Small-molecule inhibitors of histone-modifying enzymes have significant clinical utility for managing diseases such as cancer. These inhibitors are usually identified and monitored through their effects on the gain or loss of specific histone marks. In cells, multiple related enzymes can place or remove a specific mark; therefore, relying on an indirect measure of inhibitor engagement can be misleading. Mascaro et al. describe a luminescence-based ELISA approach that directly monitors binding of inhibitors to the histone lysine demethylase KDM1A.

Dynamic post-translational modifications (PTMs)\(^2\) of histones encode instructions for modulating the expression of underlying genes. In fact, the same PTM placed on different lysines of histone H3 can have opposite effects on gene expression (1). As the importance of histone PTMs in regulating gene expression became apparent, the discovery of enzymes that place or erase these marks emerged as a major goal (2). In keeping with their central role in genomic transactions, malfunctioning histone-modifying enzymes and the resulting misplaced patterns of histone PTMs have been shown to play a salient role in the etiology of a plethora of genetic diseases (3, 4). The success in blocking histone deacetylases with small molecules spurred a “gold rush” to find inhibitors of histone-modifying enzymes (5–7). The efficacy of such inhibitors is typically measured via indirect antibody-based assays of post-treatment levels of PTMs. A problem with measuring changes in PTM levels, rather than assessing inhibition of the targeted enzyme, is that multiple related cellular enzymes can place or erase a given PTM. Moreover, several forces cumulatively impact the steady-state levels of histone marks.

To circumvent these confounding effects, Mascaro et al. set out to develop an assay that measures binding of small-molecule inhibitors directly to KDM1A, a lysine demethylase that utilizes a FAD co-factor to remove methyl marks placed on lysine 4 of histone H3 (H3K4me) (Fig. 1A) (8). Armed with a method to monitor small-molecule binding directly to KDM1A, they examined several molecules that were thought to prevent demethylation by targeting this enzyme. To their surprise, several widely used and highly cited inhibitors showed little or no direct binding to KDM1A. These provocative results are consistent with other cautionary tales where citations or widespread use turned out to be unreliable indicators of a molecule’s specificity or mode of action (9). Fortunately, the approach developed by Mascaro et al. may help mitigate such indirect measures of inhibitor action because their method can be adapted to study direct binding of small-molecule inhibitors to their targeted proteins.

To measure direct interactions of KDM1A inhibitors with their target, the authors elegantly combined a highly sensitive luminescence-based ELISA approach with a reactive chemoprobe that is reminiscent of widely used activity-based probes (10) (Fig. 1B). The chemoprobe OG-881 is a bifunctional molecule that has a biotin tethered to a derivative of the authors’ previously developed KDM1A inhibitor, ORY-1001. Like the parent molecule, OG-881 covalently binds to the FAD bound to KDM1A and presents the biotin moiety as an affinity handle for a range of validation studies. After demonstrating KDM1A selectivity of their chemoprobe with standard assays, Mascaro and colleagues leveraged the aforementioned ELISA to monitor the enzyme-bound levels of the chemoprobe. Using two antibodies that bind different epitopes on KDM1A, they were able to monitor the levels of the total protein in their cellular extracts (Fig. 1B). Next, they used fluorescent beads coated with streptavidin to bind the biotin moiety of OG-881. The singlet oxygen channeled between antibody-bound donor and streptavidin-bound acceptors reveals the level of “direct engagement” between the chemoprobe and KDM1A (Fig. 1B).

More specifically, the amplified luminescence proximity homogeneous assay (AlphaLISA) utilizes tethered phthalocyanine-coated “donor” beads to detect europium-coated acceptor beads within 250 nm. Upon irradiation with a 680-nm source, the donor beads emit a singlet oxygen that, if channeled to the acceptor within 250 nm, leads to a narrow emission at 615 nm. By using acceptors that emit at different wavelengths (545 and 645 nm), the authors could multiplex the assay to simultaneously determine the total amounts of KDM1A as well as the levels at which OG-881 would bind and react with the active site (Fig. 1B). This AlphaPlex assay enabled detection of low levels of total KDM1A in cell lysates of two different cancer cell lines and from tissues of an animal model. In an important step toward monitoring direct binding of an inhibitor to KDM1A, the authors applied the AlphaPlex assay to monitor the engagement of the lead inhibitor, ORY-1001 (Fig. 1C). At different doses, ORY-1001 covalently inhibited KDM1A to differing levels in the two cancer cell types. Remarkably, the authors could...
use their AlphaPlex assay to quantitatively assess binding of ORY-1001 to KDM1A in pretreated animals. The ability to measure the level of ORY-1001 binding to KDM1A in different tissues provides invaluable pharmacokinetic (PK) and pharmacodynamic (PD) information on their lead KDM1A inhibitor.

With their assay well-validated, Mascaro and colleagues took on the challenge of testing the specificity and potency of small molecules that were thought to target KDM1A and have been widely used in highly cited studies (Fig. 1C). Pargyline (PGL), the most cited but somewhat controversial covalent inhibitor of KDM1A activity, displayed no direct binding to KDM1A. Similarly, SP2509, a noncovalent inhibitor that attenuates KDM1A activity in vitro, was found to have low potency in one cell type and was completely ineffective in another (Fig. 1C). These results add to a cautionary meme that high citation rates can propagate the use of poorly selective compounds (9). The broader message to the research community is the importance of directly monitoring target engagement with assays such as the readily adaptable AlphaPlex assay developed by Mascaro et al. Reliance on direct measures of target engagement will propel the future development of highly selective and potent small molecules as both KDM probes and inhibitor-based therapeutics.

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