

Video Article

Simultaneous Measurement of HDAC1 and HDAC6 Activity in HeLa Cells Using UHPLC-MS

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Abstract

The search for new histone deacetylase (HDAC) inhibitors is of increasing interest in drug discovery. Isoform selectivity has been in the spotlight since the approval of romidepsin, a class I HDAC inhibitor for cancer therapy, and the clinical investigation of HDAC6-specific inhibitors for multiple myeloma. The present method is used to determine the inhibitory activity of test compounds on HDAC1 and HDAC6 in cells. The isoform activity is measured using the ultra-high-performance liquid chromatography – mass spectrometry (UHPLC-MS) analysis of specific substrates incubated with treated and untreated HeLa cells. The method has the advantage of reflecting the endogenous HDAC activity within the cell environment, in contrast to cell-free biochemical assays conducted on isolated isoforms. Moreover, because it is based on the quantification of synthetic substrates, the method does not require the antibody recognition of endogenous acetylated proteins. It is easily adaptable to several cell lines and an automated process. The method has already proved useful in finding HDAC6-selective compounds in neuroblasts. Representative results are shown here with the standard HDAC inhibitors trichostatin A (non-specific), MS275 (HDAC1-specific), and tubastatin A (HDAC6-specific) using HeLa cells.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55878/>

Introduction

HDACs belong to a family of enzymes able to deacetylate histones within the chromatin structure. They also have other protein substrates in the cytosol and are located in various cell compartments. A total of 18 HDAC isoforms have been identified so far and have been related to several cell mechanisms, including the regulation of transcription factors and gene expression, as well as cell signaling and transport^{1,2,3,4,5,6,7}. HDAC catalytic inhibitors have emerged as potential therapeutic drugs for cancer therapy. Most HDAC inhibitors currently approved by the FDA are for the treatment of T-cell lymphoma and multiple myeloma, and are non-specific HDAC inhibitors, such as vorinostat (SAHA), belinostat, and panobinostat^{8,9}. However, a series of side effects have been associated with pan-inhibitors, and the search for isoform-specific small molecules is a hot topic in medicinal chemistry and drug discovery. Accordingly, the class I (HDAC1-3 and HDAC8) selective inhibitor romidepsin is an already approved drug¹⁰, while HDAC6-specific inhibitors are currently under clinical trials, with increased therapeutic potential in multiple myeloma^{11,12,13,14,15}.

Screening assays to characterize HDAC inhibitors are based on the incubation of an HDAC substrate with an enzymatic source (single isoform, nuclear extract, or cell lysate). The substrate is usually a small peptide sequence containing an acetyl lysine residue coupled to a cleavable fluorophore (e.g., coumarin), such as N-(4-methyl-7-aminocoumarinyl)-N α -(t-butoxycarbonyl)-N ω -acetyllysineamide (MAL)¹⁶. To distinguish between isoform-specific activities, separate cell-free assays involving each isoform are necessary and might not reflect the real isoform activity in living cells. Isoform-specific substrates are commercially available, such as benzyl (S)-[1-(4-methyl-2-oxo-2H-chromen-7-ylcarbamoyl)-5-propionylaminopentyl]carbamate (MOCPAC, HDAC1 specific substrate) and (S)-[5-acetylamino-1-(2-oxo-4-trifluoromethyl-2H-chromen-7-ylcarbamoyl)pentyl]carbamate (BATCP, HDAC6 specific substrate) (**Figure 1B**). However, a multi-substrate mixture containing MAL, MOCPAC, and BATCP given to living cells will not permit the detection of the individual deacetylated products by fluorometric measurement, given that they bear the same cleavable fluorophore.

The method described here allows for the detection and relative quantification of each substrate and its deacetylated product in HeLa cells using a multi-substrate assay followed by UHPLC-ESI-MS/MS analysis¹⁷. An HDAC assay is conducted on HeLa cells to enable the direct identification of HDAC inhibitory activity and of the specificity of test compounds on endogenous HDACs. There is a focus on HDAC1 and HDAC6, which are simultaneously evaluated. To achieve these enzymatic measurements in a single incubation assay, a mixture of non-specific and specific HDAC substrates is added to treated and untreated HeLa cells plated on a 96-well plate. Following an incubation step, cells are lysed to release the substrates and their respective reaction products, which are separated and detected using a UHPLC-MS method (**Figure 1C**). The

deacetylated products of the MAL, MOCPAC, and BATCP substrates are the deacetylated MAL (dMAL), deacetylated MOCPAC (dMOCPAC), and deacetylated BATCP (dBATCP), respectively. Dose-response curves can be built with active compounds.

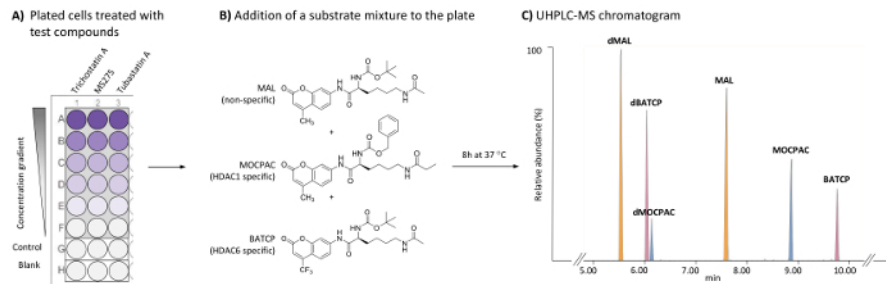


Figure 1: General scheme for the cell-based HDAC assay to identify HDAC1- and HDAC6-specific inhibitors by the UHPLC-MS analysis of multiple substrates. (A) Scheme of a typical 96-well plate containing treated (test compounds) and untreated (control) HeLa cells, as well as cell-free blanks. (B) Chemical structure of substrates added as a mixture (21 μ M each) to be deacetylated by endogenous HDACs. (C) Typical UHPLC-MS chromatogram showing the peaks of the added substrates (MAL, MOCPAC, and BATCP) and their deacetylated products (dMAL, dMOCPAC, and dBATCP, respectively). [Please click here to view a larger version of this figure.](#)

Protocol

1. Cell Culture

NOTE: The following steps are performed in a standard tissue culture hood. Familiarity with sterile technique is expected.

- Culture HeLa cells to 80% confluency in a T75 cell culture flask in MEM supplemented with 10% fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 mg/mL).
NOTE: Cell culture, treatment, and lysis steps are conducted under laminar flow, with cell incubation steps at 37 °C performed in a humidified atmosphere of 5% CO₂ under sterile conditions.
- Wash the cells twice with 5 mL of DPBS, aspirate the DPBS, and add 1 mL of cell dissociation reagent. Incubate at 37 °C for 5 min to detach the cells.
- Add 9 mL of MEM culture medium, thoroughly resuspend the cell suspension, and count the cells using a hemocytometer.
- Adjust the density of the cell suspension to 6 x 10⁴ cells/mL in MEM.
- Add 100 μ L of cell suspension to control and test wells of a sterile, flat-bottom, tissue culture-treated 96-well plate (**Figure 1A**).
- Add 100 μ L of MEM culture medium to blank wells of the 96-well plate (**Figure 1A**).
- Incubate the 96-well plate at 37 °C for 24 h.
NOTE: A 24 h incubation is a suitable time to test for endogenous HDAC activity in HeLa cells. Different cell types with unknown HDAC activity may need a timecourse evaluation of control cells.

2. Cell Treatment with Test Compounds and HDAC Substrates

- Aspirate the medium from the wells of the 96-well plate.
- Add 25 μ L of 2x test compounds (e.g., trichostatin A, MS275, and tubastatin A) in MEM to test wells. Add 25 μ L of MEM to control and blank wells.
NOTE: The concentration of test compounds is to be adjusted to provide a range of at least 5 final test concentrations (one concentration per well). The DMSO final concentration in the test compound should not exceed 0.5% and should be the same in each well, including blanks and controls. The final incubation volume is 50 μ L per well. Trichostatin A is tested at 5 concentrations ranging from 500 to 1.95 nM (1:4 dilution). MS275 and tubastatin A are tested at 5 concentrations ranging from 8,000 to 6.17 nM (1:6 dilution). The desired final test concentration for each test compound should be determined by each individual user by creating a dose-response curve.
- Add 25 μ L of the substrate mixture (42 μ M of MAL, 42 μ M of MOCPAC, and 42 μ M of BATCP in MEM) to control and test wells. Add 25 μ L of MEM to blank wells.
NOTE: The final concentration of each substrate is 21 μ M. MOCPAC and BATCP distinguish between HDAC1 and HDAC6 activity, respectively (**Figure 1B**). When no activity is observed with those substrates, the use of MAL could potentially lead to the identification of activity against another HDAC isoform.
- Incubate the 96-well plate at 37 °C for 8 h in a humidified 5% CO₂ incubator.
NOTE: This incubation time is suitable for HeLa cells and allows the endogenous HDAC to interact with the synthetic substrates. Different cell lines may require adjustments in incubation time and/or substrate concentration.

3. Cell Lysis and Sample Preparation for UHPLC-ESI-MS/MS

Caution: The sample preparation steps use organic chemicals, which are highly flammable and toxic by ingestion or inhalation. Wear appropriate personal protection, such as gloves and safety glasses.

- Add 10 μ L of 6x RIPA buffer (diluted from 10x RIPA buffer) supplemented with protease inhibitor to each well of the 96-well plate.
- Stop the reaction by adding 160 μ L of cold acetonitrile to each well and mix by pipetting up and down.
NOTE: Keep the acetonitrile at -20 °C prior to this step.

3. Place the plate in a -80 °C freezer for 10 min.
4. Remove the plate from the freezer and transfer 220 µL of the content of each well to a non-sterile, conical-bottom (V-bottom) 96-well plate. Centrifuge the plate at 5,000 x g and 4 °C for 10 min.
NOTE: This step aims at removing proteins and salts. If necessary, the solutions can be transferred to individual conical centrifuge tubes prior to centrifugation or filtered through a set of 96-well, 0.65 µm PVDF filter plates. The successful removal of the precipitate is required prior to UHPLC analysis.
5. Transfer 200 µL of supernatant (or filtrate) into a 96-well plate compatible with the UHPLC system and seal it with peelable, heat-sealing foil using a plate sealer.
NOTE: Avoid pipetting the pellets when removing the supernatants. If the UHPLC analysis cannot be immediately performed, the plate can be stored at 4 °C for a maximum of 24 h until analysis.

4. UHPLC/MS-MS Analysis

1. Prepare the UHPLC system by filling it with the mobile phase system (95% A:5% B):
A: H₂O, HPLC-grade, containing 0.1% formic acid (prepare 1 L)
B: Acetonitrile, HPLC-grade, containing 0.1% formic acid (prepare 1 L).
Caution: The mobile phase contains organic chemicals, which are highly flammable and toxic by ingestion or inhalation. Wear appropriate personal protection, such as gloves and safety glasses.
2. Place the 96-well plate into the sample manager containing a plate holder. Run the samples according to the UHPLC-ESI-MS/MS conditions provided in **Table 1**.
NOTE: Every column of the analytical 96-well plate should have one control well and one blank well (as depicted in the plate scheme of **Figure 1A**). Controls are representative of 100% enzymatic activity in the tested cell line, while blanks are there to check whether the MS source is providing consistent background. Unusual MS peaks detected in a given blank should be investigated to prevent MS sensitivity changes.

5. Data Analysis

1. Integrate the peak area of each substrate and its deacetylated product with an appropriate UHPLC integration software.
NOTE: Use automatic integration parameters with a smoothing method (Mean, window size 3, and number of smooth 2, for example). Using the chromatographic conditions provided in **Table 1**, the elution order is dMAL (5.8 min), dBATCP (6.0 min), dMOCPAC (6.2 min), MAL (7.6 min), MOCPAC (8.9 min), and BATCP (9.8 min), as depicted in **Figure 1C**.
2. For each substrate, calculate the peak area ratio (deacetylated/acetylated) in each chromatogram (test and control samples).
3. Calculate the percent HDAC inhibition of each test sample:

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{Test ratio} \times 100}{\text{Control ratio}} \right)$$

NOTE: HDAC1 inhibition is calculated using the peak area ratio of dMOCPAC/MOCPAC; HDAC6 inhibition is calculated with dBATCP/BATCP. The ratio dMAL/MAL can be used to express general HDAC inhibition.

Representative Results

To demonstrate the application of the method to identifying non-selective and selective inhibitors for HDAC1 (class I) and HDAC6 (class IIb), HeLa cells were treated with known standard compounds: trichostatin A (non-selective between HDAC1 and HDAC6)¹⁸, MS275 (HDAC1-selective inhibitor)¹⁹, and tubastatin A (HDAC6-selective inhibitor)²⁰. By using the present method (**Figure 1**), IC₅₀ values could be directly determined in living cells for HDAC1 (by looking at MOCPAC deacetylation) and for HDAC6 (by looking at BATCP deacetylation) (**Figure 2**). Trichostatin A was a potent but non-selective inhibitor towards HDAC1 and HDAC6. On the other hand, MS275 was selective for HDAC1, while tubastatin A was clearly selective for HDAC6.

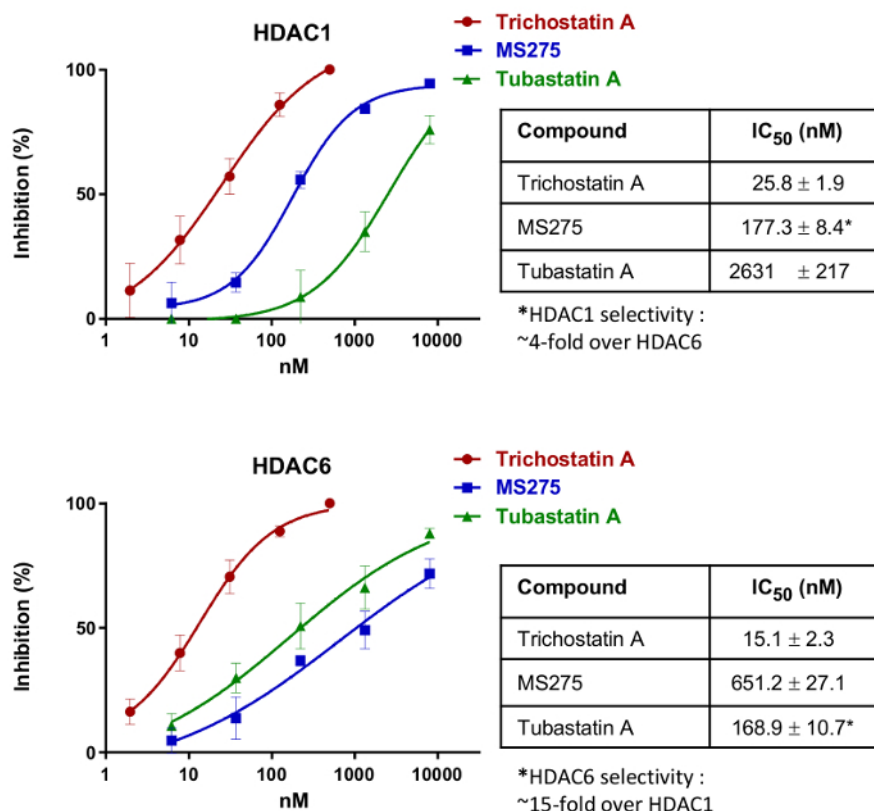


Figure 2: IC₅₀ Dose-response curves simultaneously obtained for HDAC1 and HDAC6 of three standard HDAC inhibitors: the non-selective trichostatin A, the HDAC1-selective MS275, and the HDAC6-selective tubastatin A. IC₅₀ values presented in the adjacent table are the mean ± SD of three independent measurements. [Please click here to view a larger version of this figure.](#)

Injection volume	5 µL
Column	C18 (2.6 µm, 100 mm x 3 mm i.d.)
Column temperature	40 °C
Elution flow rate	0.8 mL/min (typical pressure range: 400-600 bars)
Elution gradient	5-45% B in 10 min
	45-98% B in 2 min
Wash step	98% B for 2 min
Equilibrating step	5% B for 4 min
ESI-MS/MS	cone voltage, 30 V; capillary source voltage, 3.0 kV; capillary temperature, 350 °C; source temperature, 120 °C; sheath gas - nitrogen (600 L/h); collision gas pressure, argon set to 3 x 10 ⁻³ mbar
	Collision parameters and reaction channels optimized with a dwell time of 0.1 s, 0.01 s of interscan delays and collision energy set at 20 eV.
	MAL detection with mass transitions 446 → 346 (MAL) and 404 → 304 (dMAL).
	MOCPAC detection with mass transitions 494 → 449 (MOCPAC) and 439 → 394 (dMOCPAC).
	BATCP detection with mass transitions 500 → 400 (BATCP) and 476 → 376 (dBATCP).

Table 1: UHPLC-ESI-MS/MS conditions.

Discussion

HDAC inhibition is a hot topic in drug discovery, with a current focus on HDAC6-selective inhibitors for cancer therapy²¹. HDAC selectivity is usually assessed by a series of high-throughput, cell-free assays, which intend to determine the inhibitory potency towards individual

HDAC isoforms²¹. However, the selectivity of an inhibitor must be additionally confirmed in living cells by evaluating the acetylation status of endogenous protein substrates, such as histones (for class I HDACs) and tubulin (for HDAC6). Therefore, cell assays involving antibody recognition (e.g., immunostaining, western blot, flow cytometry, and ELISA) are usually necessary to achieve a quantitative or semi-quantitative evaluation of isoform potency of a given compound in living cells²¹. It is not rare that a compound that was selective in a cell-free enzymatic assay turns out to be non-selective in cells or tissues^{17,22}, and vice versa¹⁹. This can be explained by differences in non-standardized enzymatic sources and the absence of co-factors or protein partners in biochemical assays²¹.

The method described here is a rapid, antibody-free alternative allowing for the direct measurement of class I (HDAC1) and class IIb (HDAC6) inhibitory potency in the living cell environment¹⁷. Another advantage of the method is that it can easily be adapted to different cell lines, such as neuroblasts²³, with the possibility for miniaturization and automation, as previously described for other cell-based assays²⁴. The detection of specific substrates by mass spectrometry contributes to the accurate measurement of the deacetylated products, providing comparable quantitative results. This is the first method available for determining IC₅₀ values towards specific HDAC isoforms in living cells. As a matter of comparison, the non-quantitative selective profile of HDAC inhibitors observed by western blot have been demonstrated to be in line with the selectivity profile obtained with the method described here¹⁷. Even if this method has been developed for the discovery of new class-selective HDAC inhibitors, it could also be applied to measure HDAC activity within cell models over the course of a disease. This could contribute to the elucidation of epigenetic and molecular mechanisms in relevant diseases and could potentially identify drug candidates. In view of extrapolating this method towards HDAC isoforms other than HDAC1 and HDAC6, the development of new selective HDAC substrates is strongly encouraged. Moreover, the substrate specificity of MOCPAC and BATCP should be fully characterized in terms of targeting single isoforms. Indeed, while MOCPAC is classified as an HDAC1-specific substrate (class I over class II), it is more likely that it is a generic class I substrate (including HDAC3 isoform), as previously demonstrated²⁵. Accordingly, BATCP is a well-known HDAC6-selective substrate (class II over class I HDACs), but its specificity towards other class II HDACs, such as HDAC4, deserves further characterization²⁵.

Enzymatic activity may differ depending upon the cell line, cell number, confluency, and incubation time. When a cell line is used for the first time in the assay, it is important to test a range of substrate concentrations in control cells, at different time points, to analyze the enzyme kinetics by UHPLC-MS (e.g., through a Michaelis-Menten plot). This will allow researchers to choose the most suitable incubation time and substrate concentration. This choice relies on two major parameters: 1) the enzyme kinetics and 2) the detection of the substrates and their deacetylated products. When analyzing enzyme kinetics, it is essential to evaluate the initial velocity values (i.e., when the product formation is within the linear range) to establish the enzyme kinetics. According to the kinetics, substrate concentrations under the K_m should be chosen to provide an accurate percentage of inhibition, especially in the case of competitive and uncompetitive inhibitors²⁶. On the other hand, the chosen substrate concentration should provide detectable and quantifiable MS signals for both the substrate and the deacetylated product (signal-to-noise ratio ≥20)¹⁷. When using multiple substrates, the chromatographic method should ensure the separation of all peaks to be analyzed. Alternative chromatographic systems may require changes in the separation gradient and column type.

Disclosures

The authors have nothing to disclose.

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