A substrate-independent histone deacetylase inhibitor assay

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ABSTRACT

Developing molecularly targeted therapeutics with minimal off-target effects is facilitated by a clear understanding of compound selectivity among related targets. Histone deacetylase (HDAC) inhibitors have been pursued for various indications and recent work has focused predominantly on oncology. However, a clear understanding of the specificity of HDAC inhibitors has been challenging. Literature reports of the specificity of HDAC inhibitors have varied substantially, likely due to differences in substrate choice and enzyme sources. In particular, it has been suggested that use of non-specific substrates and the presence of multiple HDAC activities in enzyme preparations may complicate interpretation of inhibitor specificity experiments. To overcome these potential limitations of activity-based assays, we have developed an assay format based on measurement of the binding affinity of HDAC inhibitors rather than measurement of enzyme activity. A key advantage is this format is that it does not require use of a substrate and thus ameliorates concerns about lack of specificity of existing substrates. This assay is based on an Alexa Fluor 647®-labeled HDAC inhibitor or "tracer" which binds with a high affinity to Class I and Class IIb HDACs. Binding of the "tracer" to an epitope-tagged HDAC is detected by addition of a europium-labeled anti-epitope tag antibody. Binding of the tracer and antibody to the HDAC results in a high degree of FRET, whereas displacement of the tracer with an inhibitor results in a loss of FRET. Unlike activity assays, which can be affected by the presence of residual untagged endogenous HDACs from the host expression system, the signal in this format is dependent on the presence of an epitope tag on the specific HDAC of interest. We demonstrate utility of this method by determining inhibitor potencies for commonly used HDAC inhibitors for Class I and IIb HDACs.

MATERIALS AND METHODS

HDAC1-3,6,8 were obtained from BPS Bioscience as his- or GST-tagged proteins. HDAC10 (GST) was expressed and purified by Life Technologies. HDAC tracers, biotin or Eu-labeled anti-epitope tag antibodies, Eu-labeled streptavidin (Eu-SA), and assay buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 0.01% Brij-35) were from Life Technologies. White 384-well assay plates were from Corning (cat. no. 3673).

All assays were carried out using a simple three-step addition protocol:

1. 5 µL 3X test compound (3% DMSO v/v)
2. 5 µL 3X HDAC/antibody mixture
3. 5 µL 3X HDAC Tracer

Assay plates were covered and incubated at room temperature for 20-30 minutes (HDAC8) or 60 minutes (HDAC1-3,6,10) prior to measuring TR-FRET. A BMG PHERAstar was used to measure the donor (620 nm) and acceptor (665 nm) emission intensities following excitation at 340 nm.

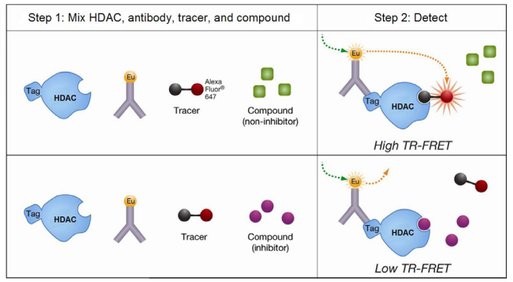
Table 1. Assay Conditions for Inhibitor Screening

HDAC	HDAC Source	HDAC Concentration	Tracer	Tracer Concentration	Antibody (2 nM)
HDAC1	BPS Bioscience	5 nM	HDAC Tracer 1	30 nM	Biotin anti-his / Eu-SA
HDAC2	BPS Bioscience	5 nM	HDAC Tracer 1	100 nM	Biotin anti-his / Eu-SA
HDAC3	BPS Bioscience	5 nM	HDAC Tracer 1	10 nM	Biotin anti-his / Eu-SA
HDAC6	BPS Bioscience	5 nM	HDAC Tracer 1	3 nM	Eu anti-GST
HDAC8	BPS Bioscience	5 nM	HDAC Tracer 1	50 nM	Biotin anti-his / Eu-SA
HDAC10	Life Technologies	5 nM	HDAC Tracer 2	30 nM	Eu anti-GST

Assay conditions for each HDAC were selected to provide a robust assay (excellent window and Z'-factor) while maintaining sensitive detection of inhibitors. The tracer concentration was selected to be at or below the measured K_d values.

RESULTS

Figure 1. LanthaScreen® HDAC Binding Assay Principle

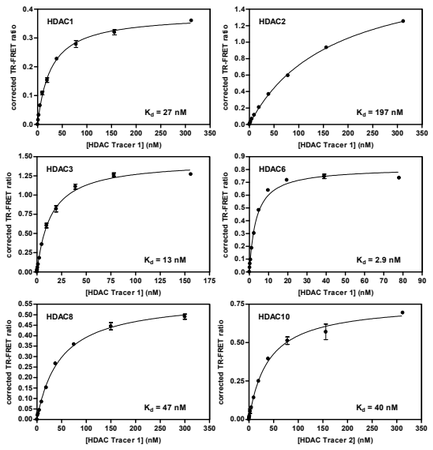


This assay is based on an Alexa Fluor 647®-labeled HDAC inhibitor or "tracer" which binds with a high affinity to Class I and Class IIb HDACs. Binding of the "tracer" to an epitope-tagged HDAC is detected by addition of a europium-labeled anti-epitope tag antibody. Binding of the tracer and antibody to the HDAC results in a high degree of TR-FRET, whereas displacement of the tracer with an inhibitor results in a loss of TR-FRET.

Key features

- Substrate-independent
- Signal is dependent on epitope-tag
- Enables evaluation of binding kinetics (can read in real-time)
- Simple format: 3 additions followed by measurement
- TR-FRET: Resistant to compound interference

Figure 2. HDAC Tracer Binding Affinity



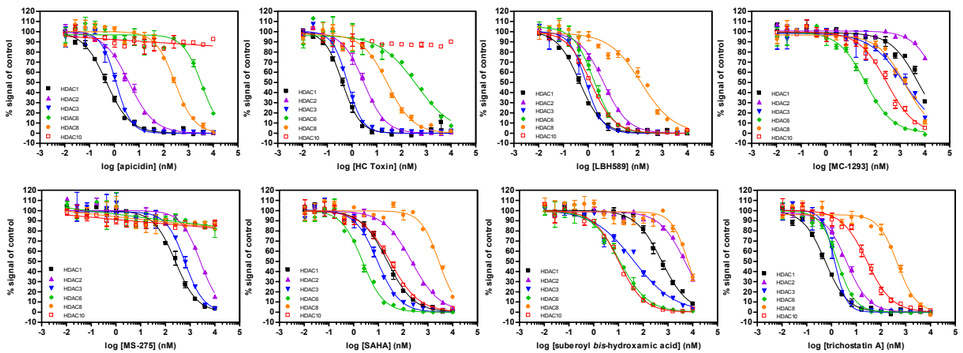
The apparent dissociation constant (Kd) for the HDAC:tracer binding interaction was measured by incubating 5 nM HDAC, 2 nM antibody, and a titration of HDAC tracer. 1% DMSO or 10 µM trichostatin A (TSA) was included to represent the total and non-specific TR-FRET signal, respectively. A corrected TR-FRET ratio was obtained from the difference between these two TR-FRET signals.

Table 2. Inhibitor Screening Results, IC_{50} Values (nM)

Compound	HDAC1			HDAC2			HDAC3			HDAC6			HDAC8			HDAC10		
	LS	Ref[1]	Ref[2]	LS	Ref[1]	Ref[2]	LS	Ref[1]	Ref[2]	LS	Ref[1]	Ref[2]	LS	Ref[1]	Ref[2]	LS	Ref[1]	Ref[2]
Apicidin	0.47	0.04	21	3.9	0.12	28	1.2	0.26	24	3223	ND	>1000	279	ND	>1000	>10,000		>1000
BML-210	1728		14,900	8220		10,700	236		2800	>10,000		>300,000	>10,000		>300,000	>10,000		>300,000
BML-281	9.7			49			5.2			1.7			1220			27		
CI-994	708	50		5806	190		711	550		>10,000	ND		>10,000	ND		>10,000		
CUDC-101	1.2			6.1			2.3			4.9			333			32		
HC Toxin	0.41	190		3.0	470		0.53	1350		397	ND		32	10,500		>10,000		
HDAC-42, (S)-	6.0			62			2.7			1.9			185			1198		
LAQ824 (Dacinostat)	0.47	0.55	12	3.8	1.4	17	0.66	4.2	42	1.4	9.5	150	213	340	>3000	1.2		205
LBH589 (Panobinostat)	0.46	1	120	4.2	0.65	140	0.81	1.1	350	1.9	1.5	>3000	231	105	>3000	1.3		1290
M344	9.8			69			4.2			0.64			1462			14		
MC 1293	5250		>600,000	>10,000		>600,000	1462		376,000	40		414,000	1780		>600,000	286		431,000
MS-275 (Entinostat)	255	22	6800	2521	65	7900	497	360	3900	>10,000	ND	>600,000	>10,000	ND	206,000	>10,000		>600,000
NCH 51	7603			>10,000			2919			1942			>10,000			>10,000		
Nullscript	4152			>10,000			492			369			5081			>10,000		
Oxamflatin	15			90			3.0			9.0			1161			137		
PXD101 (Belinostat)	9.0	0.85	290	94	0.85	350	4.3	1.5	260	2.1	1.6	530	176	25	>30,000	24		>30,000
SAHA (Vorinostat)	22	1.3	280	218	1.6	350	6.5	5	290	2.3	1.6	130	2872	480	>10,000	30		5260
Scriptaid	20	1.5	770	133	2.2	760	3.5	4.1	2220	0.75	0.25	170	882	105	>10,000	217		>10,000
Suberoyl *bis*-hydroxamic acid	539	19		4914	29		43	125		11	14.5		6691	950		10		
Trichostatin A	0.55	0.2	10	3.9	0.65	12	1.3	0.5	41	1.6	1	109	481	45	>1000	27		>1000

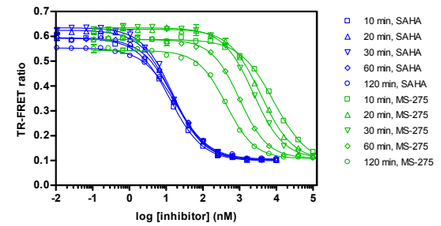
20 known HDAC inhibitors were profiled using the panel of HDAC binding assays. A 16-point dose-response curve (n=2 per data point) was generated for each compound with a maximum concentration of 10 µM.
LS: LanthaScreen HDAC Binding Assay IC_{50} Values, Ref[1]: K_i Values from enzyme assays, Ref[2]: K_d Values from mass spec-based binding assay with cell lysates.
ND indicates inhibition not detected, and blank fields indicate where a compound was not included in a particular study.

Figure 3. Inhibitor Screening Results, Sample IC_{50} Curves



Examples from the 20-compound screen were plotted to illustrate selectivity patterns of known HDAC inhibitors. Because each assay has a unique "top" and "bottom", TR-FRET values were converted to a percentage of control wells (1% DMSO as 100%, 10 µM TSA as 0%).

Figure 4. Binding Kinetics Study: HDAC3



The binding assay can be used to distinguish slow binding compounds (e.g MS-275) from fast binding compounds (e.g. SAHA) by simply reading the same assay plate at different times.

Table 3. Assay Performance: Window and Z'-Factor

HDAC	Assay Window (Fold-change)	Z'-Factor
HDAC1	2.7 ± 0.2	0.83 ± 0.03
HDAC2	5.6 ± 0.3	0.90 ± 0.01
HDAC3	6.4 ± 0.5	0.79 ± 0.10
HDAC6	5.0 ± 0.8	0.82 ± 0.04
HDAC8	2.7 ± 0.1	0.70 ± 0.02
HDAC10	3.4 ± 0.1	0.79 ± 0.01

SUMMARY OF HDAC BINDING ASSAY

- Simple, three step measurement of inhibitor potency
- Covers Class I and IIb HDACs
- Substrate-independent and therefore eliminates concerns about substrate specificity
- Binding can be observed continuously or in an end-point fashion
- Signal is specific to the epitope-tagged HDAC, which unlike activity assays, minimizes interference from copurifying HDACs from a host expression system

REFERENCES

1. Bradner *et al*., Chemical phylogenetics of histone deacetylases, *Nat Chem Biol.* (2010) 6(3):238-243.
2. Bantscheff *et al*., Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes, *Nat Biotechnol*. (2011) 29(3):255-265.

Much of the data generated for kinases using this assay format may be a useful reference for HDAC studies and can be found at invitrogen.com/bindingassay.

The HDAC tracers are not catalog products.
For more information on how to gain access to this technology, please contact us at discoveryservices@lifetech.com

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