Screening for Inhibitors of Low-Affinity Epigenetic Peptide-Protein Interactions: An AlphaScreen™-Based Assay for Antagonists of Methyl-Lysine Binding Proteins

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The histone code comprises many posttranslational modifications that occur mainly in histone tail peptides. The identity and location of these marks are read by a variety of histone-binding proteins that are emerging as important regulators of cellular differentiation and development and are increasingly becoming implicated in numerous disease states. The authors describe the development of the first high-throughput screening assay for the discovery of inhibitors of methyl-lysine binding proteins that will be used to initiate a full-scale discovery effort for this broad target class. They focus on the development of an AlphaScreen™-based assay for malignant brain tumor (MBT) domain-containing proteins, which bind to the lower methylation states of lysine residues present in histone tail peptides. This assay takes advantage of the avidity of the AlphaScreen™ beads to clear the hurdle to assay development presented by the low micromolar binding constants of the histone binding proteins for their cognate peptides. The assay is applicable to other families of methyl-lysine binding proteins, and it has the potential to be used in screening efforts toward the discovery of novel small molecules with utility as research tools for cellular reprogramming and ultimately drug discovery. (Journal of Biomolecular Screening 2010:62-71)

Key words: epigenetics, histone-binding proteins, malignant brain tumor (MBT) domains, AlphaScreen™, high-throughput screening (HTS)

INTRODUCTION

EPIGENETICS REFERS TO THE STUDY of heritable phenotypic traits that result from changes to a chromosome without alterations to the genetic code.1 The template upon which the epigenome is written is chromatin—the collection of histone proteins, DNA, and noncoding RNA that allows the genetic code to be both regulated and compressed nearly 15,000-fold into the nuclei of every cell. Environmental factors trigger intracellular pathways that initiate the chemical modification of histone proteins and DNA, including histone lysine and arginine methylation, lysine acetylation, DNA cytosine methylation, and histone sumoylation, ubiquitination, adenosine diphosphate (ADP)-ribosylation, and phosphorylation.2 These epigenetic modifications recruit complexes that cause chromatin to wind and unwind in a specific manner that controls the local access of transcription factors to the genetic code and therefore influences the gene expression profile of a cell. This is the underlying phenomenon that enables cells to possess identical genomes while having diverse functional and morphological properties. The enzymes that “write” and “erase” these marks and the “reader” proteins that recognize them have recently become the subject of intense scientific investigation, as there is no area of biology or human health where epigenetics may not play a fundamental role.1 Specifically, the link between epigenetic misregulation and afflictions such as cancer, neurological disorders, and autoimmune disease is becoming increasingly apparent, and there is growing interest in the development of pharmaceutical agents for this novel target class.4,6 However, there is a lack of chemical probes that would enable therapeutic validation of the myriad epigenetic targets.5,7,8 In this context, we are undertaking a systematic approach to the development of epigenetic chemical probes. In this report, we present the development of an AlphaScreen™ assay capable of mining chemical libraries for antagonists of histone-binding proteins.

Malignant brain tumor (MBT) domains are motifs of ~100 amino acids that are found in tandem repeats in proteins that bind the lower methylation states of histone peptides.
Structurally, MBT repeats are similar to Chromo, PWWP, Agenet, and Tudor domains, which are found in the "Royal Family" of methyl-lysine binding proteins.\(^9\) Other families of methyl-lysine (KMe) recognition motifs include the plant home-domain (PHD) and the WD40 repeat. The key KMe recognition features of these domains are an aromatic electron-rich cage interacting with the lysine cation, with additional charge neutralization and H-bonding by 0 to 2 acidic functionalities depending on the methylation state of lysine.\(^10\) The MBT family is differentiated from other KMe binding domains in 2 regards: (1) they are uniquely selective for monomethyl-lysine (KMe1) and dimethyl-lysine (KMe2), and (2) the methyl-lysine side chain is fully surrounded in a deep and narrow cavity, as opposed to the shallow surface binding motif seen in other histone-binding proteins. As a result, the binding of MBT proteins to histone peptides appears to be less dependent on sequence specificity; rather, the interaction is reliant on a "cavity insertion recognition mode."\(^3\)

From a physiologic perspective, MBT proteins are associated with chromatin condensation and act to repress the transcription of genes, ultimately affecting processes such as differentiation, mitotic progression, and tumor suppression.\(^11-14\) To date, 9 human proteins containing a total of 30 different MBT domains have been identified, demonstrating the complex precision with which this one family of histone-binding proteins regulates chromatin accessibility.\(^15\) Therefore, the development of potent and selective small-molecule probes for each of the human MBT proteins would facilitate a greater understanding of their roles in differentiation, cellular reprogramming, and disease etiology.

The unique binding mode of MBT proteins and the lack of any known chemical probes make them an ideal target class for a chemical biology approach where molecular probes are generated in parallel against as many members of the target family as possible, and used to interrogate the biological function of MBT proteins. Unfortunately, no high-throughput screening (HTS)–compatible methods for the discovery of MBT domain antagonists currently exist. Previous efforts have used isothermal titration calorimetry\(^16\) or fluorescence polarization\(^17\) to characterize the binding of various histone tail peptides with lower methylation states to human and Drosophila MBT proteins. These methods have the disadvantage of either being low throughput or requiring concentrations of protein in excess of 70 to 100 \(\mu\)M, making HTS unfeasible. This conundrum can be attributed to the binding affinities of MBT proteins for their cognate peptides, with \(K_d\) values in the micromolar range. Therefore, we have developed an HTS assay based on Amplified Luminescence Proximity Homogeneous Assay (AlphaScreen™) technology to identify small molecules that inhibit the binding of L3MBTL1, a prototypical MBT domain–containing protein, to the H3K9Me1 peptide. Due to the avidity of the AlphaScreen™ beads, this assay allows for the characterization of low-affinity (micromolar) protein-protein interactions at binding partner concentrations in the nanomolar range, making it ideally suited to tackle this and similar problems.\(^18,19\)

The specificity of this assay was demonstrated by showing that it can distinguish between the binding of L3MBTL1 to its cognate and noncognate ligands, H3K9Me1 and H3K9. The assay was miniaturized and automated in a 384-well format and proved robust and cost-efficient for use in an HTS campaign, having a \(Z'\) factor of 0.87 and a consumable cost of roughly $0.04/well. The optimized assay was used to screen 1280 compounds from the LOPAC library, identifying 5 confirmed hits, including 2 that had \(IC_{50}\) values less than 500 nM.

**MATERIALS AND METHODS**

**Reagents for the AlphaScreen™ assay**

The expression construct for the N-terminal His\(_4\)-tagged L3MBTL1 fragment containing 3 MBT repeats (residues 200-522; L3MBTL1-His) was subcloned into a pET28a-MHL vector and transformed into BL21 Codon Plus RIL *Escherichia coli* (Stratagene, La Jolla, CA), and the protein was overexpressed in 2 L of LB at 18 °C by the addition of 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) when the \(OD_{600}\) = 1.0. The cells harboring the expressed protein were pelleted using a Sorvall SS-34 rotor at 15,000 rpm for 15 min at 4 °C. The clarified lysate was filtered using a 0.45-\(\mu\)m filter and stored at -20 °C. The clarified lysate was added to 0.5 mL of 50 mM Tris-HCl (pH 8) and dialyzed against 500 mM NaCl using a 10-kDa cutoff dialysis cassette. The purified protein was analyzed by SDS-PAGE and found to be greater than 95% pure.

**Histone peptides**

Histone peptides were synthesized and high-performance liquid chromatography (HPLC) purified by the Tufts University Peptide Synthesis Core Facility (Boston, MA). L3MBTL1 has been shown to have a \(K_d\) of 26 \(\mu\)M for binding to a 15-mer representative histone H3 peptide possessing a monomethyl
lysine at lysine 9 (H3K9Me1), so 4 variations of the H3K9 peptide were used in the assay development process—(1) Biotin-H3K9Me1 (Bn-H3K9Me1); Biotin-AHA-ARTKQTARK(Me1)STGGKA-COOH; (2) Biotin-H3K9 (Bn-H3K9), Biotin-AHA-ARTKQTARKSTGGKA-COOH; (3) H3K9Me1; NH2-ARTKQTARK(Me1)STGGKA-COOH; and (4) H3K9, NH2-ARTKQTARKSTGGKA-COOH—where KMe1 indicates the presence of a monomethyl-lysine position and AHA indicates the inclusion of a 6-aminohexyl linker. Bn-H3K9Me1 serves as the AlphaScreen™ “bait,” Bn-H3K9 serves as a nonbinding control, H3K9Me1 represents an inhibitor competing with Bn-H3K9Me1 binding, and H3K9 is the unmethylated negative control inhibitor that does not bind L3MBTL1. The peptides were resuspended to 10 mM in autoclaved de-ionized water, aliquoted, and frozen at −80°C.

The AlphaScreen™ Histidine (Nickel Chelate) Detection Kit containing nickel-chelate acceptor beads and streptavidin-conjugate donor beads was purchased from PerkinElmer (Waltham, MA). Three different solid white polystyrene microplates were used in the assay development process: 96-well half-volume microplates, 384-well microplates (Greiner Bio-one, Monroe, NC) were used as compound plates. Bovine serum albumin (BSA) was purchased from New England Biolabs (Ipswich, MA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) at the highest level of purity possible. The AlphaScreen™ reactions were read on an EnVision Multilabel Reader (PerkinElmer) using a 640-nm dichroic AlphaScreen™ mirror for excitation light and a 570 ± 50-nm cutoff filter for emission.

The Library of Pharmacologically Active Compounds (LOPAC; Sigma-Aldrich), comprising 1280 biologically active compounds, was screened against L3MBTL1-His and Bn-H3K9Me1 binding using the AlphaScreen™ assay. The compounds were stored at a stock concentration of 10 mM in 100% DMSO in 384-well V-bottom polypropylene microplates.

### Assay development and optimization

For initial assay characterization, L3MBTL1-His and Bn-H3K9Me1 were titrated from 0 to 200 nM and 0 to 1666 nM, respectively, in 20 mM Tris-HCl (pH 8), 2 mM DTT, 0.1% BSA in a final volume of 30 µL in 96-well half-volume Optiplates. Reactions were incubated for 30 min at 25°C, then nickel-chelate acceptor and streptavidin-conjugate donor beads were simultaneously added to a final concentration of 30 µg/mL, incubated for 30 min, and subsequently read in the EnVision.

The assay buffer was optimized to increase the signal to background of the assay. The concentrations of L3MBTL1-His and Bn-H3K9Me1 were maintained at 50 and 150 nM, respectively, and a factorial design of experiments analysis was performed on 3 different agents known to reduce nonspecific interactions: NaCl, Tween 20, and BSA. All permutations of 0 or 50 mM NaCl, 0% or 0.05% Tween 20, and 0% or 0.1% BSA were added as components to a basic buffer of 20 mM Tris-HCl (pH 8) and 2 mM DTT in a final volume of 30 µL in 96-well half-volume Optiplates. After a 30-min incubation at 25°C, the donor and acceptor beads were added to a final concentration of 30 µg/mL and incubated at 25°C for another 30 min before being read in the EnVision. Once it was determined that BSA had a negative effect on the assay and that NaCl and Tween 20 interacted in an additive manner, the latter 2 components were subject to a 3-point titration to determine the optimal concentration of each component in the assay buffer. In addition, DMSO was separately titrated in basic buffer (20 mM Tris-HCl [pH 8] and 2 mM DTT) using 50 nM L3MBTL1-His and 150 nM Bn-H3K9Me1 to examine the effect on the binding signal.

To confirm that the optimized assay buffer delivered a high signal and low background, we performed a grid titration of L3MBTL1-His and Bn-H3K9Me1 in 30 µL in 96-well half-volume Optiplates. Based on this optimized assay buffer, the fixed single-point screening assay concentrations of 50 nM L3MBTL1-His and 150 nM Bn-H3K9Me1 were selected for further optimization and validation.

To demonstrate the ability of the assay to distinguish between cognate and noncognate histone peptide ligands of L3MBTL1, we performed a competition experiment between Bn-H3K9Me1 and H3K9Me1 or H3K9. L3MBTL1-His (50 nM) and Bn-H3K9Me1 (150 nM) were preincubated in optimized 1 × assay buffer (20 mM Tris-HCl, 2 mM DTT, 0.05% Tween 20, and 25 mM NaCl) and added to wells containing a titration of the peptides from 150 to 0.78 µM in a final volume of 30 µL. After a 30-min incubation period at 25°C, acceptor and donor beads were then added to a final concentration of 20 µg/mL and read after a 30-min incubation at 25°C.

To explore the effect of order of addition, we investigated 2 sequences of reagent mixing schemes in a 30-µL reaction in optimized assay buffer in half-volume 96-well Optiplates. In one set of reactions, 20 µg/mL each of donor and acceptor beads were separately prebound to 50 nM L3MBTL1-His and 150 nM Bn-H3K9Me1, then added to a plate containing the inhibitor H3K9Me1 (100 µM) and incubated for 30 min at 25°C. Beads were then added to 20 µg/mL and incubated for 30 min at 25°C before being read. In the other set of reactions, 50 nM L3MBTL1-His and 150 nM Bn-H3K9Me1 were premixed and added to H3K9Me1 (100 µM), then the beads were added and the plate was read after a 30-min incubation at 25°C.

### Assay miniaturization and automation

The stability of the signal was determined over a 24-h period by examining the signals of 3 conditions at various time points: minimum and maximum signal controls and a reaction inhibited by 50 µM H3K9Me1. Eight identical reactions (1 per time point) were tested for each condition in 384-well microplate columns.
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(16 points per reaction) and incubated for 30 min at 25 °C before the addition of acceptor and donor beads. Following the addition of beads, the plate was sealed. Time points were taken by unsealing the plate and reading 1 column of each reaction. The plate was sealed between readings, and a new column was read for each time point to avoid photobleaching the beads.

The assay was performed using a multidrop dispenser (Thermo Fisher, Waltham, MA) to add 1 μL of 1% DMSO in 1× assay buffer to the wells to simulate the presence of test compound. Then, 9 μL of reaction cocktail containing L3MBTL1-His and Bn-H3K9Me1 (or Bn-H3K9 for the negative control cocktail) in 1× assay buffer was added using a Multimek NSX-1536 fitted with a 384-channel head (Nanoscreen, Charleston, SC) so that the final concentration in 10 μL was 50 nM L3MBTL1-His and 150 nM peptide. The plates were incubated for 30 min at 25 °C, then 2 μL of an acceptor and donor beads cocktail was added so that the final concentration of beads would be 10 μg/mL each, and the plates were incubated for 30 min at 25 °C before reading. The interday and interplate variation of the automated assay was examined over the course of 3 days by running 2 identical 384-well plates per day, where columns 1 and 2 were the minimum signal control and columns 3 to 24 were the maximum signal control.

LOPAC screen

The automated, miniaturized assay was used to screen a library of 1280 compounds from the LOPAC library. The LOPAC library was obtained as 10-mM stocks in DMSO and stored at −20 °C. Previously prepared 1-μL samples of the library in 384-well V-bottom polypropylene microplates were thawed and diluted to 100 μM in 1× assay buffer over 2 steps using the Multimek, and 1 μL of this stock was spotted into the wells of a 384-well Proxiplate. In the assay, the final concentrations of compound and DMSO were 10 μM and 0.1%, respectively. The assay was performed as described above in the automation and miniaturization section.

Counterscreen

The entire LOPAC library was counterscreened to determine compounds that interfered with the AlphaScreen™ beads. The compounds were diluted and spotted as described in the preceding section, and the assay was performed in a 10-μL volume by adding 9 μL Biotin-His₆ peptide (PerkinElmer) to a concentration of 62 nM in 1× assay buffer and incubating for 30 min at 25 °C prior to addition of the beads to 10 μg/mL each. The minimum signal control wells contained 3 mM EDTA. The reactions were incubated for 30 min at 25 °C before being read in the EnVision.

Data analysis

Factorial design of experiment analysis, full plate control runs, screening assay runs, and dose-response runs were analyzed using ScreenAble software (Screening Solutions LLC, Chapel Hill, NC). Curve fitting for Kᵢₚ and IC₅₀ values were done using a 1-site specific binding with Hill slope and 4-parameter fits, respectively, using Prism software (GraphPad Software, San Diego, CA). The quality and robustness of the assay were determined by analysis of the Z’ factor:

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Z' = 1 - \frac{3\sigma_{\text{max}} + 3\sigma_{\text{min}}}{|\mu_{\text{max}} - \mu_{\text{min}}|},
\]

where σ_{\text{max}} and σ_{\text{min}} are standard deviations of the respective maximum and minimum signal controls, and μ_{\text{max}} and μ_{\text{min}} are the averages of the respective maximum and minimum signal controls.

RESULTS AND DISCUSSION

Characterization of protein-peptide interaction and buffer optimization

We are actively pursuing the development of chemical probes that antagonize the interaction of methyl-lysine binding proteins with histone tail peptides in a parallel, protein family-based approach. However, we were confronted with a challenge presented by a lack of known HTS-compatible methods for the detection of these interactions. Here, we investigated the use of an oxygen tunneling assay platform, Alphascreen™, to improve the detection sensitivity of the protein-peptide interaction. Alphascreen™ is a bead-based technology that is designed to measure the proximity of donor and acceptor beads conjugated to biomolecules of interest. When excited at 680 nm, the donor beads convert ambient oxygen to singlet oxygen. If the beads have been brought within 200 nm of each other, then the singlet oxygen initiates a chemiluminescent reaction in the acceptor bead, which emits light in the 520- to 620-nm range. Donor beads can produce thousands of singlet-oxygen molecules per second, resulting in the amplification of the binding signal. Due to the avidity of the Alphascreen™ beads, binding partners appear to have a higher affinity for one another, which is a property of bead-based assay technology that is not well suited for measuring direct binding kinetics. However, this phenomenon, in addition to the amplification of the signal, makes Alphascreen™ ideal for use as a screening assay in the discovery of inhibitors of binding partners that interact with Kᵢ values in the micromolar range. In our Alphascreen™ assay, we investigated the binding of L3MBTL1 to the H3K9Me1 peptide, an interaction that has a measured Kᵢ of 26 μM. L3MBTL1 was obtained in >98% purity by nickel affinity and size exclusion chromatography, and the His₆ tag was not cleaved from the protein following purification (L3MBTL1-His), retaining a site on the protein for binding to the nickel-chelate acceptor beads. The donor beads were streptavidin-conjugates that interact with the peptide through biotin attached by an N-terminal 6-aminohexyl linker designed...
to distance the methylated lysine from the bead, reducing the effect of steric hindrance upon binding (Fig. 1).

We performed an initial titration of L3MBTL1-his and Bn-h3K9Me1 in basic assay buffer and observed a binding signal when L3MBTL1-his was present in excess of 50 nM and Bn-h3K9Me1 was present above 100 nM. Although binding could be observed, a high background signal indicated that nonspecific binding was occurring between the binding partners and the beads. To reduce the nonspecific binding observed, we examined the effect of BSA, Tween 20, and NaCl on the signal-to-noise ratio. A factorial design of experiments was performed on all buffer components, and it was determined that Tween 20–NaCl interacted synergistically in an additive manner to reduce background and increase signal, whereas BSA–Tween 20 and BSA–NaCl respectively gave a counterproductive effect or an insignificant increase in signal (data not shown). Therefore, Tween 20 and NaCl concentrations were chosen as variables in further optimization experiments to increase the signal-to-noise ratio, and they were titrated across 3 concentration points to determine an optimal balance of each additive (Fig. 2). A substantial effect was observed on the relative increase in signal to background at 25 mM NaCl and 0.05% Tween 20, so these concentrations were selected for further optimization.

In other studies, DMSO was titrated from 0% to 5%, and it was observed that this assay was very sensitive to DMSO concentrations above 0.1%, which was in agreement with the effect of DMSO observed in a fluorescence polarization assay (see Supplementary Figure S1 at http://jbx.sagepub.com/supplemental). Therefore, any HTS assay of this binding interaction needs to be configured so that the DMSO concentration is approximately 0.1%. Once the buffer was optimized (20 mM Tris-HCl [pH 8], 2 mM DTT, 0.05% Tween 20, and 25 mM NaCl) to achieve the highest signal-to-noise ratio, we performed another titration on L3MBTL1-His and Bn-H3K9Me1 (Fig. 3). Smooth and reproducible binding curves were obtained, delivering $K_{d}^{app}$ values ranging from 54 to 88 nM and showing a marked increase in the signal-to-noise ratio. Based on these binding curves, 50-nM L3MBTL1-His and 150-nM Bn-H3K9Me1 points were selected as appropriate concentrations that balanced the need to conserve reagents while maintaining a high signal window in HTS screening activities.

**FIG. 1.** AlphaScreen™ assay platform for discovery of L3MBTL1 antagonists. Interaction of His$_{6}$-tagged L3MBTL1 and biotinylated H3K9Me1 peptide brings the donor and acceptor beads in proximity. Excitation light causes the donor bead to generate thousands of singlet-oxygen molecules per second, which travel up to 200 nm and interact with the acceptor beads, eliciting a chemiluminescent reaction that is measured as the AlphaScreen™ signal. Inhibitors of this biomolecular interaction will lead to a reduction in the AlphaScreen™ signal by competing with the biotinylated peptide.

**FIG. 2.** Optimization of Tween 20 and NaCl in the optimized assay buffer. To decrease the nonspecific binding observed when the assay was performed in basic buffer (20 mM Tris-HCl [pH 8] and 2 mM DTT), we employed Tween 20 and NaCl to reduce this effect. Tween 20 and NaCl were each titrated over 3 concentration points, and the Relative Effect, defined as the signal-to-background window relative to that obtained using basic buffer not containing either agent, is plotted. We determined the optimum signal was attained at 0.05% Tween 20 and 25 mM NaCl. The use of NaCl below 25 mM and above 75 mM resulted in the collapse of the signal-to-background window. However, it was possible to use Tween 20 at a concentration of as little as 0.025% while maintaining a large relative effect.

**Competition experiments of Bn-H3K9Me1 with cognate and noncognate peptides**

Next, we sought to demonstrate that this assay could distinguish between L3MBTL1’s cognate and noncognate peptides. We performed competition experiments between Bn-H3K9Me1 and the unbiotinylated peptides H3K9Me1 and H3K9, where H3K9Me1 was predicted to compete with Bn-H3K9Me1 for L3MBTL1-His. The unmethylated peptide, H3K9, was not predicted to bind to L3MBTL1-His. As expected, H3K9Me1 inhibited this interaction with an $IC_{50}$ of 22 ± 3 μM, in agreement with the previously reported $K_{d}$ of 26 μM measured by fluorescence polarization (Fig. 4). The H3K9 peptide showed...
no binding even at concentrations of 200 μM, and as a result, the biotinylated H3K9 peptide, Bn-H3K9, was selected as a negative control for all further assay validation.

The order of addition of the inhibitor, binding partners, and beads was also examined. H3K9Me1 fully inhibited the Alphascreen™ signal when it was preincubated with L3MBtL1-his and Bn-H3K9Me1 before the addition of the beads. However, if the beads were added before H3K9Me1, it failed to inhibit the Alphascreen™ signal (Fig. 5). Therefore, we determined that the inhibitor must be added and allowed to reach equilibrium prior to bead addition, as the beads appear to prevent access of the H3K9Me1 peptide once they are added. This effect can be attributed to the phenomenon of bead avidity. The multiple binding sites on the beads allow multiple L3MBtL1-his and Bn-H3K9Me1 peptides anchored to the same bead to bind as the interaction of 1 L3MBtL1-his and 1 Bn-H3K9Me1 places other protein and peptide molecules in close proximity, increasing their local concentrations. The resulting binding constant is therefore the cooperative sum of multiple L3MBtL1-his and Bn-H3K9Me1 interactions.

Assay miniaturization and automation

Following optimization of the assay buffer and screening concentrations of L3MBtL1-His and Bn-H3K9Me1, the assay was converted to a 384-well format and miniaturized to increase throughput and reduce consumable costs. We investigated the assay performance in 384-well microplates while simultaneously exploring the possibility of lowering the bead concentration and assay volume to optimize the signal-to-cost ratio for an HTS campaign. Various bead concentrations and assay volumes were tested in 384-well low-volume microplates designed to bring the beads closer to the microplate reader’s PMT detector. We determined that an HTS-worthy assay signal could be obtained using 10 μg/mL each of donor and acceptor beads in an assay volume of 10 μL (data not shown).

Considering the time (~4.5 min) it takes for an EnVision plate reader to read 1 Alphascreen™ 384-well assay and the need to read multiple plates in a single batched run, we tested the stability of the assay signal over time. We examined the signal of 3 conditions over the course of 24 h: (1) maximum signal control, (2) binding inhibited by 50 μM H3K9Me1, and (3) minimum signal control. All 3 were stable over the course of 24 h, with the maximum signal control only showing a slight reduction in signal beyond 10 h, and the midpoint inhibitor signal was consistent over the course of the experiment, with the observed inhibition increasing 15% by the 24-h time point (Fig. 6). This indicated that we could batch process plates for reading in a stacker-equipped microplate reader.

To evaluate the robustness of the assay for HTS, we studied interplate and interday variation in assay plate replicates containing 32 wells of minimum and 352 wells of maximum signal control. Initial validation runs were performed using a multichannel dispenser, but we observed spurious low signal wells among the maximum signal control wells, which would lead to false positives in a production screen. This problem was experimentally attributed to the Tween 20 in the assay buffer acting as a foaming agent and interfering with protein dispensing from the multichannel dispensers. Therefore, the protocol was altered to use a Multimek 384-channel pipet head equipped with a stacker to add the protein (Fig. 7).

The average signal for interplate assays performed on the same day had a maximum signal average of 547,926 (coefficient
of variation [CV] 4.1%), 521,016 (CV 4.0%), and 499,836 (CV 5.1%), respectively. On the same pairs of assay plates, the minimum signal averages were 8711 (CV 3.9%), 10,320 (CV 8.1%), and 24,890 (CV 12.1%). The Z’ values were 0.87, 0.88, and 0.86 for each respective run, and the average signal-to-background ratio was 52. Interday variability in the maximum signal control was less than 10%. Overall, these results indicate a high-quality assay suitable for HTS.

**Pilot screen of LOPAC library**

A pilot screen was performed using the validated assay on the LOPAC library, which contains 1280 compounds that we compressed into four 384-well plates. To ensure that we would be at least 3 standard deviations from the minimum signal control and to identify only high-quality hits, we used a selection criterion of >40% inhibition at ~10 μM compound and found 47 primary hits in this library. A counterscreen was performed on the entire LOPAC library to identify compounds that interfered with the assay by acting as metal chelators, biotin mimetics, or singlet-oxygen quenchers. A His₆-biotin fusion peptide served as the substrate for the nickel-chelate acceptor and streptavidin-conjugate donor beads, and using identical 10-μM compound concentrations, assay buffer, and liquid handling protocol as the MBT assay, we were able to eliminate 21 compounds that interfered with the AlphaScreen™ assay, including several flavonoids. The remaining 26 compounds were examined to remove compounds that were most likely acting through protein aggregation.
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or nonspecific protein binding mechanisms, resulting in the elimination of polysulfated aromatic compounds such as Suramin in the follow-up screen (see Supplementary Figures S2 and S3 at http://jbx.sagepub.com/supplemental). In addition, the remaining flavonoid compounds that did not show up as hits in the counter-screen were eliminated from consideration, leaving 17 compounds for follow-up. These compounds were clustered into 9 groups by chemotype class, and 1 representative member from each chemotype class was purchased from Sigma for IC₅₀ analysis. In total, 5 of 9 compounds were confirmed as hits, and 2 compounds, Cefsulodin and I-Ome Tyrphostin AG 538, had IC₅₀ values in the nanomolar range (Fig. 8A). These compounds were tested in a fluorescence polarization (FP) assay (data not shown) and did not greatly diminish the FP signal. One primary difference between the AlphaScreen™ assay and the FP assay is the concentration of MBt protein, which is typically 1500- to 2000-fold higher in the FP format. In addition, the FP assay has been used only to quantify the interaction of various histone peptides with MBt proteins, and it has never been used in small-molecule screening efforts.

To investigate whether these compounds were inhibiting the AlphaScreen™ assay by a protein-specific mechanism of action, we assayed them again, using either a 10-fold excess L3MBTL1 (10 × L3MBTL1) or 3.3-fold excess Bn-H3K9Me1 peptide (3.3 × Bn-H3K9Me1) over normal assay conditions (normal conditions), and the large proportional shift in the IC₅₀ curves for the 10 × L3MBTL1 condition over the normal conditions indicated that the compounds were binding to the protein. IC₅₀ values were obtained as the average of duplicate runs, and after 4-parameter curve fitting they were calculated as follows: Cefsulodin—normal = 98 nM, 10 × L3MBTL1 = 2.5 μM, 3.3 × BnH3K9Me1 = 171 nM; I-Ome Tyrphostin AG 538—normal = 282 nM, 10 × L3MBTL1 = 3.7 μM, and 3.3 × Bn-H3K9Me1 = 489 nM.

Taken together, these results indicate that the compounds were inhibiting the interaction between L3MBTL1-His and the Bn-H3K9Me1 peptide through a mechanism that involved specific binding to L3MBTL1-His rather than the peptide or nonspecific protein binding (Fig. 8B).

CONCLUSIONS

Using AlphaScreen™ technology, we have established the first reported HTS assay to identify compounds that inhibit the interaction of L3MBTL1 and one of its cognate histone peptides, H3K9Me1. The assay demonstrates that L3MBTL1 is capable of distinguishing between histone H3 peptides having monomethyl versus unmethylated lysine residues. The Z’ value for the assay was 0.87, indicating that this assay can easily be adapted to HTS. The assay was used to screen 1280 compounds from the LopAc library and resulted in the identification of 5 confirmed hits, each from a different chemotype class. Two of the hits were active with IC₅₀ values in the nanomolar range, and we determined they acted via a mechanism that involved binding to L3MBTL1.

Many proteins that read the histone code individually bind their cognate histone peptides with low affinity, but the combinatorial effect of multiple interactions on various lysine marks leads to high binding affinity and specificity in vivo. As a result, it is challenging to subject individual reader proteins to biochemical screening assays in an effort to discover selective and potent molecular probes. The avidity of the AlphaScreen™ beads artificially enhances the binding affinity of histone peptides and reader proteins to values in the nanomolar range. This phenomenon makes it possible to
reduce the required protein for an assay signal to be observed from 100 μM to 50 nM, a 2000-fold decrease. A major advantage of this assay is its versatility in the screening of chemical libraries against entire families of histone-binding proteins. We have successfully applied this assay to 4 other MBT proteins (SFMBT1, MBTD1, L3MBTL3, and L3MBTL4), a Tudor domain protein (SGF29), and a PHD finger protein (PHF13), a chromodomain (CBX7), and the Heightgroup (Structural Genomics Consortium, Oxford, UK) has applied it to several members of the bromodomain family of acetyl-lysine binding proteins (unpublished results). We have also been able to interrogate the binding specificity of other MBT domains for various histone peptides containing a variety of modified lysine residues and are encouraged that the results will permit a comprehensive view of the cellular substrates for the MBT family of proteins. Such broad application of this technology could pave the way for a systematic chemical biology study of histone reader proteins to understand their roles in development and differentiation and disease states related to epigenetics. Eventually, such small molecules could be optimized to leads that would enable cellular reprogramming, or could be developed into pharmaceutical agents for the treatment of diseases rooted in epigenetic misregulation.

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