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Screening for lysine-specific demethylase-1 inhibitors using a label-free high-throughput mass spectrometry assay

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ABSTRACT

Posttranslational modifications on the N terminus of histone H3 act in a combinatorial fashion to control epigenetic responses to extracellular stimuli. Lysine-specific demethylase-1 (LSD1) represents an emerging epigenetic target class for the discovery of novel antitumor therapies. In this study, a high-throughput mass spectrometry (HTMS) assay was developed to measure LSD1-catalyzed demethylation of lysine-4 on several H3 substrates. The assay leverages RapidFire chromatography in line with a triple stage quadrupole detection method to measure multiple LSD1 substrate and product reactions from an assay well. This approach minimizes artifacts from fluorescence interference and eliminates the need for antibody specificity to methylated lysines. The assay was robust in a high-throughput screen of a focused library consisting of more than 56,000 unique chemical scaffolds with a median Z' of 0.76. Validated hits from the primary screen were followed up by successive rounds of virtual and HTMS screening to mine for related structures in a parent library consisting of millions of compounds. The screen resulted in the rapid discovery of multiple chemical classes amenable to medicinal chemistry optimization. This assay was further developed into a generic platform capable of rapidly screening epigenetic targets that use the N-terminal tail of histone H3 as a substrate.

In eukaryotes, DNA is wrapped around octameric structures consisting of two tetramers of histone proteins H3, H4, H4A, and H2B, forming nucleosomes [1]. Nucleosomes are the fundamental structures of chromatin that condense the genetic code and control a variety of cellular processes such as gene expression, DNA replication, and repair. These processes are tightly regulated by nonrandom posttranslational modifications (PTMs)[2] on amino acid residues that are generally localized to the N-terminal region of histone proteins. A large number of histone PTMs have been identified, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and ribosylation [2]. These PTMs are often referred to as the histone code and act in a combinatorial manner to allow for the alteration of nucleosome structure that dictates gene expression patterns in responses to extracellular stimuli. Histone H3 contains a majority of the histone code in the N-terminal 50 residues [3]. In particular, the methylation pattern on specific H3 lysine residues correlates with the overall structure of chromatin. Methylation on H3 lysine-9 and -27 is associated with transcriptionally repressed genes in regions of highly condensed heterochromatin [4], whereas methylation on H3 lysine-4, -36, and -79 is associated with transcriptionally active genes in euchromatin, defined as regions where DNA is loosely packed around nucleosomes and accessible to transcription factors [4]. The reversible regulation of histone methylation enables gene expression to be dynamically regulated during cellular differentiation, growth, and proliferation.

The regulation of histone methylation requires a balance of activities between H3 histone lysine demethylases (LSD1, JMJD2C, JMJD2D) and methyltransferases (G9a, SMYD, NSD2, EZH2), many of which appear to be attractive targets for drug discovery because they have been linked to aberrant gene expression in various diseases, including cancer [5–9]. In 2004, lysine-specific demethylase-1 (LSD1) was discovered to be a flavin adenine dinucleotide (FAD)-dependent amine oxidase [9]. LSD1 interacts with CoREST, a 33-kDa cofactor, and localizes to the N terminus of H3 in nucleosomes [10–12]. LSD1 specifically catalyzes demethylation of H3 di- and monomethylated lysine-4. H3 dimethylated lysine-4 is a

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[2] *Abbreviations used:* PTM, posttranslational modification; LSD1, lysine-specific demethylase-1; FAD, flavin adenine dinucleotide; AR, androgen receptor; APAO, acetylpolyamine oxidase; SMO, spermine oxidase; MAO, monoamine oxidase; 2-PCPA, tranylcypromine; H3K4, H3 lysine-4; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; cDNA, complementary DNA; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; AUC, area under the curve; HTMS, high-throughput mass spectrometry; SRM, selective reaction monitoring; SAR, structure–activity relationship.

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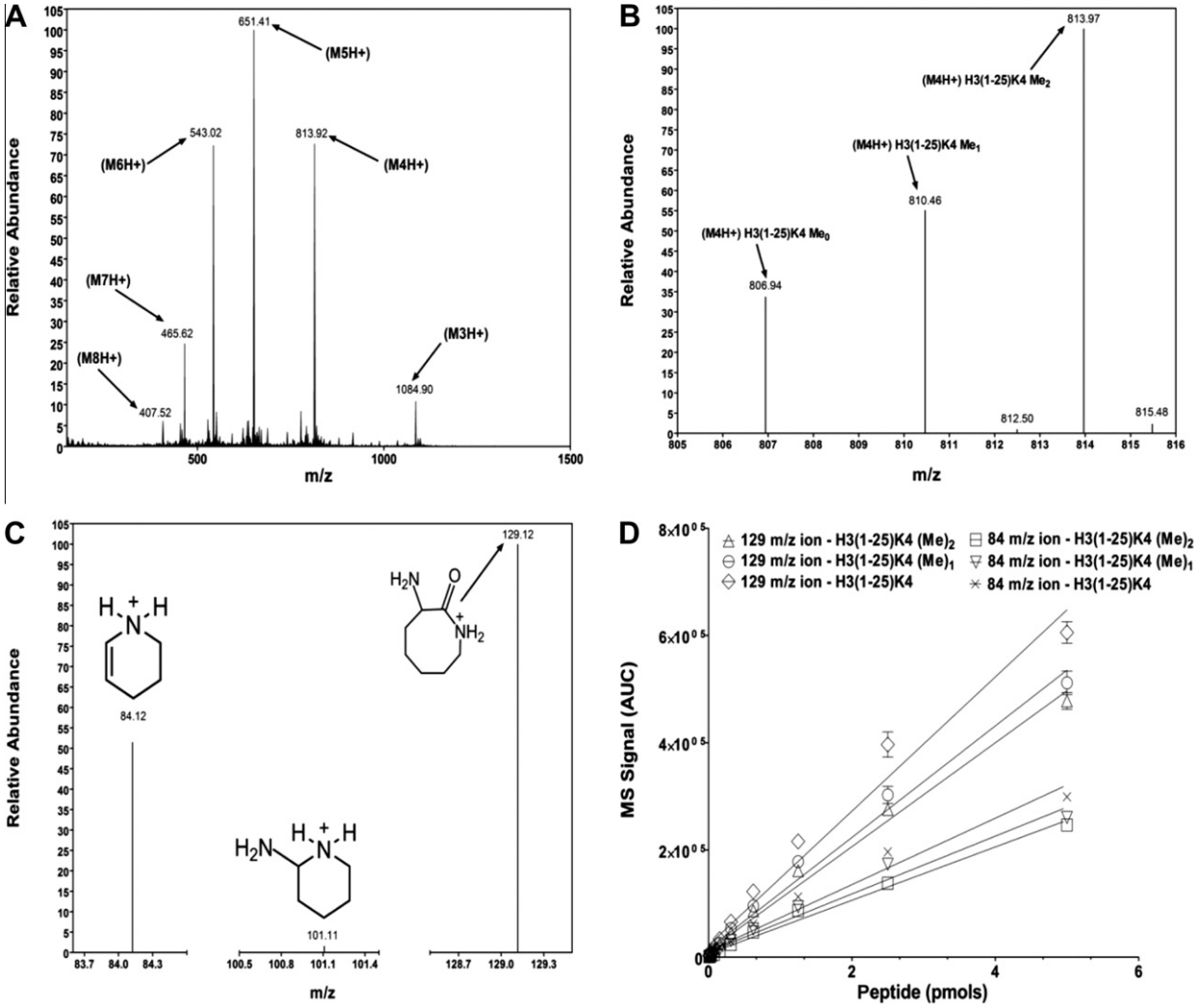

Fig.1. Analysis of LSD1 substrate and product standards by mass spectrometry. Peptide standards corresponding to unmethyl-, monomethyl-, and dimethyl-H3(1–25) were analyzed using a TSQ Vantage triple stage quadrupole system. (A) Full precursor ion scan (Q1: m/z = 175–1500) of 4 μM dimethyl-H3K4(1–25) peptide standard analyzed by static electrospray in positive ion mode. The extensive charge state distribution observed is characteristic of the H3(1–25) peptide. (B) Precursor ion scan (Q1: m/z = 805–816) for the M4H+ charge states of unmethyl-, monomethyl-, and dimethyl-H3K4(1–25) standards mixed at 1, 2, and 4 μM, respectively. The data were acquired by static electrospray in positive ion mode. (C) Fragment ion scans (Q3: m/z values = 84, 101, and 129) showing the M4H + precursor breakdown products corresponding to the lysine immonium ion (–NH_3), immonium ion, and acylium ion formed by gas-phase rearrangement, respectively. The data were acquired on dimethyl-H3K4(1–25) by static electrospray in positive ion mode. (D) Quantitative analysis of the linearity and dynamic range for the lysine immonium ion (–NH_3) (m/z = 84) and the lysine acylium ion (m/z = 129) used for SRM scanning in Q3. The data were acquired on a range of concentrations for each the unmethyl-, monomethyl-, and dimethyl-H3K4(1–25) peptide standards using RapidFire chromatography as described in Materials and Methods. MS, mass spectrometry.

key modification that is associated with promoters of active genes. Overexpression of LSD1 leads to excessive demethylation of H3 lysine-4 and is thought to contribute to the development of cancer, in part, by preventing the expression of tumor suppressor genes. Several studies with colorectal, breast, lung, and neuroblastoma cancer cell lines report that small molecule inhibitors of LSD1 inhibit cell proliferation [13–17]. A corresponding reexpression of aberrantly silenced tumor suppressor genes, such as secreted frizzled-related proteins (SFRP family) and the GATA family of transcription factors, has been observed [13,14]. Several of these LSD1 inhibitors were further demonstrated to have in vivo antitumor activity in neuroblastoma and colorectal cancer xenograft models [13,17]. In addition, reduction of LSD1 using small interfering RNA (siRNA) or small molecule inhibitors of LSD1 transcription reduces androgen receptor (AR)-activated gene expression [18]. Recently, a mechanism for several histone deacetylase inhibitors was proposed and is based on inhibition of LSD1 transcription levels in prostate carcinoma cells [19]. These pharmacological agents were previously shown to reduce prostate tumorigenesis in a murine transgenic model [20,21]. Collectively, these studies highlight the importance of LSD1 as an emerging target for the development of novel antitumor therapeutics.

A limited number of LSD1 inhibitor classes have been reported. Several of these inhibitor classes are designed based on a high degree of sequence homology between the catalytic domains of LSD1 and other oxidases. The polyamine class is derived from inhibitors of N^1-acetylpolyamine oxidase (APAO) and spermine oxidase (SMO) and inhibit LSD1 through a substrate competitive mechanism [13] On the other hand, the related bisguanidine and biguanide polyamines inhibit LSD1 through a noncompetitive mechanism [14]. The monoamine oxidase (MAO) inhibitors of LSD1 are designed around tranylcypromine (2-PCPA), which is used clinically as an antidepressant. Members of the 2-PCPA class act as suicide inhibitors of LSD1 by covalently linking FAD through a single electron transfer mechanism [22–24]. A third class of LSD1 inhibitors is designed from an H3 peptide substrate in which lysine-4 is mutated to a methionine [12] or derivatized with *N*-methylpropargyl, the latter of which forms a covalent adduct with FAD