Histone deacetylase selectivity screening by ultra-fast SPE-MS/MS

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Overview

- Selectivity screening can require the testing of whole families of enzymes, necessitating the analysis of thousands of reactions
- Mass spectrometry provides robust, reliable data, but is limited in throughput due to the required upstream sample cleanup
- SPE-MS/MS can replace standard liquid chromatography in applications that do not require separation, allowing individual sample analysis at a rate of ~10-12 seconds per sample
- Here, we demonstrate an increase in effective analysis rate to ~3 seconds per sample by analyzing 4 reactions in multiplex, allowing selectivity screens to be analyzed by MS in a timeframe similar to that afforded by optical methods

Introduction

Selectivity screening of a promising new compound requires testing against a host of enzymes related to the target of interest. Mass spectrometry offers robust, dependable data and is less prone to artifacts than optical screening methods; however, the utility of MS can be limited by the comparatively low throughput necessitated by the slow, serial desalting step that is required. The RapidFire high-throughput mass spectrometry system addresses this bottleneck by employing SPE upstream of MS analysis, allowing speeds of ~10-12 seconds per well. Multiplexed analysis of reactions can increase this speed even further, in this example to an effective rate of ~3 seconds per sample, opening up the possibility of generating ~10,000 MS data points in one 8 hour shift, with no loss of data quality or alteration of results. This increased analysis rate enables the acquisition of MS-quality selectivity screen data at speeds comparable to optical methods.

Methods

Hardware

The Agilent RapidFire High-throughput Mass Spectrometry System is an ultra-fast liquid handler that quickly desalts biological samples upstream of mass spectrometric detection. The instrument aspirates a portion of each well of a 96- or 384-well assay plate and applies the sample to an SPE column chosen to retain the analyte(s) of interest. After a washing step, the sample is eluted and sent to a mass spectrometer for detection. The entire cycle time between aspiration of one well and the next is usually between 7 and 12 seconds, as opposed to the several minutes required by standard HPLC methods. The RapidFire-MS system can be interfaced with an array of mass spectrometer types, depending on assay requirements.

Assay setup

Biochemical assays, including enzyme linearity, substrate K_m, and reference inhibition experiments, were developed for four histone deacetylases, SIRT1, SIRT3, HDAC1, and HDAC6. Acetylated peptide substrates representing a portion of p53 (SIRT1, HDAC1, and HDAC6) or histone 4 (SIRT3) were chosen as follows:

Enzyme	Peptide Substrate	Molecular Weight
SIRT1	Ac-GQSTSRHK-K(Ac)-LMFKTEG-NH2	1918
HDAC1	Biotin-LC-KKGQSTSRHK-K(Ac)-LMFKTEG-NH2	2472
HDAC6	KKGQSTSRHK-K(Ac)-LMFKTEG	2133.5
SIRT3	SGRGKGGKGLGKGGA-K(Ac)-RHRC	2009.5

Reactions were carried out at room temperature in 50 mM Tris pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.05% BSA, with 250 µM NAD^+ added for SIRT reactions, and quenched with a final concentration of 1% formic acid. Reference inhibitors were suramin sodium (SIRT1), nicotinamide (SIRT3), valproic acid (HDAC1), and tubacin (HDAC6).

Methods

Analysis conditions

Samples were analyzed as is (individual) or post-reaction pooled (multiplex) on a RapidFire 365 system coupled to an Agilent 6490 iFunnel QQQ MS. The SPE method was as follows:

Cartridge	A (C4)
Buffer A	ddH_2O + 0.1% formic acid
Buffer B	70% acetonitrile + 0.1% formic acid
State Timings	1: 600 2: 3000 3: 8000 4: 500
Pump Flow Rates	1: 1.5 mL/min 2: 1.25 mL/min 3: 0.6 mL/min

Eight transitions were monitored in an MS method as follows:

Drying Gas	17 L/min, 290 ºC
Sheath Gas	12 L/min, 400 ºC
Nebulizer	45 V
Capillary	4000 V
Nozzle	2000 V
Delta EMV	100 V

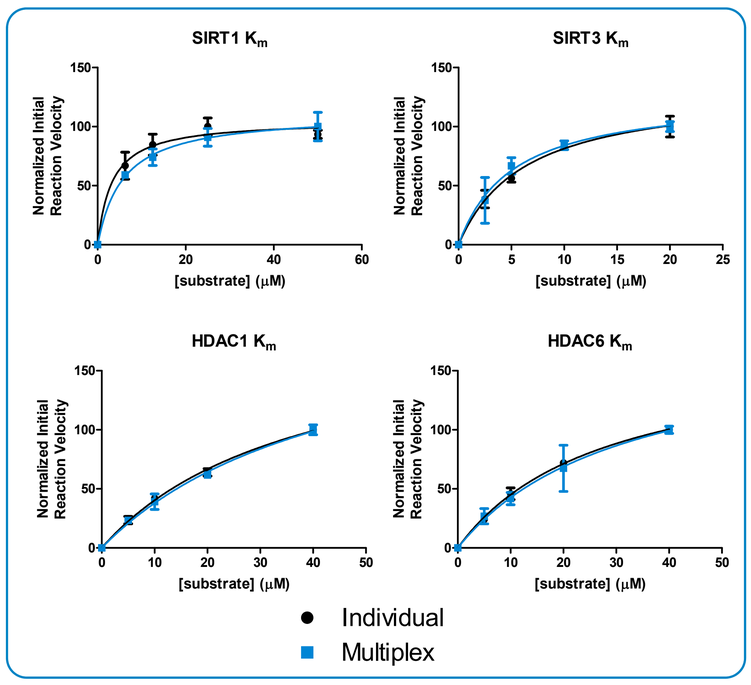
Compound ID	Q1	Res	Q3	Res	Dwell	CE	CAV	Polarity
HDAC6 sub	427.6	unit	424	unit	20	9	3	pos
HDAC6 prod	419.1	unit	415.5	unit	20	9	3	pos
SIRT1 sub	480.4	unit	564.4	unit	20	12	2	pos
SIRT1 prod	469.9	unit	550.3	unit	20	12	2	pos
HDAC1 sub	495.3	unit	226.9	unit	20	25	3	pos
HDAC1 prod	608.4	unit	226.9	unit	20	25	3	pos
SIRT3 sub	340.0	unit	84	unit	20	42	3	pos
SIRT3 prod	333	unit	84	unit	20	42	3	pos

Data were analyzed using Microsoft Excel 2010, RapidFire Integrator v4.0 and GraphPad Prism 5.0. All experiments were carried out in triplicate, and error bars represent one standard deviation.

Results and Discussion

Substrate K_m curves

Timecourses through 2 hours (SIRTs) or 4 hours (HDACs) at various substrate concentrations were analyzed individually or in multiplex and found to be linear (data not shown). The initial velocities were plotted against substrate concentration to allow for the calculation of a K_m value. All best-fit K_m value pairs matched well within 2-fold, and all multiplex values fell within the 95% confidence interval generated from the individual values, indicating that they are statistically indistinguishable.

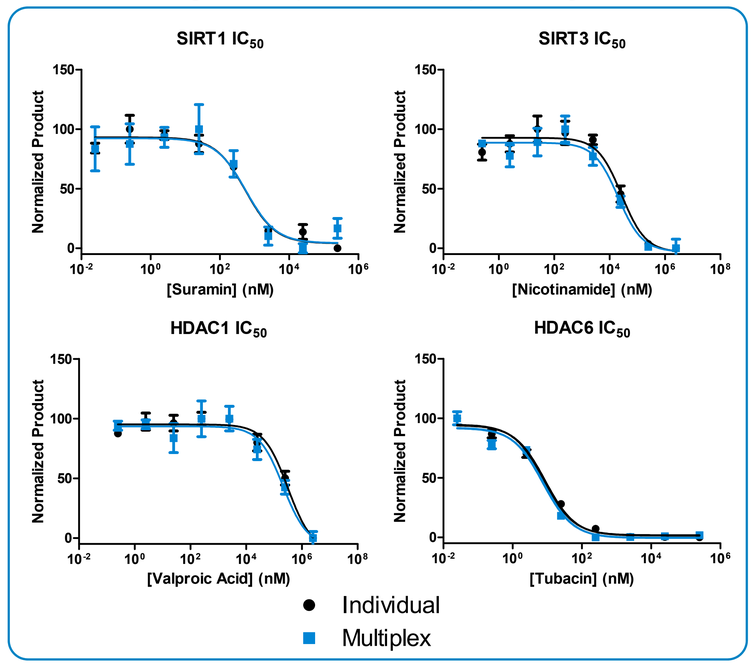


K_m (µM)	SIRT1	SIRT3	HDAC1	HDAC6
Individual	3.3	6.3	38.0	27.7
Multiplex	5.8	5.1	46.2	32.0

Results and Discussion

Reference inhibition curves

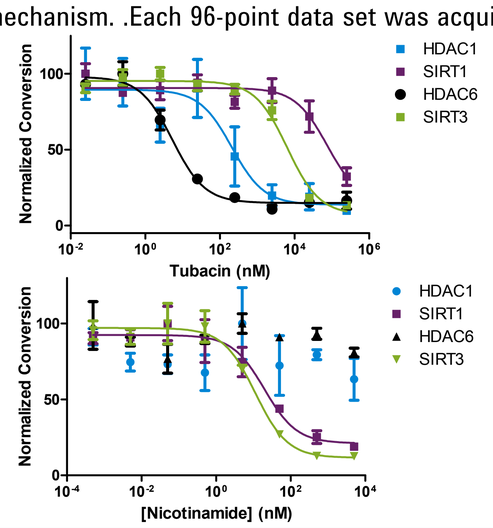
Inhibition curves utilizing a different inhibitor for each enzyme and substrate concentrations of 5 µM (SIRTs) or 10 µM (HDACs) were analyzed individually or in multiplex. All best-fit IC_{50} value pairs matched well within 2-fold, and all multiplex values fell within the 95% confidence interval generated from the individual values, indicating that they are statistically indistinguishable.



IC_{50} (nM)	SIRT1	SIRT3	HDAC1	HDAC6
Individual	547	27354	316978	8.6
Multiplex	564	20534	212729	7.4

Isoform-specific inhibition

Inhibition curves utilizing tubacin, an HDAC6-specific inhibitor[1], or nicotinamide, an NAD^+-compeitive inhibitor, against all four enzymes were analyzed in multiplex. As expected, orders of magnitude differences are observed in tubacin's inhibition potency against the four enzymes, and inhibition curves could be fit to the data for the NAD^+-dependent SIRT1 and SIRT3, but not for HDAC1 and HDAC6, which do not utilize NAD^+ in their reaction mechanism. .Each 96-point data set was acquired in < 5 minutes.



Conclusions

- Robust biochemical assays were developed for four histone deacetylase enzymes acting on four peptide substrates
- Pairing the RapidFire high-throughput system with MS solves the time bottleneck associated with MS detection, allowing an analysis rate of ~10-12 seconds per well
- Multiplexed analysis of deacetylation reactions by SPE-MS/MS allows data acquisition at an effective rate of 3 seconds per sample
- This faster analysis does not affect the quality of the data, as statistically indistinguishable K_m and IC_{50} values were achieved in the multiplexed analysis as compared to the individual analysis
- Isoform-selective inhibitors are easily detected, suggesting great utility of the method in selectivity screening
- Multiplexed analysis of biochemical reactions using the RapidFire system allows ~10,000 data points to be generated in 8 hours, which is on par with the speed of optical methods and allows rapid selectivity screening based on MS-quality data

References

1. Regna NL, Reilly CM *Isoform-Selective HDAC Inhibition in Autoimmune Disease* J Clin Cell Immunol (2014) 5:207