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No Taste for Methyl: Methylation Sensitive Proteolysis in Drug Screening

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Methyltransferases and demethylases are emerging targets for epigenetic therapy. Wigle et al. (2010) present a new assay for the enzyme inhibition of methyltransferases and demethylases based on selective enzymatic cleavage of unmethylated versus methylated peptides and their subsequent electrophoretic separation.

The therapeutic potential of drugs that influence posttranslational modifications of histones is vast, as there are many diseases, especially cancer, that have been linked to aberrant gene expression patterns or deregulated histone modifications. Therefore, it is of particular importance to find the inhibitors of histone-modifying enzymes in order to target these diseases; usually, as a first step in epigenetic drug discovery, biochemical in vitro assays are the tools of choice. Wigle et al. (2010) have presented a new assay for studying enzyme and inhibition kinetics of methyltransferases and demethylases that is expanding the possible ways to explore these enzymes. Histone methylation and demethylation are two of the most interesting posttranslational modifications, which may also occur on nonhistone substrates. Histone methylation is mediated by histone methyltransferases and is involved in several important processes, such as heterochromatin formation and maintenance, X chromosome inactivation, transcriptional regulation, DNA repair, RNA maturation, and genomic imprinting (Spannhoff et al., 2009). Histones can be methylated either on lysine or on arginine residues. Depending on the site of methylation, lysine methylation can be linked either to transcriptional activation or repression. Methylation on H3K4 (throughout the text, specific histone residues will be labeled as follows: histone H3 lysine 4, H3K4, etc.), H3K36, and H3K79, for example, is considered to be activating, whereas methylated H3K9, H3K27, and H4K20 are regarded as repressive marks (Martin and Zhang, 2005). The loss of trimethylation at H4K20 is a general hallmark of human cancer, together with the loss of acetylation at H4K16 (Fraga et al., 2005).

Histone methyltransferases are by far not restricted to histones as substrates. Besides histone proteins, many other targets for histone methyltransferases have been identified thus far—e.g., the tumor suppressor protein p53. Both lysine and arginine residues within p53 are methylated by different enzymes. To give an example, the lysine methyltransferase G9a (KMT1C), also chosen by the authors as a representative methyltransferase in the newly established assay, has recently been shown to dimethylate p53 on K373. This was correlated with inactivity of p53 (Huang et al., 2010). Its impact on apoptotic processes, as well as its overexpression in various cancer types, suggests that G9a is a putative oncogene (Huang et al., 2010). Demethylation of methylated lysines is catalyzed by LSD1, which is able to demethylate H3K4 me1/me2 (Shi et al., 2004) and JmjC-domain-containing enzymes that are able to turn over trimethylated lysines (Xiang et al., 2007). LSD1 colocalizes with the androgen receptor and participates in androgen-receptor activation. Thus, the inhibitors of LSD1 activity could be valuable drugs that affect androgen-receptor functions (Metzger et al., 2005).

As for probing enzyme activity and the potency of inhibitors, some assays are already available for methyltransferases (reviewed in Spannhoff et al. [2009]), which are now complemented by Wigle et al. (2010) with their newly presented assay. For methyltransferases, the incorporation of radioactivity by radioactively labeled cofactor *S*-adenosyl-[methyl-^{3}H] can be measured. The protocols using radioactivity are sensitive, but they bear the disadvantage of hazards for the user and additional measures and costs regarding waste disposal. Also for a high throughput, specialized equipment is necessary (Scintillation proximity assay). Furthermore, the turnover of SAM to *S*-adenosyl homocysteine (SAH) can be used to detect methyltransferase activity. In a corresponding assay, the byproduct SAH is hydrolyzed to *S*-ribosylhomocysteine and adenine by a nucleosidase. The formed adenine is then hydrolyzed by a second enzyme to homoxanthine. This is accompanied by a decrease in absorbance at 265 nm that can be monitored. The low wavelength is a disadvantage due to a high risk for compound interference. Another drawback is the need for several enzymes in the procedure. A different method to detect SAH is to use the ThioGlo® reagent that is based on detecting reactive sulfhydryl groups. The caveat of this assay is that thiols are also present in many buffers, especially those for methyltransferases, and therefore this assay might not always be eligible. A further possibility to conduct a homogeneous assay is to use an AlphaScreen (*A*mplified *L*uminescent *P*roximity *H*omogeneous *A*ssay) setup (Quinn et al., 2010).

Antibody-based assays, such as the one in AlphaScreen approaches, are a good tool for the determination of enzyme activity and inhibition properties of different compounds. These assays can be set up like a construction kit, using peptide or native substrates, a number of primary antibodies for the respective target, and also different secondary antibodies for the different readout methods.

For the measurement of in vitro methyltransferase activity, a heterogeneous assay using biotinylated histone peptide as a substrate was presented by our group (Spannhoff et al., 2007). The peptide is immobilized on streptavidine-coated microtiter plates. The methylation can be recognized by using antibodies directed against the methyl mark transferred by the respective methyltransferase. Subsequent incubation with an europium-labeled secondary antibody allows the measurement of time-resolved fluorescence (TRF) to detect the methylation level. These heterogeneous assays are rather time demanding compared with the homogeneous assays.

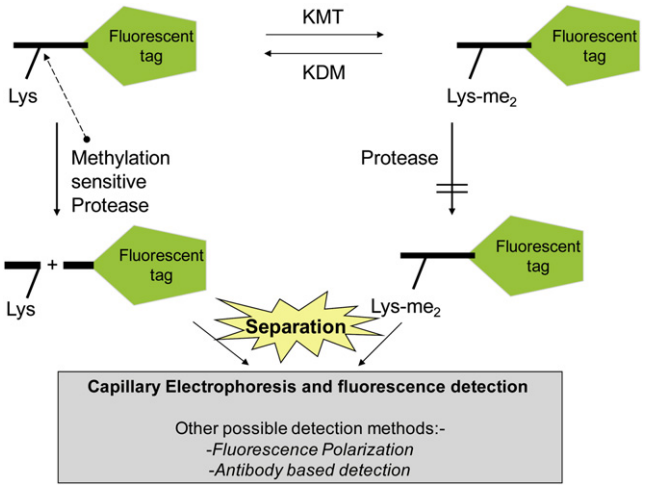

Figure 1. Screening Assay for Lysine Methyltransferases and Demethylases
Methylation-sensitive proteolysis leads to fluorescent peptides with different size and hence mass-to-charge ratio. These can be separated by capillary electrophoresis.

The newly published assay shows potential for drug screening, as it is miniaturized and can be easily automated. It is based on capillary electrophoresis, in which charged molecules are separated through their charge and size. As a disadvantage, specialized and expensive equipment (Caliper Ez Reader II) is necessary for performing the assay and fluorescence-labeled peptides have to be used. As methylated and unmethylated peptides do not differ in their charge, they normally would be difficult to separate. To solve this problem, Wigle et al. (2010) used a protease (Endo-LysC), which is sensitive to the methylation mark and does not digest methylated peptides (see Figure 1). This is the aspect of the paper with the greatest relevance for a broader research community. The methylation-sensitive proteolytic cleavage enables testing of the activity of methyltransferases and demethylases as long as a suitable peptide substrate is known and available. Because of the good solubility of the peptides and because it is not necessary to immobilize substrates, enzyme kinetics can be studied better, as with antibody-based procedures, which is another benefit of this method. The assay was set up for a lysine methyltransferase G9a and the demethylase LSD1 so far, but it could be used as well for other lysine methyltransferases. With a suitable protease, application for arginine methyltransferases such as PRMT1 should be possible as well. The cleavage reaction might also be exploited in other assay formats such as AlphaScreen, fluorescence energy transfer (FRET), or fluorescence polarization (FP) methods, similar, for example, to an established protocol for sirtuins (Milne et al., 2007).

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