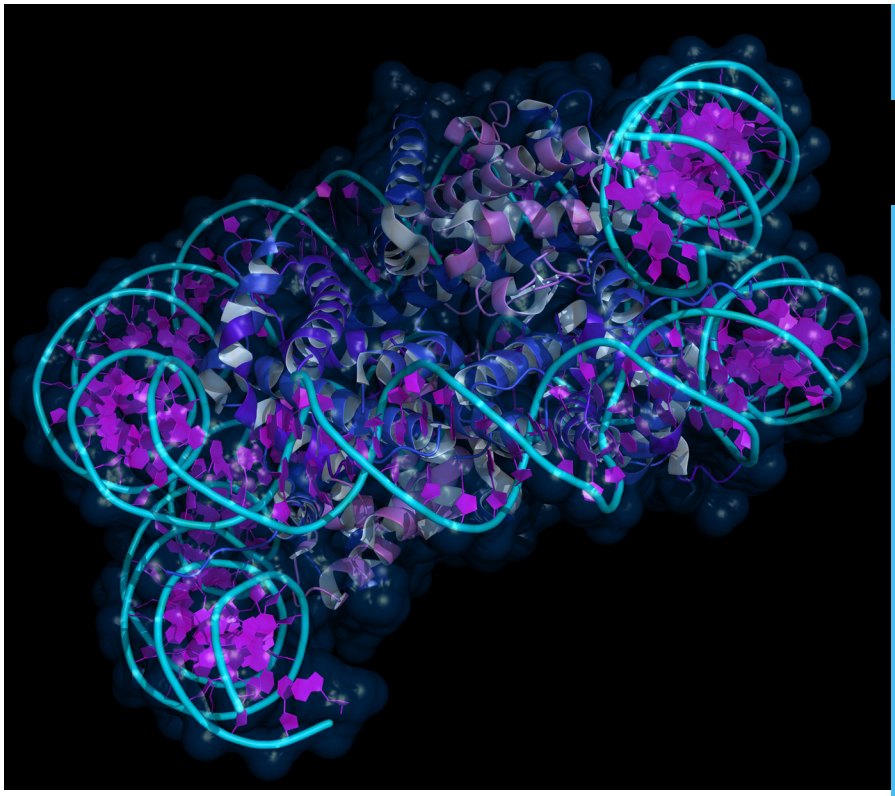

APPLICATION NOTE

AlphaLISA Technology

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Development and Utilization of Bromodomain and Extra Terminal Domain (BET) Assays Using AlphaLISA Technology

Introduction

Lysine acetylation is a post-translational signal modification with repercussions on physiochemical properties such as charge, thereby influencing protein conformation and protein-protein interactions. The acetylation state of histone lysine residues is critical in the epigenetic regulation of gene transcription.[1] Bromodomains are small protein domains that recognize acetylated residues, thereby modulating protein-protein interactions essential for epigenetic mechanisms of transcriptional control.

The role of bromodomains in transcriptional regulation directly influences numerous disease pathways in cancer, viral infection, and inflammation/immune response. There are 57 bromodomains, with bromodomain and extra-terminal (BET) being a subset that is currently a primary focus of cancer research. The BET family (BRD2, BRD3, BRD4 and BRDT) is easily targeted by small molecule inhibitors to regulate various transcription factors in disease.[2] Isolated, individual, or tandem bromodomains of BRD2 and BRD4 have been shown to bind to acetylated histone tails. Small molecule inhibitors, such as (+)JQ-1, can be used to disrupt this interaction and therefore serve as potential therapeutics.[2,3]

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Available assays for BET family bromodomain activity detect mainly tetra acetylated peptides used as surrogates for the full nucleosome, rather than the weaker binding mono- or di- acetylated peptide surrogates that are more challenging to study.[4,5] More recently, a suite of novel chromatin and histone-binding assays combining a modified AlphaLISA® assay platform, in situ cell extraction, and fluorescence-based high content imaging was developed.[6] AlphaLISA technology can be used to screen inhibitors specific to bromodomains which have all been identified as potential cancer targets and are implicated in disease states.[7]

We chose to develop AlphaLISA assays based on BRD4 bromodomain BD1, which recognizes acetylated lysines on the tail of histone H4. By combining modified peptides that mimic the histone tail of H4 with various acetylation states (mono- through tetra-), GST-tagged bromodomain protein BRD4 (BD1) and AlphaLISA bead-based technology, we demonstrate biochemical bromodomain binding assays.

Importantly, AlphaLISA assays can be used to detect low affinity binding interactions, allowing for screening of bromodomain inhibitors specifically targeted to mono- and di-acetylation states of the histone. AlphaLISA technology allows for the detection of molecules of interest in a homogeneous, no-wash format with a high sensitivity and is amiable to high throughput screening.

As illustrated in Figure 1, glutathione (GSH) AlphaLISA Acceptor beads are used to capture the GST-tagged bromodomains and streptavidin-coated Donor beads are used to capture the biotinylated peptides. Donor and Acceptor beads come into proximity through BRD4 domains binding to specific acetylated peptides derived from histone H4. Excitation of the Donor beads at 680 nm results in the release of singlet oxygen thereby triggering energy transfer to the Acceptor beads, if within 200 nm of each other, resulting in a sharp emission peak at 615 nm. This light emission can then be detected on an Alpha-enabled reader. Here, it is demonstrated that by using AlphaLISA technology assays specific for BRD4 bromodomain BD1 and various acetylated H4 histone-derived peptides, it is possible to screen and validate such targets and measure inhibitor potency.

Materials and Methods

Reagents

All assays were performed using GST-tagged Bromodomain-containing protein 4, bromodomain 1 (BRD4 (BD1), #15-0012) from EpiCypher. The concentration of protein was optimized for binding with each peptide and is therefore listed in the each figure. Peptides used were biotinylated tetra-acetylated (H4 (K5,8,12,16Ac), #12-0034), tri-acetylated (H4 (K5,8,12Ac), #12-0047), di-acetylated (H4 (K8,16Ac) #12-0046), di-acetylated (H4 (K5,12Ac), #12-0045), mono-acetylated (H4 (K5Ac), #12-0030), mono-acetylated (H4 (K12Ac), #12-0032), mono-acetylated (H4 (K8Ac), #12-0031), and non-acetylated (#12-0029) histone H4 peptides from EpiCypher.

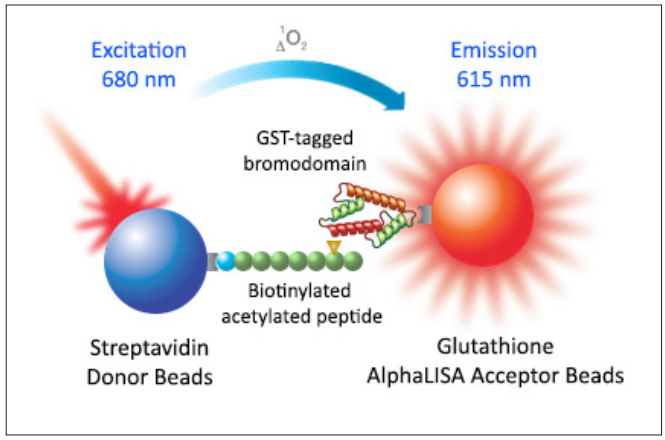

Figure 1. AlphaLISA assay principle for bromodomain binding.

BET inhibitors were sourced from Tocris ((+)JQ-1, #4499, (-)JQ-1, #5630, CPI 203, #5331, XD 14, #5489, I-BET 151, #4650, PFI 1, #4445, LY303511, #2418, PFI 3, #5072). Inhibitors were serially diluted in 100% DMSO, then diluted 1:10 into 1X AlphaLISA Epigenetics Buffer 1 and added to the plate at 10X concentration, for a final assay DMSO concentration of 1%. All assays were run using Streptavidin Donor beads (PerkinElmer, #6760002) and Glutathione (GSH) AlphaLISA Acceptor beads (PerkinElmer, #AL109C).

Proteins, peptides, and compounds were all diluted into Epigenetics Buffer (PerkinElmer, #AL008). All assays were performed in PerkinElmer AlphaPlate™-384, Shallow Well (#6008350) or ProxiPlate™-384 (#6008280).

AlphaLISA Bromodomain Assay Protocols

Two different protocols were used for this application note, and are outlined in Figure 2. Protocol 1 was used for saturation binding studies, while Protocol 2 was used as a more HTS-amenable format for screening BRD4 (BD1) inhibitors.

Instrumentation

For the assays, plates were mixed slowly during the incubation on a rotating shaker (DELFIA® PlateShake). All AlphaLISA bromodomain assays were measured on a standard PerkinElmer EnVision® multimode plate reader using standard settings for Alpha detection (Figure 3). In addition to standard fluorescence, luminescence, absorbance, fluorescence polarization, and time-resolved fluorescence measurements, the EnVision multimode microplate reader system offers fast, sensitive Alpha detection technology. The incorporation of unique temperature controls within the system ensures accurate, reproducible results for Alpha and AlphaPlex™ assays.

Data Analysis

Data were plotted in GraphPad Prism® version 7.0. For cross-titration and saturation experiments, curves were fit using a one-site fit saturation binding curve. Inhibition curves were generated using a four-parameter (variable slope) inhibition dose-response curve fit.

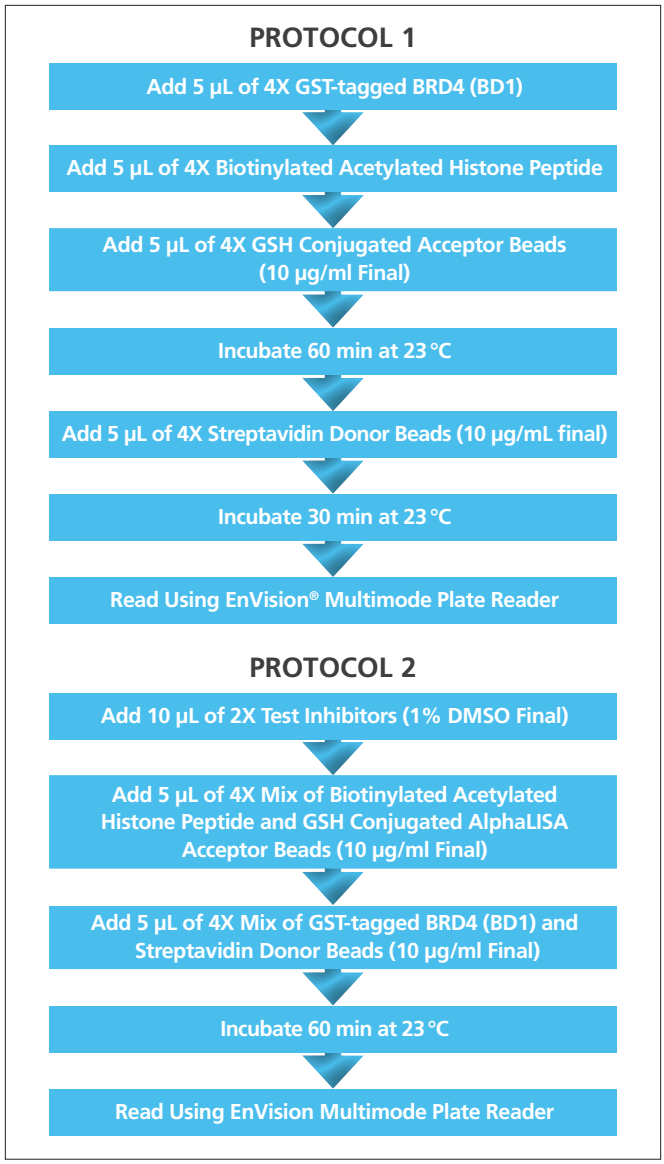

Figure 2. Protocols for AlphaLISA bromodomain assays.

Figure 3. EnVision multimode plate reader for Alpha detection.

Results and Discussion

Saturation Binding Curves

The AlphaLISA bromodomain assay utilizing acetylated histone peptides and BRD4 (BD1) was tested for its ability to specifically detect acetylated versions of the histone peptides. Figure 4 shows that when BRD4 (BD1) is exposed to the acetylated histone peptide, H4 (K5,8,12,16Ac), a concentration-dependent binding signal is generated that saturates at ~100 nM. Conversely, when BRD4 (BD1) is exposed to the non-acetylated peptide, H4, no signal is generated. This is in agreement with published reports.[8] This experiment demonstrates the specificity of BRD4 (BD1) for acetylated versions of the histone H4-derived peptide.

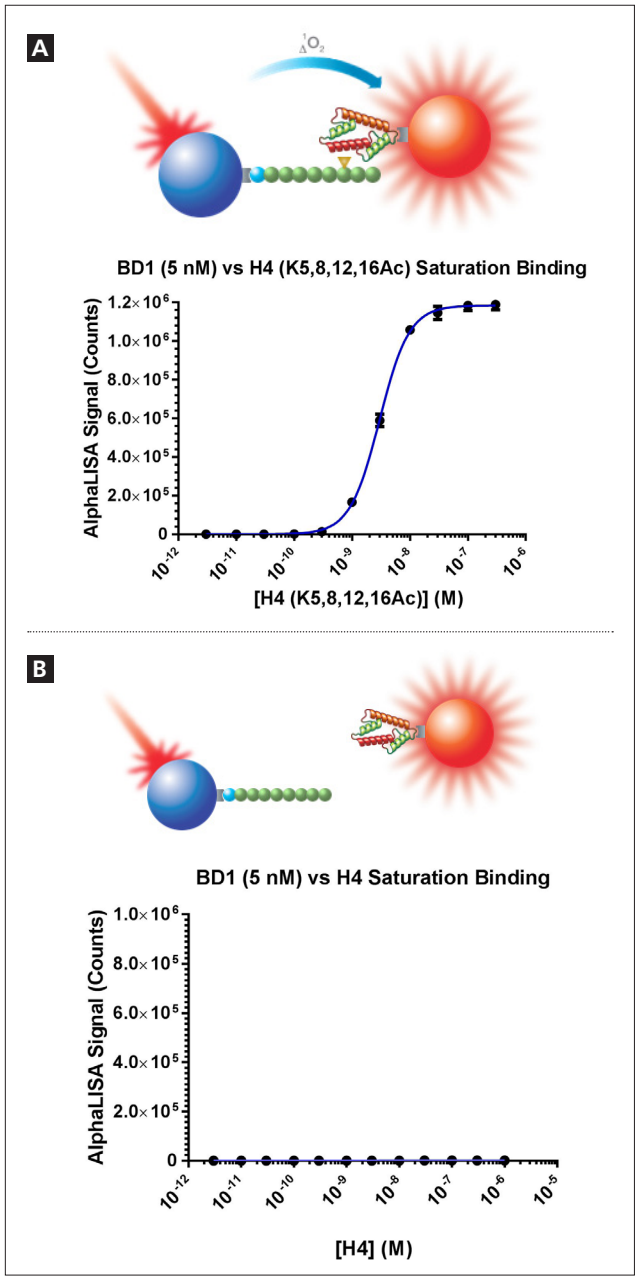

Figure 4. The AlphaLISA BRD4 (BD1) binding assay specifically detects binding of acetylated histone peptides. (A) Specific binding of acetylated H4 (K5,8,12,16Ac) peptide to BRD4 (BD1) is observed whereas non-acetylated H4 does not interact with BRD4 (BD1), resulting in no signal (B).

Acetylation State Specificity

AlphaLISA was shown to detect bromodomain binding to different H4 acetylation states (mono-, di-, tri, and tetra-). We tested BRD4 (BD1) binding to peptides with various acetylation states. Representative curves for a few of these experiments are shown in Figure 5. Table 1 shows the complete list of peptides tested. From these data, an "apparent" dissociation constant (EC_{50}) was determined. This EC_{50} is dependent upon both the binding constant of the complex as well as the bead capacity. The data presented here show how AlphaLISA can measure binding events for both strong (tetra-acetylated peptides) and weak binders (H4 (K16Ac)).

Screening of BET Inhibitors

In order to highlight the ability of this AlphaLISA assay to be utilized for screening BET inhibitors, we profiled a variety of inhibitors for BRD4 (BD1) (Table 2). Representative curves of the data generated for an inhibition of BRD4 (BD1) binding to various peptides are shown in Figure 6. As can be seen from these inhibition graphs for BRD4 (BD1), a large range of IC_{50} values (~30 nM – 9 µM) can be measured with AlphaLISA technology. Data generated allowed compounds that inhibit the BRD4 (BD1)/H4 (K5,8,12,16Ac) or H4 (K5,12Ac) interaction to be ranked according to potency, with CPI 203 > (+)JQ-1 > PFI 1 > I-Bet 151 > XD 14/LY 303511 > (-)JQ-1 > PFI 3.

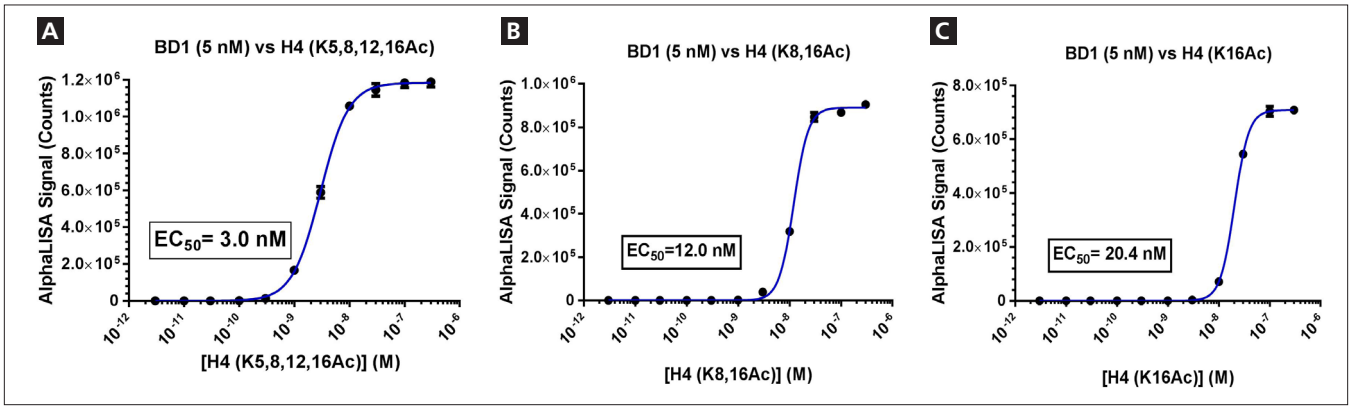

Figure 5. AlphaLISA detects bromodomain binding to different histone H4 acetylation states and allows for apparent dissociation constants to be calculated. A tetra-acetylated peptide, H4 (K5,8,12,16Ac) (A), a di-acetylated peptide, H4 (K8,16Ac) (B), and a mono-acetylated peptide H4 (K16Ac) (C) were tested for their association with bromodomain, BRD4 (BD1). For all BRD4 (BD1) experiments, 5 nM (final) protein concentrations were used.

Table 1. Apparent EC_{50} values of different acetylation states to BRD4 (BD1).

Peptide	BRD4 (BD1) (5 nM)	
	EC_{50} (nM)	Ratio
H4 (K5,8,12,16Ac)	2.99	2055
H4 (K5,8,12Ac)	3.71	2009
H4 (K5,12Ac)	6.66	2148
H4 (K8,16Ac)	12.04	2014
H4 (K5Ac)	11.5	1924
H4 (K8Ac)	14.84	1900
H4 (K12Ac)	6.65	2162
H4 (K16Ac)	20.36	1441
H4	ND	3

Table 2. BET inhibitors.

Inhibitor	Description
(+)JQ-1	Highly specific potent bromodomain inhibitor, binds both BD1 and BD2
(-)JQ-1	Exhibits no significant interaction with BRD4
CPI 203	Potent BET bromodomain inhibitor, binds both BD1 and BD2
XD 14	BET bromodomain inhibitor, higher specificity for BD1 than BD2
I-Bet 151	BET bromodomain inhibitor
PFI 1	BET bromodomain inhibitor
LY 303511	BET bromodomain inhibitor, specific for BD1
PFI 3	No specific interaction with bromodomains

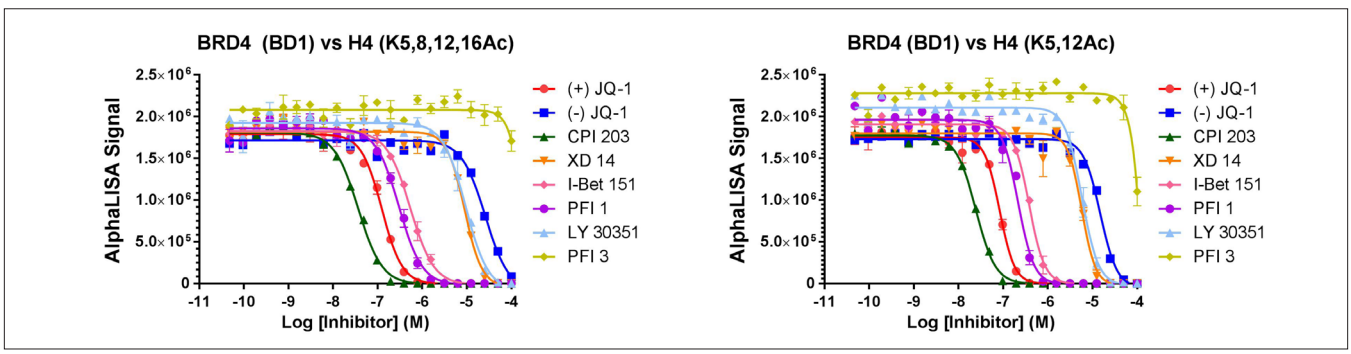

Figure 6. AlphaLISA was used to profile a variety of BET inhibitors. The graphs demonstrate different inhibition profiles for BRD4 (BD1) association with H4 (K5,8,12,16Ac) peptide or H4 (K5,12Ac) peptide. The concentration used for BRD4 (BD1) is 2.5 nM (final) for binding to H4 (K5,8,12,16Ac) peptide and 10 nM (final) for binding to H4 (K5,12Ac) peptide.

Conclusions

Here, we developed AlphaLISA assays with various acetylated histone H4 peptides, some of which are known to have low affinities to BRD bromdomains. Despite this, our platform was able to detect binding of these low affinity peptides and show high signal to background and low CVs, making AlphaLISA assays excellent candidates for high throughput screening methods. By demonstrating specificity of the AlphaLISA bromodomain binding assay to the known BET family inhibitors, we have shown the relevance and robustness to screen next generation drugs for specific acetylation states of histone peptides.

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