --- |
| KESDVE--- | ---RPF EEPEEDEE--- --- --- --- --- --- |
IQTPLASSTD HQTPPTSPVQ GTTPQISPST LIGSLRTELE GSESQGASES
LNIRGKEAAA LSMFHVSTPL PVMTGGLSCL ILGLVLPAY GFTPDLVLVA

QAPGEENLLG EAAAGQDMDA SM-----LMQ GSRGLTDQAI FYAVTLPWC
LGPHGHLQGP HAALLAAMLR GlAGGGVLALE LEENSTPQLA GILARVLNGE

PHLVAVCPIP AAAGLVTQPC GDCGTIQENW VCLSCYQVYC GRYINGHMLQ
APPSSLGPSSV ASPEDVQALM YLRGQLEPWQ KMLQCHP---

HHGNSGHPLV LSYIDLSAWC YYCQAYVHHQ AILDVKNIAH QNKFGEDMPH

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II. Supplementary Methods

Reagents. All chemical reagents were purchased from Lancaster, Sigma-Aldrich, Alfa-Aesar, or Fluka Chemicals and were used as received unless otherwise stated. Amino acids were purchased from Bachem. Substrates for biochemical assays were synthesized by RM. Antibodies for immunoblotting were obtained from Sigma (Acetyl-tubulin, 6-11B-1) and Upstate Biotechnology (Acetyl-H3K18, 07-354). Recombinant, human histone deacetylases were expressed purchased from BPS Biosciences. Trypsin and buffer components were purchased from Sigma-Aldrich. Black, opaque-bottom microtiter plates were purchased from Corning.

Instrumentation. Proton and carbon nuclear magnetic resonance ($^1$H & $^{13}$C NMR) spectra were recorded on Varian AS-500 (500 MHz), Varian AS-400 (400MHz) or Bruker 300 (300 MHz) spectrometers. Chemical shifts for protons are reported in parts per million (ppm) downfield from tetramethylsilane and are referenced against the dimethylsulfoxide lock signal (1H, 2.50; 13C, 39.52 ppm). Data is reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet) and coupling constants (Hz). Preparatory and analytical LCMS were performed on mass directed Autopurification systems from Waters Co. (Milford, MA), operated by Fractionlynx 4.0 or Masslynx software with Waters Xterra columns (C18, 10 μm, 19 x 50 mm for preparatory; C18, 5 μm, 4.6 x 50 mm for analytical). High-resolution mass spectra were acquired on a Bruker Daltonics APEXIV 4.7 Tesla Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR-MS), with ESI (Electro Spray Ion) source.
Synthetic Procedures

Scheme S1. Synthesis of hydrazide S-5\(^1\).

\((E)-4-(3\text{-ethoxy-3-oxoprop-1-enyl})\text{benzoic acid (S-1)}\)

To a flask was added 4-formylbenzoic acid (1.5 g, 10 mmol), 3-ethoxy-3-oxopropanoic acid (2.0 g, 15 mmol), piperidine (0.08 mL, 0.81 mmol), and pyridine (4 mL) at room temperature. The reaction mixture was heated to 100 °C for 18 h under a steady flow of nitrogen gas, cooled to room temperature, and poured into 2 M aqueous HCl (100 mL). The resulting mixture was cooled to 0 °C and filtered. The filter cake was washed with acetonitrile (2 x 10 mL) and dried in vacuo. Cinnamyl ester S-1 (1.63 g, 74%) was isolated as a white solid and carried on to hydrazide formation without further purification.

\((E)-\text{ethyl 3-}(4\text{-hydrazinecarbonyl})\text{phenyl} \text{acrylate (S-2)}\)

To a solution of S-1 (0.44 g, 2.0 mmol) in dichloromethane (10 mL) was added triethylamine (0.36 mL, 2.0 mmol) and methyl chloroformate (0.19 mL, 2.0 mmol) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C before hydrazine (0.30 mL, 6.0 mmol)

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\(^1\) The routes to S-1 - S-10, S-5\(^*\)HCl, S-10\(^*\)HCl, S-5, and S-10 were proposed by the authors and the syntheses were performed at Medicilon Shanghai. The characterization data for S-5 and S-10 were collected and compiled by the authors.
was added. The resulting solution was stirred for an additional 2 h at 0 °C. Saturated aqueous NaHCO₃ (10 mL) was added to the reaction mixtures and the resulting biphasic solution was stirred for 30 min at room temperature. The organic layer was separated, dried, and the solvent removed via rotary evaporation. The resulting residue was purified by flash chromatography on silica (eluting with EtOAc) to yield compound S-2 (0.23 g, 49%) as a white solid.

(E)-tert-butyl 2-(4-(3-ethoxy-3-oxoprop-1-enyl)benzoyl)hydrazinecarboxylate (S-3)

To a solution of hydrazide S-2 (6.00 g, 25.6 mmol) in dichloromethane (300 mL) was added Boc anhdyride (5.40 g, 26.2 mmol) and DMAP (12.5 g, 103 mmol). The mixture was stirred at room temperature for 3 h, concentrated, and loaded directly on to silica. Flash chromatography, eluting with 1:1 EtOAc / petroleum ether, yielded S-3 (5.76 g, 67.3%).

(E)-tert-butyl 2-(4-(3-(hydroxyamino)-3-oxoprop-1-enyl)benzoyl)hydrazinecarboxylate (S-4)

To a solution of S-3 (5.76 g, 17.2 mmol) in methanol (300 mL) was added a solution of hydroxylamine hydrochloride (11.9 g, 171 mmol) in 1 M NaOH / ethanol (341 mL). The reaction mixture was stirred for 18 h and concentrated. The residue was dissolved in water to yield a colorless homogenous solution, which was neutralized to pH 7 by the addition of aqueous 1 M HCl. The resulting suspension was extracted with ethyl acetate. The combined organic extracts were dried and concentrated via rotary evaporation. Crude S-4 was loaded on to silica and purified via flash chromatography, eluting with ethyl acetate, to yield S-4 (3.80 g, 68.8%).

(E)-3-(4-(hydrazinecarbonyl)phenyl)-N-hydroxyacrylamide hydrochloride (S-5·HCl)

Boc protected hydrazide S-4 (3.50 g, 10.9 mmol) was dissolved in 6 M HCl / methanol (20 mL) and stirred at ambient temperature for 1 h, while a white precipitate formed. The reaction mixture was filtered to yield the title compound as a white solid (2.38 g, 84.9%).

(E)-3-(4-(hydrazinecarbonyl)phenyl)-N-hydroxyacrylamide (S-5)

A solution of 1 M aqueous NaOH was added dropwise to a suspension of S-5·HCl (1.8 g, 7.0 mmol) in water (200 mL) until the pH reached 11. The colorless, homogeneous solution was neutralized with dilute aqueous HCl. The resulting precipitate was isolated via filtration and dried in vacuo to yield S-5 (1.2 g, 78%) as a gray solid. ¹H NMR (500 MHz, DMSO) δ 10.85 (s, 1H), 9.84 (s, 1H), 9.12 (s, 1H), 7.85 (d, J = 7.8, 2H), 7.63 (d, J = 7.8, 2H); 13C NMR (126 MHz, DMSO) δ 165.92, 163.13, 138.08, 138.03, 134.44, 128.22, 128.07, 121.36. HRMS (ESI+) found: 222.0876s [M+H] calculated: 222.0873 [M+H].
Scheme S2. Synthesis of hydrazide S-10.

(E)-3-(3-ethoxy-3-oxoprop-1-enyl)benzoic acid (S-6)

To a flask was added 3-formylbenzoic acid (1.5 g, 10 mmol), 3-ethoxy-3-oxopropanoic acid (2.0 g, 15 mmol), piperidine (0.08 mL, 0.81 mmol), and pyridine (4 mL) at room temperature. The reaction mixture was heated to 100 °C for 18 h under a steady flow of nitrogen gas, cooled to room temperature, and poured into 2 M aqueous HCl (100 mL). The resulting mixture was cooled to 0 °C and filtered. The filter cake was washed with acetonitrile (2 x 10 mL), and dried in vacuo. Cinnamyl ester S-6 (2.20 g, 100%) was isolated as a white solid and carried on to hydrazide formation without further purification.

(E)-ethyl 3-(4-(hydrazinecarbonyl)phenyl)acrylate (S-7)

To a solution of S-6 (0.44 g, 2.0 mmol) in dichloromethane (10 mL) was added triethylamine (0.36 mL, 2.0 mmol) and methyl chloroformate (0.19 mL, 2.0 mmol) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C before hydrazine (0.30 mL, 6.0 mmol) was added. The resulting solution was stirred for an additional 2 h at 0 °C. Saturated aqueous NaHCO₃ (10 mL) was added to the reaction mixture and the resulting biphasic solution was stirred for 30 min at room temperature. The organic layer was separated, dried, and the solvent removed via rotary evaporation. The resulting residue was purified by flash chromatography on silica (eluting with EtOAc) to yield compound S-7 (0.26 g, 56%) as a white solid.

(E)-tert-butyl 2-(4-(3-ethoxy-3-oxoprop-1-enyl)benzoyl)hydrazinecarboxylate (S-8)
To a solution of hydrazide S-7 (6.00 g, 25.6 mmol) in dichloromethane (200 mL) was added Boc anhdyride (5.40 g, 26.2 mmol) and DMAP (12.5 g, 103 mmol). The mixture was stirred at room temperature for 3 h. The mixture was concentrated and loaded directly on to silica to yield S-8 (7.2 g, 84%) following flash chromatography (eluting with 1:1 EtOAc / petroleum ether).

\((E)\)-\textit{tert}-butyl 2-(4-(3-(hydroxyamino)-3-oxoprop-1-enyl)benzoyl)hydrazinecarboxylate (S-9)

To a solution of S-8 (7.0 g, 20.8 mmol) in methanol (300 mL) was added a solution of hydroxylamine hydrochloride (14.5 g, 208 mmol) in 1 M NaOH in ethanol 420 mL). The reaction mixture was stirred for 18 h and then concentrated. The residue was dissolved in water to yield a colorless homogenous solution, which was neutralized to pH 7 by the addition of aqueous 1 M HCl. The resulting suspension was extracted with ethyl acetate. The combined organic extracts were dried and concentrated via rotary evaporation. Crude S-9 was loaded on to silica and purified via flash chromatography, eluting with ethyl acetate, to yield S-9 (5.2 g, 78%).

\((E)\)-3-(4-(hydrazinecarbonyl)phenyl)-N-hydroxyacrylamide hydrochloride (S-10*HCl)

Boc protected hydrazide S-9 (4.50 g, 14.0 mmol) was dissolved in 6 M HCl / methanol (30 mL) and stirred at ambient temperature for 1 h, while a white precipitate formed. The reaction mixture was filtered to yield the title compound as a white solid (3.0 g, 83%).

\((E)\)-3-(3-(hydrazinecarbonyl)phenyl)-N-hydroxyacrylamide (S-10)

A solution of 1 N aqueous NaOH was added dropwise to a suspension of S10*HCl (2.0 g, 7.8 mmol) in water (100 mL) until the pH reached 7. A precipitate formed and was isolated via filtration and dried in vacuo to yield S-10 (1.1 g, 63%) as a gray solid. \(^1\)H NMR (500 MHz, DMSO) \(\delta\) 10.81 (s, 1H), 9.85 (s, 1H), 9.08 (s, 1H), 8.02 (s, 1H), 7.80 (d, \(J = 7.5, 1H\)), 7.69 (d, \(J = 7.4, 1H\)), 7.63 – 7.36 (m, 2H), 6.55 (d, \(J = 15.8, 1H\)), 4.58 (s, 2H); \(^13\)C NMR (126 MHz, DMSO) \(\delta\) 166.12, 163.22, 138.37, 135.63, 134.60, 131.04, 129.72, 128.45, 126.28, 120.75. HRMS (ESI+) found: 222.0879 [M+H] calculated: 222.0873 [M+H].
Scheme S3. Library synthesis.

Each well of a 96-well microtiter plate was charged with 10 μL of a distinct, commercially-available aldehyde (0.2 M in DMSO) and 190 μL of a stock solution of the appropriate hydrazide (S-5 or S-10) in DMSO (0.0105 M). The plate was heated at 70 °C for 36 h. LCMS analysis confirmed that a sampling of acyl hydrazone products were analytically pure (>95%). This stock plate of m- and p- substituted cinnamyl acyl hydrazones was used in screening, as described. Library compounds were enumerated using Reactor 5.2, ChemAxon, Budapest, Hungary. www.chemaxon.com/products.html

Scheme S4. Synthesis of Pandacostat 22.

\[(E)-N\text{-hydroxy-3-}(4-((E)-2-(2,3,4\text{-trihydroxybenzylidene) hydrazinecarbonyl) phenyl) acrylamide (22)}\]

Compound 22 was resynthesized and purified to be re-subjected to the biochemical assay to confirm the results from the initial library screen. To a 4 dram vial charged with 2,3,4-trihydroxybenzaldehyde (25.9 mg, 0.168 mmol) was added 420 L of a 200 mM solution of hydrazide S-5 (0.084 mmol) in DMSO. The solution was heated on a rotating heating block at 70 °C for 16 h. Reaction progress was monitored via LCMS. Following purification by reverse phase preparatory LCMS (44 mL / min, CH3CN / H2O with 1% formic acid, 5 min gradient), 22 (7 mg) was isolated as a yellow powder (98% pure, by analytical LCMS). \(^1\)H NMR (300 MHz, DMSO) δ 12.01 (s, 1H), 11.51 (s, 1H), 10.84 (s, 1H), 9.49 (s, 1H), 9.13 (s, 1H), 8.54 (s, 1H), 8.48 (s, 1H), 7.96 (d, J = 8.3, 2H), 7.73 (d, J = 8.2, 2H), 7.53 (d, J = 16.2, 1H), 6.80 (d, J = 8.6, 1H), 6.59 (d, J = 8.6, 1H), 6.40 (d, J = 8.4, 1H); \(m/z\) (ES) 356 ([M-H]). \(^{13}\)C NMR (126 MHz, DMSO) δ 163.1, 162.6, 151.0, 149.5, 148.2, 138.8, 138.0, 134.0, 133.4, 128.9, 128.2, 121.9, 121.8, 111.5, 108.4. HRMS (ESI+) found: 358.1033 [M+H] calculated: 358.1034 [M+H].

(S)-2-amino-N-(4-methyl-2-oxo-2H-chromen-7-yl)-6-(2,2,2-trifluoroacetamido)hexanamide (ε-trifluoroacetyl-L-lysine-AMC) S-11
To a solution of Boc-ε-trifluoroacetyl-L-lysine-AMC (4.6 g, 9.2 mmol) in dry dichloromethane at 0 °C was added 5 mL of a 4 M solution of HCl / dioxane. The reaction mixture was warmed to room temperature and stirred over night. The solvent was evaporated under reduced pressure to afford the desired product in quantitative yield (4.0g) and excellent purity as white powder, which was used without further purification. **1H NMR (400 MHz, DMSO)** δ 11.53 (s, 1H), 9.47 (s, 1H), 8.47 (s, 3H), 7.95 – 7.80 (m, 1H), 7.76 (d, J = 8.8, 1H), 7.58 (d, J = 8.1, 1H), 6.30 (s, 1H), 4.12 (s, 1H), 3.17 (d, J = 5.5, 2H), 2.41 (d, J = 6.7, 3H), 1.88 (s, 2H), 1.61 – 1.46 (m, 2H), 1.38 (d, J = 6.4, 2H); **13C NMR (101 MHz, DMSO)** δ 168.33, 159.93, 156.15 (q, J = 35.8), 153.54, 153.09, 141.46, 126.11, 115.64, 115.59 (q, J = 289 Hz), 115.47, 112.69, 106.07, 52.93, 38.79, 30.60, 27.75, 21.45, 18.04. **HRMS (ESI+)** found: 400.1469 [M+H] calculated: 400.1479 [M+H].

(S)-2-(2-((S)-2-acetamido-4-methylpentanamido)acetamido)-N-(4-methyl-2-oxo-2H-chromen-7-yl)-6-(2,2,2-trifluoroacetamido)hexanamide (6)

(S)-11 hydrochloride (1.35 g, 3.10 mmol) was added to a solution of N, N-diisopropylethylamine (2.5 mL) and Ac-Leu-Gly-OH (805 mg, 3.50 mmol) in 100mL anhydrous dichloromethane followed by PyBop (1.8 g, 3.5 mmol) in dichloromethane (5 mL). After stirring over night at room temperature the reaction mixture was diluted with dichloromethane (200 mL) and washed with dilute HCl and then saturated aqueous sodium bicarbonate solution. The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified on silica gel (dichloromethane, MeOH 10:1) to yield the desired product as off-white solid (1.57 g, 83%). **1H NMR (400 MHz, DMSO)** δ 10.39 (s, 1H), 9.42 (t, J = 5.6, 1H), 8.35 (t, J = 5.8, 1H), 8.11 (d, J = 7.3, 1H), 8.05 (dd, J = 7.9, 4.0, 1H), 7.79 (d, J = 2.0, 1H), 7.71 (d, J = 8.7, 1H), 7.51 (dd, J = 8.7, 2.0, 1H), 6.26 (d, J = 1.2, 1H), 4.46 – 4.28 (m, 1H), 4.22 (dd, J = 15.0, 7.3, 1H), 3.85 – 3.63 (m, 2H), 3.16 (dd, J = 13.1, 6.8, 2H), 2.39 (d, J = 1.1, 3H), 1.86 (s, 3H), 1.81 – 1.19 (m, 9H), 0.85 (dd, J = 17.5, 6.5, 6H); **13C NMR (101 MHz, DMSO)** (mix of confomers) δ 172.93, 172.31, 171.38, 169.75, 169.13, 169.07, 166.34,
160.04, 156.10 (q, J = 36Hz), 153.65, 153.10, 142.13, 125.95, 115.98 (q, J=288Hz), 115.30, 115.16, 105.76, 53.53, 51.49, 50.88, 45.57, 44.89, 42.03, 41.24, 40.96, 40.52, 31.30, 27.98, 25.63, 24.18, 23.74, 23.09, 22.95, 22.72, 22.52, 22.49, 21.64, 21.55, 18.02. HRMS (ESI+) found: 612.2645 [M+H] calculated: 612.2640 [M+H].
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