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Small molecule-based inhibition of histone demethylation in cells assessed by quantitative mass spectrometry

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Abstract

Post-translational modifications on histones are an important mechanism for the regulation of gene expression and are involved in all aspects of cell growth and differentiation as well as pathological processes including neurodegeneration, autoimmunity, and cancer. A major challenge within the chromatin field is to develop methods for the quantitative analysis of histone modifications. Here we report a mass spectrometry (MS) approach based on ultra-performance high/low collision switching (UPLC-MS^E) to monitor histone modifications in cells. This approach is exemplified by the analysis of trimethylated lysine-9 levels in histone H3 following a simple chemical derivatization procedure with deuterated acetic anhydride. This method was used to study the inhibition of histone demethylases with pyridine-2,4-dicarboxylic acid (PDCA) derivatives that inhibit histone demethylases in cells. Our results show that the PDCA-dimethyl ester inhibits JMJD2A catalyzed demethylation of lysine-9 on histone H3 in human HEK 293T cells. Demethylase inhibition, as observed by MS analyses, was supported by immunoblotting with modification-specific antibodies. The results demonstrate that PDCA derived small molecules are cell permeable demethylase inhibitors, and reveal that quantitative MS is a useful tool for measuring post-translational histone modifications in cells.

Synopsis

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Supporting Information Available

The contents of Supporting Information includes the following: (1) Synthesis of 2,4-PDCA derivatives, (2) MS/MS spectra of the peptide standards K9acSTGGK14acAPR, K9me3STGGK14acAPR and K9acSTGGK14 me3APR.

Keywords

Demethylases; epigenetics; histone demethylation; JMJD2A; mass spectrometry; proteomics; 2-oxoglutarate oxygenases; post-translational modifications

INTRODUCTION

Histones undergo a diverse range of dynamic and reversible post-translational modifications that regulate gene expression and act as mechanisms for epigenetic control ^{1, 2}. These modifications include acetylation / ubiquitination / SUMOylation of lysine residues, phosphorylation of serine / threonine / tyrosine residues, methylation of lysine / arginine residues, ADP-ribosylation and citrullination of methylarginine residues ³. Combinations of these modifications enable regulation in some cases, leading to the proposal of an epigenetic 'code' ^{4, 5}. Modifications to the *N*-terminal tails of histones have been identified as both transcriptionally activating and deactivating (for reviews see ^{6, 7}). The combinatorial complexity arising from the possibility of different types of modifications at the same and different sites together with the possibility of cross-talk between different sites, poses a major analytical challenge.

Methylated lysines in histone tails are involved in the establishment of different chromatin states, and contribute to both gene silencing and activation ^{1, 2}. The available evidence suggests that histone lysines residues undergo the widest variety of identified modifications, with all three possible N^ε-methylation states known to occur. Lysine methylation was originally thought to be irreversible, however, following the discovery of the lysine specific demethylase (LSD1) and the 2-oxoglutarate (2OG)-dependent oxygenase histone demethylase (HDM) subfamily, it is now recognized to be a dynamic modification ⁸.

The 2OG oxygenases are the largest group of identified HDMs and can be subdivided into subfamilies ⁸. The JMJD2 subfamily catalyses preferentially demethylation of tri- and dimethylated histone lysines. Various HDMs have been implicated in disease with the JMJD2 HDM subfamily being linked to prostate and oesophageal cancers ^{9, 10}. The JMJD2A (JHDM3A) catalyses demethylation of histone H3 lysines 9 and 36, an activity which abrogates recruitment of heterochromatin protein 1 (HP1), leading to reduced transcription of JMJD2A target genes, including ASCL2 ¹¹.

Histone modifying enzymes, in particular, histone deacetylases, are being actively pursued as targets for cancer therapy¹². Recently, histone demethylases have also been identified as cancer targets¹³⁻¹⁹. However, as with work on the inhibition of other types of histone modifying enzymes, analyses on the cellular effects of these HDM inhibitors is hampered by the lack of quantitative methodology for the analysis of histone modifications.

The analysis of histone modifications is normally performed using antibody-based methods by immunoblotting, immunoprecipitation, ELISA or immunofluorescence, and is constrained by problems such as specificity, availability, limited dynamic range and by problems in detecting multiple modifications on different sites at the same time. Mass spectrometry (MS) provides an alternative experimental approach that can address the complexity of histone PTMs in single experimental sets and overcome some of the limitations of antibody-based methods. Recently, MS-based methods have been developed and applied to study histone post-translational modifications including histone lysine methylation^{20, 21}. We are interested in developing small molecules that regulate histone modifying enzymes, in particular histone demethylases (HDMs) and have initially characterized a number of HDM inhibitors *in vitro*^{17, 18, 22}. Here we report a quantitative MS-based method for the analysis of inhibition of the HDM JMJD2A in cells, focusing on monitoring levels of trimethylation at lysine-9 on histone H3 since JMJD2A has a preference for N^ε trimethylated substrates. This approach provided insights into the HDM inhibitory capacities of pyridine-2,4-dicarboxylic acid (PDCA) derivatives in living cells.

MATERIALS AND METHODS

Chemicals were from Sigma unless stated otherwise. pcDNA3 Flag-*JMJD2A* plasmid was a gift from Dr. Robert J Klose, Department of Biochemistry, University of Oxford. β-Glycerophosphate and sodium orthovanadate used in the histone acid-extraction were kindly gifted by the Mahadevan group in the Department of Biochemistry, University of Oxford. The 2,4-PDCA derivatives²³ were prepared as described in Supporting Information. For use in cells, sterile 100 mM stock solutions of the 2,4-PDCA (pyridine-2,4-dicarboxylic acid) derivatives were prepared in 25% ethanol in water for the diethyl ester, phosphate buffer saline (PBS) buffer for the *N,N'*-bis(2-methoxyethyl)-2,4-pyridine dicarboxamide (henceforth referred to as the diamide and also known as HOE 077²⁴), and DMSO for the dimethyl ester.

Synthesis of peptide standards

The peptides K9me3STGGK14acAPR, K9acSTGGK14acAPR and K9acSTGGK14me3APR were prepared by Fmoc-based solid phase peptide synthesis using Wang resin (100-200 mesh, Novabiochem) on a CSep336X peptide synthesizer (CSBio). Peptides were cleaved from the resin using CF₃CO₂H/triisopropylsilane (97.5%/2.5% w/v) to yield C-terminal acids which were purified by reverse-phase HPLC (Agilent 1200) on an Agilent C-18 column (4.6 × 250 mm) using a flow rate of 1 ml/min and a gradient of: 2% B for 1 min, 2-15% B from 1-40 min, 15-95% B from 40-43 min, 95% B for 43-48 min, 95%-2% B from 48-49 min and 2% B from 49-60 min where solvent A is 0.1% trifluoroacetic acid (TFA) and solvent B is 98% acetonitrile in 0.1% TFA. Identification of

fractions containing the final peptide products was carried out using a MALDI-TOF mass spectrometer in the linear mode (Ultraflex, Bruker Daltonics).

Cell culture

HEK (human embryonic kidney) 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml of penicillin G and 100 µg/ml of streptomycin antibiotics at 37°C in a humidified 5% CO₂ atmosphere.

Cytotoxicity assay

The effect of 2,4-PDCA derivatives on cell viability was measured as described²⁵ using the CellTiter 96 AQueous One Solution Cell Proliferation kit (Promega). Varying concentrations 10-0.15 mM of the compounds were prepared from the stock solutions by two-fold serial dilution in complete growth medium to a final volume of 100 µl in each well of a 96-well microtiter plate. Wells were then seeded with 100 µl of HEK293T cells (1-2×10⁵ cells/ml). Controls with HEK293T cells only and a background (no cells) were included for each sample. Assays were performed in triplicate (37°C with 5% CO₂). After 3 days, 40 µl of MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] was added to each well and further incubated at 37°C, 5% CO₂ for 1-3 hr. Absorbance was measured at 490 nm with a microplate reader (Perkin Elmer Victor 3); the amount of formazan produced from the reduction of MTS is proportional to the number of viable cells. Cytotoxicity was calculated as CC₅₀, i.e. the concentration required to reduce the absorbance of treated cells by 50% with reference to the untreated control after background correction using the curve-fitting function in GraphPad Prism 4 (GraphPad Software).

Cell transfections and inhibitor assays

293T cells grown to 80% confluence were harvested and seeded in 6-well plates (3 ml / well at a density of 2 × 10⁵ cells/ml) for 24 h under the usual cell culture conditions. Cells were then transiently transfected with 5 µg pcDNA3-empty or -Flag-*JMJD2A* expression vectors per well for another 24 h using 10 µg polyethylene imine per well (Sigma). 2,4-PDCA compounds (0.3 mM) were then added (0.3mM and 1 mM of the 2,4-PDCA diamide) and incubated for 72 h. Cells were harvested and extracts prepared for immunoblotting or MS analysis (described below in the histone acid-extraction section). For the immunoblotting, harvested cells were rinsed in ice-cold phosphate-buffered saline (PBS) and then lysed in urea-sodium dodecyl sulfate (SDS) buffer (6.7 M urea, 10 mM Tris-Cl [pH 6.8], 1 mM dithiothreitol, 10% glycerol, 1% SDS) supplemented with protease inhibitors (cComplete™, Roche). The lysates were heated at 95°C for 10 min, and then disrupted using an ultrasonicator probe (Model CV 33 Vibra-Cell, Sonics) for a few seconds, followed by centrifugation. Protein concentrations of the lysates were determined using the BCA protein assay kit (Thermo/Pierce). Whole-cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (26 well 4-12% gradient Bis-Tris Criterion™ XT precast gel, Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (PVDF 0.2 µm pore, Millipore). The membrane was stained with Ponceau S solution which was used to verify equal loading. The

antibodies and dilutions used were: anti-rabbit H3K9me3 (1:1000; 07-442, Millipore) and anti-rabbit β -actin (1:25,000; A2066, Sigma) primary antibodies, monoclonal anti-Flag M2 horseradish peroxidase (HRP)-conjugated antibody (1:2000; A8592, Sigma), and HRP-conjugated anti-rabbit secondary antibody (1:20'000; P0399, DAKO). The immunoblots were developed using the SuperSignal® West Pico and West Dura kits (Thermo/Pierce) and visualized on film (Kodak X-Omat LS).

Histone acid-extraction

The method for extraction of histones in acid was adapted from reported protocols ^{3, 26}. 293T cells were washed three times with PBS buffer and scraped from the 6-well plates into 15 ml falcon tubes and spun (1500 rpm, 4 min, 4°C). The supernatant was discarded and cell pellet washed with 2 ml PBS followed by centrifugation as above. The supernatant was then discarded again. The cell pellet was then resuspended in 1 ml ice-cold lysis buffer 90.2% Triton X-100, 10 mM HEPES pH 7.6, 1.5 mM MgCl₂, 1 mM KCl, 100 μ M sodium orthovanadate, 10 mM sodium butyrate, 20 mM β -glycerophosphate and protease inhibitors (Roche), transferred into 1.5-ml microcentrifuge tubes on ice, and then rotated for 30-60 min at 4°C. These tubes were then centrifuged (3000 rpm, 4 min, 4°C) to yield nuclear pellets. The supernatant was removed and the nuclear pellets resuspended in 1 ml of the same lysis buffer and spun as in the previous step. Ice-cold 0.4 N HCl (100 μ l) was added to the nuclear pellets which were then kept on ice for 1 hr or a rotator for 30 min. Following centrifugation (13000 rpm, 20 min, 4°C), the supernatant was transferred to 1.5-ml microcentrifuge tubes. 10 volumes (1 ml) of ice-cold acetone were then added and the tubes were left overnight at -20°C. Precipitated histones were then recovered by centrifugation (13000 rpm, 20 min, 4°C) and the supernatant removed using a micropipette. Ice-cold acetone (1 ml) was added as follows: 0.5 ml was first added and dispersed using the tip of pipette and the remaining 0.5 ml then added. Samples were then centrifuged (13000 rpm, 10 min, 4°C) and resuspended in 1 ml ice-cold acetone followed by centrifugation for an additional 10 min. The resulting histone extract pellets were dried by allowing residual acetone to evaporate and then stored at -20°C.

1D In-gel chemical derivatization and trypsin digestion

Acid-extracted core histones from 293T cells were separated on 18% SDS-PAGE gels. Chemical derivatization using either deuterated acetylation or propionylation was performed as described ^{27, 28} with some modifications. Histone H3 gel bands stained with Coomassie Blue (Imperial™ Protein Stain, Thermo/Pierce) were cut into small pieces (ca. 1 mm³). Destaining solution (50% methanol, 5% acetic acid) was added, and the solution was shaken (~ 350 rpm) for 2-3 hr, the de-staining solution was then removed, 200 μ l of the same solution added and left overnight under the same conditions. The de-staining solution was then removed, 200 μ l of acetonitrile (MeCN) was added and the solution left for 5-15 min (until the gel pieces had shrunk and whiten). The MeCN was then pipetted off and the gel pieces dried by centrifugation *in vacuo* (Heto/Eppendorf vacuum concentrator) for 5 min. The gel pieces were then washed: (1) three times with 100 mM ammonium bicarbonate (20 min, 200 μ l each time), once for 15 min with 100 mM MeCN/ammonium bicarbonate (50:50 v/v); and once for 15 min with MeCN which was then removed and dried in a concentrator for 5 min. Deuterated acetic anhydride (10 μ l) was added to acetylation buffer (990 μ l of 2

M sodium chloride, 50 mM sodium bicarbonate, pH 8) to prepare a 100 mM solution from which 10 μ l was added to gel pieces in each tube and incubated on ice for 5 min. Acetylation buffer (90 μ l) was added and left for another 25 min on ice. After this, the solution was removed and the reaction was quenched with 200 μ l of 50 mM Tris buffer (pH 8) for 15 min. The quenching solution was then removed, 200 μ l of MeCN added and left at room temperature for 5-15 min. This was followed by 5 min of evaporation in a concentrator. Trypsin digestion for MS analysis was then carried out essentially as described^{29, 30} but without reduction and alkylation. In brief, the excised gel pieces were subjected twice to hydration and dehydration using de-staining solution and MeCN, respectively, followed by overnight incubation at 37°C with trypsin (20 ng/ μ l) in 100 mM ammonium bicarbonate (pH 7.8) digestion buffer. The same procedure was also carried out using propionic anhydride substituting for deuterated acetic anhydride. The final samples were redissolved in 0.1% formic acid (HCOOH), 2% MeCN and stored at -20°C until analysis.

Protein identification and quantitative analysis by mass spectrometry

The digested material was subjected to nano-ultra performance liquid chromatography tandem MS analysis (nano-UPLC-MS^E or -MS/MS) using a 75 μ m-inner diameter $\times$ 25 cm C₁₈ nanoAcquityTM UPLCTM column (1.7- μ m particle size; Waters) and a 90 min gradient of 2–45% solvent B (solvent A: 99.9% H₂O, 0.1% HCOOH acid; solvent B: 99.9% MeCN, 0.1% HCOOH acid) on a Waters nanoAcquity UPLC system (final flow rate, 250 nl/min; 7000 p.s.i.) coupled to a Q-TOF Premier tandem mass spectrometer (Waters) run in positive ion mode. Data were acquired in high definition low/high collision energy MS (MS^E) mode (low collision energy, 4 eV; high collision energy ramping from 15 to 40 eV, switching every 1.5 s). Alternatively, MS analysis was performed in data-directed analysis (DDA) mode (MS to MS/MS switching at precursor ion counts greater than 10 and MS/MS collision energy dependent on precursor ion mass and charge state). All raw MS data were processed using the PLGS software (version 2.2.5) including deisotoping and deconvolution (converting masses with multiple charge states to $m/z = 1$). For MS^E data MS/MS spectra were reconstructed by combining all precursor and fragment masses with identical retention times. The mass accuracy of the raw data was corrected using Glu-fibrinopeptide (200 fmol/ μ l; 700 nl/min flow rate; 785.8426 Da [$M + 2H$]²⁺) that was infused into the mass spectrometer as a lock mass during analysis. MS, MS^E, and MS/MS data were calibrated at intervals of 30 s. The UniProtKB/Swiss-Prot database (Release 14.8; 408, 099 entries) was searched for each run with the following parameters: peptide tolerance, 15 ppm for MS^E and 0.2 Da for DDA; fragment tolerance, 15 ppm for MS^E and 0.1 Da for DDA; trypsin missed cleavages, 2; fixed modification, Met oxidation; and variable modifications, acetylation (K), tri-, di- and mono-methylation (K/R), and deuterated acetylation (K and N-terminal). Alternatively MS/MS spectra (peak lists) were also searched against the above database using Mascot version 2.2.04 (Matrix Science) and the following parameters: peptide tolerance, 0.2 Da; ¹³C = 2; fragment tolerance, 0.1 Da; missed cleavages, 2; instrument type, ESI-Q-TOF. All database searches were restricted to human species because of the complexity of the searches when combined with multiple modifications. The interpretation and presentation of MS/MS data were performed according to published guidelines³¹. Assignments of trimethylation and acetylation sites by PLGS/Mascot for the sequence ⁹KSTGGKAPR¹⁷ were verified by manual inspection. Relative quantitative

analysis of the different conditions measured changes in abundance of the selected post-translationally modified (PTM) histone peptides using raw data from the alternate high/low collision energy MS^E experiments which were processed with MassLynx version 4.1. Quantitation was based on peak heights from extracted ion chromatograms using observed precursor ions and retention time data of the selected PTM histone peptides normalized to an internal standard corresponding to a non-PTM tryptic digest fragment. Ion chromatograms were extracted using the mass windows of ± 0.1 Da and 0.05 Da, for the PTM fragment of interest and an internal standard, respectively. The internal standard used was the histone H3 tryptic peptide fragment ⁴¹YRPGTV⁴⁹ALR for which no modifications were observed.

RESULTS

To assess demethylase inhibition in cells, HEK293T cells were transfected either with control or *JMJD2A* vectors, followed by treatment with the different 2,4-PDCA derivatives for three days (outlined in Figure 1). The analysis of histone H3 K9 trimethylation was performed either by antibody based detection or by mass spectrometry using a chemical derivatization strategy. Initially, we treated HEK 293T cells with 2,4-PDCA derivatives to determine the non-cytotoxic concentrations for use in the HDM inhibitor assays (Figure 2). The concentrations required to 50% of cell death (CC₅₀) for the diethyl, diethyl and diamide derivatives were 1.4 mM, 3.9 mM and 4.6 mM, respectively. No cytotoxicity was observed at the concentration of 0.3 mM for the diethyl and dimethyl esters, and up to 1 mM for the diamide derivative.

Subsequently, the effects of the 2,4-PDCA derivatives on the inhibition of H3K9 demethylation were determined in cells that were treated in duplicates for three days. HEK 293T cells were transfected with either control vector (EV) or *JMJD2A* for 24 hours prior to exposure to either 0.3 mM or 1 mM inhibitor (Figure 1), then harvested after three days. Cell extracts were prepared by lysis with SDS-urea buffer and separated by SDS-PAGE, followed by immunoblotting using an anti-H3K9me3 antibody.

Histone demethylase (*JMJD2A*) inhibition by 2,4-PDCA derivatives in cells

Consistent with previous reports ^{11, 32}, overexpression of *JMJD2A* led to a marked reduction in H3K9me3 levels compared to control cells (Figure 3). In cells overexpressing, *JMJD2A*, treatment with the 2,4-PDCA dimethyl ester showed a marked increase in the level of H3K9me3 consistent with the inhibition of *JMJD2A*. Increased H3K9me3 trimethylation levels were also seen with the diethyl ester and diamide derivatives, but to a lesser extent (Figure 3). Semi-quantitation by densitometric analysis suggested a greater than four fold increase of H3K9me3 levels upon 2,4-PDCA dimethyl ester treatment in *JMJD2A* overexpressing cells. Accumulation of the trimethylated H3K9 was also observed following treatment with 1 mM 2,4-PDCA dimethyl ester for 12 hours, but to a lesser extent (data not shown). Compared to the *JMJD2A* transfected cells, only marginal differences in H3K9me3 levels were seen between inhibitor-treated and -untreated cells that were not overexpressing *JMJD2A*.

Core histone extraction and chemical derivatization of H3 histone for MS analysis

We then developed an MS based approach for analyzing the methylation status of H3K9. After treatment with the 2,4-PDCA derivatives, histone proteins were isolated by acid extraction³ from HEK 293T cells (normal and overexpressing JMJD2A). The core histones in the acid extracts were separated into their different isoforms (H1, H2, H3 and H4) by SDS-PAGE followed by excision of histone H3 containing gel bands (Figure 4A). Analyses of HeLa H3 histone tryptic fragments without prior chemical derivatization failed to yield fragments containing the lysine 9 residue (data not shown). To overcome this problem, samples were treated prior to trypsin digestion with deuterated acetic anhydride to introduce the trideuterated-acetyl group ($\text{CD}_3\text{CO}-$) at positions of unmodified and monomethylated lysine residues, a procedure reported to increase recovery and detection of *N*-terminal histone peptides^{27, 33}. Acetylation rendered the peptides more hydrophobic thereby increasing their retention time by reverse-phase chromatography and eliminated trypsin cleavage sites on the unmodified and monomethylated lysine residues^{27, 33-35}. Derivatization led to complete acetylation of unmodified K14, which was thus observed in both deuterated and non-deuterated forms. The gel-excised d_3 -acetylated samples were subjected to a standard trypsin digestion procedure followed by extraction of peptides from the gel pieces.

We also attempted an approach using derivatization with propionic anhydride. However, detection of the propionylated material was not pursued because the MS analysis was complex due to isobaric redundancy (see below), a previously reported problem for propionylated histone peptide material²⁷. The H3 $^9\text{Kme3STGGKacAPR}^{17}$ H3 9-17 peptide was selected for quantitation because the trimethyl modification on K9 could be used as an indicator of JMJD2A HDM activity.

MS-based identification of trimethylation and acetylation modifications on the lysine residues of the H3 $^9\text{KSTGGKAPR}^{17}$ peptide fragment

The eluted tryptic histone fragments were then analyzed for identification by ultra-performance nano-liquid chromatography in data-directed analysis (DDA) mode using the conditions described above³⁶. The majority of the derivatized tryptic fragment peptides were doubly- and triply-charged which facilitated sequencing and identification. This procedure yielded a tryptic fragment peptide with a sequence (KSTGGKAPR) corresponding to residues 9-17 from histone H3 (other fragments from the PTM region of H3 e.g. 1-8, 18-26, 19-26, 27-40 and 73-83 were also detected). The MS analyses revealed that this peptide contained combinations of deuterio/acetylated and trimethylated modifications at K9 and K14 (Table 1 and Figure 4), all of which gave Mascot scores between 28-50 and delta masses (i.e. mass differences) between expected and theoretical masses of 0.01 Da or less (Table 1).

The presence of isobaric peptides, generally on *N*-terminal histone tails, including the H3 9-17 peptide, is a significant challenge for MS-analyses. Redundancy was particularly pronounced in the peptides produced by a propionylation procedure. Propionylated peptides showed identical masses for several combinations of PTMs on the H3 9-17 peptide, for example, m/z 1012.57 for (propionylated)KmeSTGGKacAPR and

K(propionylated)STGGK- (propionylated) APR or m/z 984.54 for KacSTGGKacAPR and Kme2STGGK- (propionylated)APR (data not shown). Isobaric redundancy was less of a problem for the d_3 -acetylated material.

The mass difference between Kme3STGGKacAPR and KacSTGGKacAPR peptides is 0.036 Da (acetyl group, 42.0106 Da and trimethyl group, 42.0470 Da) (Table 1). Although, it is possible to distinguish these two peptides using this small mass difference, the Mascot-based assignment alone does not directly distinguish them. Identification of these two peptides required the manual inspection of the Mascot-processed MS/MS spectra which showed smaller delta masses for the correct assignments for all the precursor and fragment ions. Despite the utility of this type of analysis, it was desirable to distinguish the peptides by other parameters. Previous work has shown that differences in retention times and fragmentation patterns can be used to distinguish these peptides³⁷⁻⁴⁰. We therefore used these parameters to assign these two PTMs on K9 and K14 as described below.

MS/MS analysis of the synthetic and biological sample derived H3 ⁹KacSTGGKacAPR¹⁷, ⁹Kme3STGGKacAPR¹⁷ and ⁹KacSTGGKme3APR¹⁷ peptides

Three peptide standards were synthesized, i.e. KacSTGGKacAPR, Kme3STGGKacAPR and KacSTGGKme3APR, in order to enable PTM assignments of histone H3 based on differences in retention time and fragmentation patterns. Extracted ion chromatograms were used to compare retention times for the three standards. A clear shift to a later retention time was observed for the peptide derivative containing two acetyl groups as compared to the trimethyl/acetyl containing peptide counterparts (Table 1 and Figure 4B). The isobaric modified peptides K9me3K14ac and K9acK14me3 have very close retention times (data not shown) so other parameters are required to differentiate between these two species.

Significant differences were observed in the MS/MS spectra of the peptides Kme3ATGGKacAPR, KacSTGGKme3APR, and ⁹KacSTGGKacAPR¹⁷. The MS/MS spectrum of KacSTGGKme3APR showed the accumulation of several y -NH₃ and y -59 ions ($y_{6,8}$) likely resulting from a Hoffman type elimination⁴⁰ of the C-terminal N^{ϵ} trimethyllysine on the KacSTGGKme3APR peptide (Figure S2). The Hoffman-type elimination involved the loss of trimethylamine, N(CH₃)₃ corresponding to a mass loss of -59 Da. In contrast, the MS/MS spectrum of Kme3STGGKacAPR (Figure S1) was characterized by the presence of intense y -type ions ($y_{5,8}$) and an accumulation of the b_2 -59 (199.09) ion. The MS/MS spectrum of KacSTGGKacAPR (Figure S3) displayed strong y -series ions with the $y_{6,8}$ ions showing similar intensities unlike the varying intensities seen for these ions resulting from the fragmentation of the Kme3STGGKacAPR peptide. Accumulation of the b_2 -59 ion (m/z 199.09) was not observed in either the KacSTGGKacAPR or Kme3STGGKacAPR peptides. Therefore, their different fragmentation patterns resulting from Hofmann elimination could be used to distinguish all three peptides.

MS/MS spectra and LC retention times from extracted ion chromatograms of the relevant H3 fragment peptides from the histone extracts were consistent with the synthetic standards for either acetylation/acetylation or trimethylation/acetylation on K9 and K14. Mascot identically assigned both the ⁹Kme3STGGKacAPR¹⁷ and ⁹Kac STGGKme3APR¹⁷

peptides. However, the presence of the latter was ruled out based on a comparison of the MS/MS spectra between the synthetic standards for both peptides and the histone extracts. The MS/MS fragmentation pattern obtained for the KacSTGGKme3APR synthetic standard was not observed in the histone H3 preparations isolated from cells.

MS-based quantitation of histone demethylase (JMJD2A) inhibition by 2,4-PDCA derivatives using high/low collision switching mass spectrometry (nano-UPLC-MS^E)

Having established the methodology for discriminating between acetylation/acetylation and trimethylation/acetylation of the H3 9-17 peptides by MS, it was then possible to quantify the relative levels of Kme3STGGKacAPR in HEK 293T cells treated with 2,4-PDCA derivatives by alternating low/high collision LC-MS (nano-UPLC-MS^E). The intensities of the H3 ⁹Kme3STGGKacAPR¹⁷ mass peak were obtained from the extracted ion chromatograms from both the triply and doubly charged ions for the 2,4-PDCA treated and untreated samples (Figure 5). Kme3STGGKacAPR levels were normalized using an internal standard from a tryptic fragment to correct for the variations in the amounts of histone in the acid extracts. The analysis of the various tryptic fragments for H3 showed that the sequences ⁴¹YRPGTVALR⁴⁹ (doubly charged, 516.80 Da) and ⁷³EIAQDFK⁷⁹ (doubly charged, 425.71 Da) were unmodified in every sample examined, which allowed their consideration as internal standards. Analysis of the MS/MS fragmentation spectra using Mascot did not assign modifications to these two sequences (Ac, *d*₃-Ac, Me, Me₂, Me₃ were considered as potential variable modifications). Furthermore, no modifications have been observed at these sites in the literature (UniProtKB and the Swiss-Prot database, Release 14.8, 408, 099 entries). The ⁴¹YRPGTVALR⁴⁹ sequence was chosen as the internal standard because peak intensities were within the dynamic range of the mass spectrometer (intensity <1×10⁴ ion counts). The relative ion intensities of Kme3STGGKacAPR, corrected using ion intensities of the internal standard peptide, were then compared to the untreated control in the empty vector or JMJD2A overexpressed sets (+/- inhibitor) (untreated control corresponded to an arbitrary unit of 1.0) once the quantitation method has been established.

Using this MS approach, we observed an accumulation of Kme3STGGKacAPR levels compared to untreated control (relative value > 1) which was consistent with an inhibitory effect of JMJD2A HDM (Figure 6). Treatment with 2,4-PDCA dimethyl ester showed the highest levels of Kme3STGGKacAPR in both normal and JMJD2A overexpressing cells. The observed levels of Kme3STGGKacAPR peptide were higher than the untreated control by 3.9- and 13-fold, at endogenous and overexpressed levels of JMJD2A, respectively. Additionally, the values for relative Kme3STGGKacAPR levels were similar for the doubly- (m/z 493.28) and triply- (m/z 329.20) charged states for each treatment (Figure 6). One representative out of two independent experiments is shown. Note that the levels of Kme3STGGKacAPR represent the combined results for histone fragments from all H3 variants (mainly H3.1, H3.2 and H3.3). The results did not take into account any modifications within residues 9-17, including phosphorylation at serine 10 ⁴¹ or threonine 11⁴².

DISCUSSION

Our work has focused on the inhibition of the HDM JMJD2A by a set of likely cell-penetrating derivatives of pyridine-2,4-dicarboxylic acid, a recently identified ‘template’ for HDM inhibitors that inhibit JMJD2 with IC₅₀ values in the micromolar range^{14, 17}. The results demonstrate the viability of quantitative MS-based methodology for the analysis of small molecules that inhibit histone modifying enzymes in cells. Following initial antibody-based work that verified that 2,4-PDCA derivatives inhibit JMJD2A (and likely other HDMs) in cells, we optimized an MS-based protocol to monitor effects on histone modifications directly. We focused on a known substrate for JMJD2A, i.e. the trimethylated form at H3K9. In order to detect a H3K9-containing tryptic fragment, we employed a chemical derivatization protocol using deuterated acetic anhydride.

Based on the work of Zhang et al.^{39, 40}, we developed an MS-based assay to monitor the relative extent of H3K9 trimethylation in differently treated cells. The optimal procedure employed chemical derivatization by acetylation of lysine residues to enable detection of the target fragment peptide by increasing retention time and blocking trypsinolysis at unmodified lysines as the histone N-terminal tail is rich in lysine and arginine residues. The use of deuterated acetic anhydride enabled the identification of unmodified lysine residues that could otherwise be overlooked by conventional proteolytic digestion.

Assignment of the target peptide over other isobaric sequences can be achieved by exploiting the high resolution of the UPLC-MS to discriminate between small differences in the absolute masses of H3 ⁹Kme3STGGKacAPR¹⁷ and ⁹Kac3STGGKacAPR¹⁷, such as MS/MS fragmentation of the peptide backbone, further fragmentation of the side chain (Hofmann elimination and immonium ions) and retention time differences. The combination of these parameters enabled us to distinguish between the Kme3STGGKacAPR, KacSTGGKacAPR and KacSTGGKme3APR peptides. The extent of HDM inhibition can be ascertained by measuring the relative levels of the histone H3 ⁹Kme3STGGKacAPR¹⁷ tryptic fragment using alternating low/high collision LC-MS, indicative of trimethylation levels at H3K9.

Notably we were able to distinguish *N*^ε-trimethyl lysine from *N*^ε-acetyl lysine residues by observing the Hoffman type fragmentation in the former but not the latter as reported by Zhang and colleagues^{39, 40}. Additionally, these two peptides could be distinguished based on the presence of the b₂-59 ion resulting from Hofmann elimination (described above) in the former peptide^{39, 40}. Consistent with these reports, our data shows that the b₂-59 ion peak at m/z 199.09 Da was only detected in the MS/MS spectrum of the synthetic and biological Kme3STGGKacAPR, but not the KacSTGGKacAPR peptide. Additionally, both peptides could also be distinguished based on pronounced differences in the pattern of the y-ion series from y_{6,8} (stronger peaks of comparable intensity for ac/ac and strong y₈ for me3/ac on lysines 9 and 14), and the presence of an intense acetyl lysine immonium ion at m/z 143.10 Da. Furthermore, the related base peak ion resulting from the loss of ammonia at m/z 126.06 Da was predominantly observed for KacSTGGKacAPR. This ion was detected at a much weaker intensity in the spectrum of the Kme3STGGKacAPR standard, which was dominated by the lysine immonium ion at m/z 84.07 (base peak). Immonium ions have also

been used previously to assign histone modifications on acetyllysine^{40, 43}. The increased retention time of the acetylated peptide (KacSTGGKacAPR) compared to the trimethylated peptide (Kme3STGGKacAPR) is also consistent with previous reports on the effects of these two modifications.

Although the isobaric peptides Kme3STGGKacAPR and KacSTGGKme3APR were identically assigned by Mascot and showed similar retention times, the presence of the latter was ruled out based on the comparison of the MS/MS spectra between the synthetic standards for both peptides and the biological samples (–59 Da Hoffmann elimination occurred on different fragment ions). All the biological samples displayed the MS/MS spectra of Kme3STGGKacAPR only, which is supported by the fact that existence of the H3K14me3 modification has not been reported in the literature.

In addition to trimethylation, the Mascot searches also detected both mono- and dimethylation on the H3K9 peptide (data not shown). These methylation states were not included in our quantitative analyses because H3K9me3/me2 are both substrates for JMJD2A (note that like the trimethyl N^ε-lysine, the N^ε-dimethylated state could not react with the acetic anhydride to give a stable product, whereas the monomethylated state will likely do so) and contribute to possible mixtures of methylation states, thereby complicating the analysis considerably. It should also be taken into account that the digestion of H3 histone material with trypsin produces ⁹Kme3STGGKacAPR¹⁷ fragments resulting from all H3 variants including H3.1, H3.2 and H3.3 and their derivatives. Therefore, H3 ⁹Kme3STGGKacAPR¹⁷ peptide levels should be interpreted as a ‘global’ average of an asynchronous cell population. PTMs on core histones have been reported to be mainly identical throughout the various stages of the cell cycle except during the short period of mitosis⁴⁴. During mitosis, drastic changes occur on H3 and H4 involving phosphorylation on serine 10 in H3. However, this and other modifications such as on threonine 11 were excluded from the quantitative analysis of the H3 9-17 peptide fragment.

Relative quantitation of ⁹Kme3STGGKacAPR¹⁷ levels to evaluate the effect of cell-permeable 2,4-PDCA derivatives on H3K9me3 HDM inhibition using alternating low/high collision nano-UPLC-MS^E analysis^{45, 46} on the chemically derivatized H3 material represents a useful alternative to other labeling techniques such as stable isotope labeling by amino acids in cell culture (SILAC)⁴⁷, tandem mass tags (TMT)⁴⁸, isotope-coded affinity tags (ICAT)⁴⁹, and isotope tags for relative and absolute quantification (iTRAQ)⁵⁰. Levels of the ⁹Kme3STGGKacAPR¹⁷ peptide in all samples were normalized using the internal tryptic fragment ⁴¹YRPGTVALR⁴⁹ as a protein loading control and their levels compared between cells treated with 2,4-PDCA derivatives and untreated control.

The 2,4-PDCA dimethyl ester was found to be the most potent inhibitor of H3K9 demethylation in cells overexpressing JMJD2A as compared to endogenous levels, with 13- and 4-fold increases being observed in the trimethylated state, respectively, although the observed levels of increase did vary between independent experiments. Although the diethyl ester and diamide derivatives showed between a 2-3 fold inhibition in cells overexpressing JMJD2A, no notable effect was observed in control cells. These results observed in normal

cells may be due to the inhibition of other targets such as prolyl hydroxylases as previously demonstrated for HOE 077²⁴.

The results of the immunoblotting analysis supported and gave credence to the MS-approach describe here, both corroborating the inhibitory effect of the 2,4-PDCA derivatives on HDMs. Unlike the MS data, densitometric analysis of band intensities for semi-quantitation is hampered by saturation effects and the narrow dynamic range. The methodology developed here will be applicable not only to HDM inhibitors, but also to methyltransferase inhibitors, in which the levels of H3 K9me3ATGGKacAPR¹⁴ (or other suitably derivatized peptides) can function as a readout for lysine methyltransferase activity. Thus, with appropriate optimization and automation, it is likely that such an MS-based approach will be useful for detailed quantitative studies on the effect of small-molecules on histone methylation states. Although we have focused on a single modification, MS-based methods also have the potential to be developed for the quantitative analysis of complex combinations of histone modifications, which is difficult or often not possible to be achieved by antibody based methodologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HMT	histone methyltransferase
HDM	histone demethylase
JMJD2A	JMJD2A histone demethylase
LC-MS/MS	liquid chromatography tandem mass spectrometry
MS	mass spectrometry
UPLC-MS^E	ultra-performance liquid chromatography high/low collision switching mass spectrometry
2,4-PDCA	pyridine-2,4-dicarboxylic acid

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Figure 1.

Scheme for the analysis of demethylase inhibition in cells. Treatment with propionic anhydride was also carried out but not used for the final inhibitor analysis (see Results and Discussion sections).

Figure 2.

Cytotoxicity of small molecule demethylase inhibitors. **A**, 2,4-PDCA diethyl ester. **B**, *N,N'*-bis(2-methoxyethyl)-2,4-pyridine dicarboxamide (referred to as 2,4-PDCA diamide in this study). **C**, 2,4-PDCA dimethyl ester. HEK 293T cells were incubated for three days with inhibitors at the concentrations indicated. Cell viability was measured by adding MTS reagent for 1-3 hours followed by measuring the absorbance at 490 nm. The CC_{50} values (50% cytotoxicity) are indicated.

Figure 3. Inhibition of H3K9 demethylation by 2,4-PDCA derivatives assessed by immunoblotting. HEK 293T cells were transfected either with empty vector (EV) or JMJD2A for 24hr followed by incubation with inhibitors for three days. **A**, Immunoblotting analysis of whole cell lysates from HEK 293T cells using antibodies specific for H3K9me3, Flag-tagged JMJD2A and β-actin. **B**, Relative levels (normalized) of H3K9me3 from densitometric analysis of autoradiographic exposures (set to 1.0 for the untreated control cells).

Figure 4.

MS-based detection of H3K9 modifications. **A**, SDS-PAGE gel (18%) of core-histone enriched extract indicated the presence of H3 polypeptide material. **B**, Sequence of histone H3 showing the peptides used for quantitative analysis. **C**, (i) MS/MS spectrum of triply charged K9me3STGGK14acAPR (ii) MS/MS spectrum of doubly charged K9acSTGGK14acAPR (iii) MS/MS spectrum of the doubly charged internal peptide standard ⁴¹YRPGTVALR⁴⁹. The b and y fragment ions are indicated. (iv) Chromatography

traces of synthetic standards K9me3STGGK14acAPR and K9acSTGGK14acAPR (ion extraction chromatograms).

Figure 5.

MS-based quantitative measurement of H3K9 demethylation in living HEK 293T cells overexpressing JMJD2A. Extracted ion chromatograms of **A**, K9me3STGGK14acAPR (329.2 ± 0.1 Da) level in 2,4-PDCA dimethyl ester (0.3 mM) treated cells; **B**, Internal peptide standard $^{41}\text{YRPGTVLR}^{49}$ (516.8 ± 0.05 Da) level in 2,4-PDCA dimethyl ester (0.3 mM) treated cells; **C**, K9me3STGGK14acAPR level in untreated control cells; and **D**, Internal peptide standard $^{41}\text{YRPGTVLR}^{49}$ level in untreated control cells.

Figure 6.

Inhibition of H3K9 demethylation by 2,4-PDCA derivatives assessed by quantitative MS. The black bars indicate ion intensities of the doubly charged precursor ion (493.28 Da) and the grey bars the ion intensities of the triply charged precursor ion (329.20 Da) of the peptide $^9\text{Kme3STGGKacAPR}^{17}$. The peptide ion intensities are normalized relative to the ion intensities of the peptide standard $^{41}\text{YRPGTVALR}^{49}$ observed in the same sample (set to 1.0 for the untreated control cells). One representative of two independent experiments is shown.

Table 1

Post-translational modifications on the ⁹KSTGGKAPR¹⁷ histone peptide from H3.2 assessed by MS

Mod on K9	Mod on K14	Observed mass (m/z)	Charge state	Expected mass	Theoretical mass	Delta mass*	Retention Time [min]	Mascot Score
Me ₃	Ac	329.2016	+3	984.5830	984.5716	0.0114	24.4	41
Me ₃	d ₃ -Ac	330.2019	+3	987.5824	987.5905	-0.0081	24.4	28
Ac	Ac	493.2783	+2	984.5420	984.5352	0.0068	28.7	48
Ac	d ₃ -Ac	494.7877	+2	987.5608	987.5540	0.0068	28.7	34
d ₃ -Ac	Ac	494.7877	+2	987.5608	987.5540	0.0068	28.7	34
d ₃ -Ac	d ₃ -Ac	496.2943	+2	990.5752	990.5740	0.0012	28.7	34

* Difference between expected and theoretical mass