An Automated Profiling Application using a Direct, Cell-Based, Target-Compound Interaction Assay for G9a Histone Methyltransferase and Bromodomain Proteins

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Introduction

Histone modifications play an essential role in the regulation of eukaryotic gene expression and are driven by histone writer, eraser and reader proteins. These changes to the N-terminus of histone tails are dynamic in nature and are a normal part of the embryonic differentiation of cells from their original totipotent state. However, aberrant modifications have been linked to numerous disease states, and many human cancers. The SET domain-containing G9a methyltransferase writer protein is over-expressed in various cancers, and is also deficient in CD4+ T helper cells, leading to increased rates of intestinal infection (Lehnertz *et al.*, 2010). In addition, the BET bromodomain reader protein Brd4 was implicated in NUT midline carcinoma (NMC) through a fusion of the protein and NUT (French *et al.*, 2007). Due to these and other findings, G9a and BET bromodomain proteins have become the target for numerous drug discovery projects.

Current assay technologies exist in a biochemical format to examine enzyme activity and inhibition of G9a and BET bromodomain proteins. The existing cell-based assays for these epigenetic players are only limited to the detection of specific histone modifications using antibodies, with antibody specificity being an issue. Hence there still exists a need for a robust and high throughput assay that can identify the binding of compounds to the catalytic domain of these proteins in a cell-based format. In this study, we describe a novel, cell-based assay platform that uses the robust, yet simple Enzyme Fragment Complementation (EFC) Technology. The assay measures compound binding to an intracellular target by detecting changes in protein stability. In this assay, the target is fused to the enhanced ProLabel™ (ePL) enzyme fragment and expressed in the selected cell background. The amount of fusion is quantified through the addition of substrate and complementary enzyme acceptor fragment (EA). The assay can be used to detect the specific binding and direct protein engagement of potential small molecule inhibitors to the G9a methyltransferase and Brd2, Brd4, and BrdT bromodomain proteins.

The assay procedures were carried out in 384-well format using high throughput liquid handling and detection instrumentation. Initial experiments included optimization of cell number and post-plating incubation time, as well as a Z'-factor validation. Screening of a small focused library was then carried out with each target assay. Finally, dose response testing was completed using "hits" from the compound screen, as well as known inhibitor positive control compounds. Experimental results confirm the ability of the automated cellular assays to accurately detect the target specific inhibitory characteristics of test compounds, with a low false positive rate, in a simple, yet robust manner for these important epigenetic targets.

BioTek Instrumentation

Precision™ Microplate Pipetting System. The Precision combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to dilute the Epigenetics library and transfer the final 5X concentrations to the 384-well assay plates.

MultiFlo™ Microplate Dispenser. The dispenser offers fast, accurate plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 0.5-3000 µL. The instrument was used to dispense all assay components including cells and prepared detection reagent in 384-well format.

Synergy™ NEO HTS Multi-Mode Microplate Reader. The patented Hybrid Technology™ of the reader combines filter-based and monochromator-based detection systems in one unit. The dedicated, high-performance luminescence detection system was used to quantify the luminescent signal from each assay well.

InCELL Hunter™ eXpress Epigenetics Assays

InCELL Hunter™ eXpress assays are pre-validated, target-specific assays for epigenetics that can be used to investigate cellular permeability, and measure compound-target engagement inside intact cells as well as cellular potency of compounds.

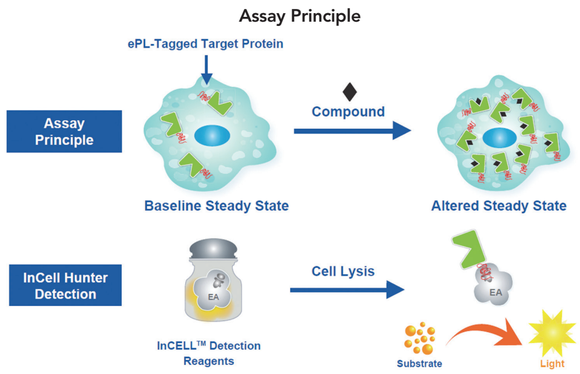

Figure 1 – *Cells are engineered to express the target protein fused to an enhanced ProLabel (ePL) tag. In the absence of a binding compound, the target-ePL fusion reaches a steady state, which increases when a compound binds the target. The signal is detected by adding the lysis buffer and Enzyme Acceptor (EA) fragment. This creates an active β-galactosidase enzyme, which converts added substrate to generate a luminescent signal.*

Materials and Methods

Materials

Assay Components. InCELL Hunter eXpress Brd2(1) Bromodomain Assay (Cat. No. 96-0004E1CP0S), InCELL Hunter eXpress Brd4(1) Bromodomain Assay (Cat. No. 96-0005E1CP0S), and InCELL Hunter eXpress G9a Methyltransferase Assay (Cat. No. 96-0003E15CP7S) kits, containing assay-ready InCELL Hunter cells expressing the appropriate epigenetic target protein, Cell Plating (CP) Reagent, and Detection Reagent, were donated by DiscoveRx Corporation (Fremont, CA).

Compounds. The 43 compound Screen-Well® Epigenetics Library, Version 1.0 (Cat. No. BML-2836-0500) was donated by Enzo Life Sciences (Plymouth Meeting, PA). (+)-JQ1 (Cat. No. 92-1149) was donated by DiscoveRx Corporation (Fremont, CA). Sinefungin (Cat. No. S8559) and 2,4-Pyridinedicarboxylic acid monohydrate (Cat. No. P63395) were purchased from Sigma-Aldrich Co. (Saint Louis, MO). UNC 0646 (Cat. No. 4342) and UNC 0638 (Cat. No. 4343) were purchased from R&D Systems (Minneapolis, MN).

Methods

Cell Preparation. 500 µL of the appropriate CP Reagent is added to 100 µL of assay-ready InCELL Hunter cells ($12x10^6$ cells/mL) to make 600 µL of $2.0x10^6$ cells/mL. The cells are further diluted by transferring an aliquot of cells to additional CP Reagent. For screening, the final concentration of cells was $5.0x10^4$ cells/mL or 1000 cells/20 µL.

Assay Optimization

Cell Concentration and Reaction Time Optimization

Optimization of cell number per well and post-plating incubation time was performed in order to maximize the signal to background between wells containing compound bound target wells with target alone, while still minimizing cell number. InCELL Hunter HEK293 Brd2(1) cells were plated into two separate 384-well plates in concentrations ranging from 16,000 to 0 cells/well. The plates were incubated in a 37°C/5% CO_2 cell incubator for either 24 or 48 hours. Following the post-plating incubation, 10 µM (+)-JQ1 (1X) was added to one half of the wells at each cell concentration, while plating medium was added to the other half. The plates were returned to the cell incubator for 6 hours. Detection reagent was then added to each well, the plate was incubated at room temperature for 30 minutes, and the luminescent signal was then quantified.

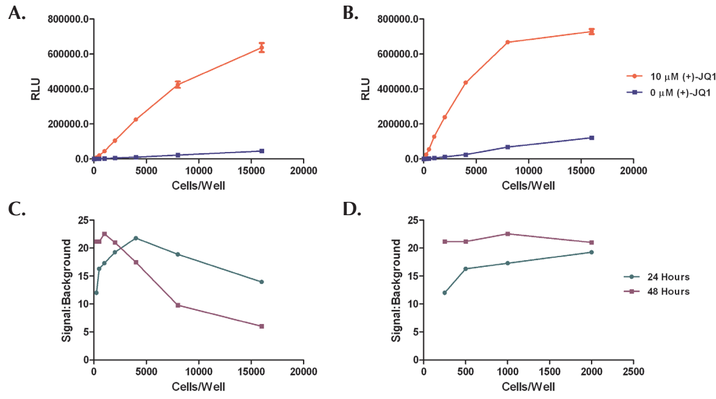

Figure 2 – Cell Concentration/Post-Plating Incubation Results. *RLU values plotted for wells containing either 10µM or 0 µM (+)-JQ1 from cells incubated for 24 hours* ***(A.)*** *or 48 hours* ***(B.)*** *post-plating. Signal to background values calculated at each cell concentration via the following formula: $RLU_{(10\ \mu M\ (+)\text{-}JQ1)}$ / $RLU_{(0\ \mu M\ (+)\text{-}JQ1)}$. Calculated values then plotted for all cell concentrations* ***(C.)*** *as well as for 250-2000 cells/well only (D.).*

Computed signal to background ratios (S:B) between wells containing 10 or 0 µM (+)-JQ1 remain steady to slightly increasing for both cell plating times up to 2000 cells/well (Figure 2D). When comparing results from cells incubated for either 24 or 48 hours following plating, across the same cell concentrations, S:B data from cells incubated for 48 hours is consistently higher than from cells incubated for 24 hours, with S:B values >20. Therefore a cell plating concentration of 1000 cells/well, coupled with a 48 hour post-incubation time was chosen for use in subsequent experiments in order to ensure a high S:B ratio, while also minimizing cell usage.

Automated InCELL Hunter Assay

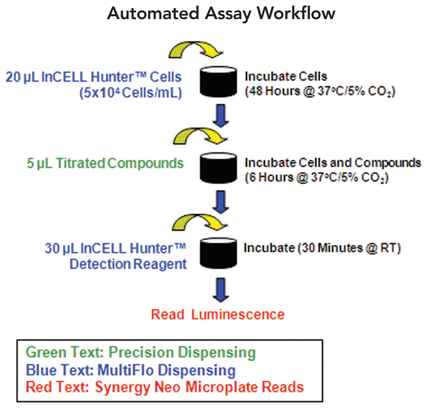

Figure 3 – Automated 384-Well InCELL Hunter assay workflow. *Synergy NEO settings used for luminescent signal detection were as follows: Integration Time=0.2 seconds; Gain=120.*

Automated Assay Z'-factor Validation

The automated Brd2(1), Brd4(1), and G9a assay procedures were then validated using the previously optimized conditions. 10 and 0 µM concentrations of either (+)-JQ1 (Brd2 and Brd4) or UNC 0638 (G9a) were used as the positive and negative controls in a Z'-factor experiment to measure assay robustness. The Z'-factor[1] value takes into account the difference in signal between a positive and negative control, as well as the variation in the signal amongst replicates. A scale of 0-1 is incorporated, with values ≥ 0.5 being indicative of an excellent assay.

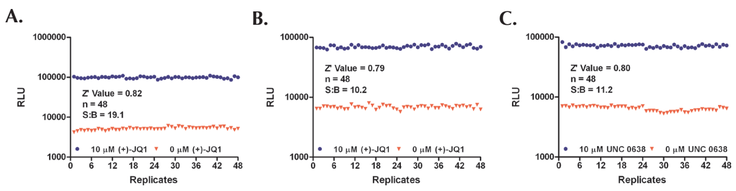

Figure 4 – Z'-factor results. *Forty-eight replicates of 10 µM and 0 µM (+)-JQ1 were run with the Brd2(1)* ***(A.)*** *and Brd4(1)* ***(B.)*** *assays. Forty-eight replicates of 10 µM and 0 µM UNC 0638 were also run with the G9a assay* ***(C.)*** *Assay component additions and incubation times were as previously indicated in Figure 3.*

The Z' values generated with the three automated assays, ranging from 0.79-0.82, as explained previously, are indicative of an excellent, robust assay. The instrument methods for the MultiFlo and Precision, and detection settings on the Synergy NEO used for this experiment would then be used to perform a compound library screen to look for potential inhibitors of these target proteins.

Screen-Well Epigenetics Compound Library Screen

The 43 compound Screen-Well Epigenetics Library V. 1.0 (BML-2836), generously donated by Enzo Life Sciences, was then screened using the validated, automated Brd2(1), Brd4(1) and G9A assays. Five other known inhibitors for various epigenetic targets were also screened, including Sinefungin, 2,4-PDCA, (+)-JQ1, UNC 0646, and UNC 0638. All compounds were diluted in the appropriate CP Reagent from their original 10 mM concentration. The final 1X compound concentrations used in the screen were 20 µM, 2 µM, and 200 nM.

	Brd2(1) Assay			Brd4(1) Assay			G9a Assay		
Compound	20 µM	2 µM	0.2 µM	20 µM	2 µM	0.2 µM	20 µM	2 µM	0.2 µM
Trichostatin A	3.5	2.1	1.7	1.2	1.3	1.1	1.4	1.6	1.5
2,4-Pyridinedicarboxylic Acid	1.1	1.0	1.1	1.2	1.0	1.0	0.9	0.9	1.2
Garcinol	0.4	1.2	1.2	0.3	1.4	1.2	1.0	1.0	1.1
Splitomicin	1.3	1.1	1.1	1.3	1.1	1.2	1.0	1.2	1.1
BML-210	1.7	1.1	1.1	1.9	1.3	1.1	2.0	1.0	1.1
Apicidin	3.6	2.1	1.6	0.6	1.3	1.3	1.8	1.6	1.1
Suberoyl bis-hydroxamic acid	1.1	1.0	1.2	1.0	0.6	1.0	1.0	0.9	1.0
Scriptaid	2.4	1.7	1.1	1.3	1.0	0.8	1.4	1.1	1.3
Nullscript	1.5	1.1	1.2	1.0	0.9	0.8	0.9	1.1	1.2
5-Aza-2'-deoxycytidine	1.1	1.1	1.2	0.8	0.9	0.9	1.1	1.0	1.1
Zebularine	1.1	1.0	1.2	0.9	0.9	1.0	1.0	1.1	1.1
Sulfo	2.2	1.5	1.0	1.0	1.0	0.9	1.6	1.3	1.1
Isonicotinamide	1.0	0.9	0.9	1.2	1.0	0.9	0.9	1.0	1.0
ITSA-1	1.2	0.9	1.1	1.4	0.9	0.9	0.9	0.9	1.0
Phenylbutyrate-Na	1.0	1.1	1.1	1.0	0.9	0.9	1.1	1.1	1.1
Tranylcypromine hemisulfate	1.1	1.1	1.2	0.9	0.9	0.9	1.1	1.1	1.1
Valproic acid	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.0	1.1
EX-527	1.3	1.0	1.0	1.1	0.9	1.1	1.1	1.1	1.0
Resveratrol	1.0	1.0	1.0	1.0	0.9	1.0	0.8	1.2	1.0
M-344	2.0	1.4	1.1	1.2	1.1	0.9	1.6	1.4	1.1
Nicotinamide	1.1	1.1	1.0	1.0	0.9	1.0	1.1	1.1	1.0
BML-266	0.7	1.1	1.0	0.7	1.0	1.0	0.8	1.1	1.2
Piceatannol	1.0	1.1	1.1	1.0	1.0	1.1	1.0	1.0	1.1
Fluoro-SAHA	1.7	1.4	1.0	1.2	1.1	0.9	1.3	1.0	1.0
Valproic acid hydroxamate	1.0	1.0	1.1	1.1	1.0	1.0	1.0	1.1	1.1
AGK2	1.1	1.2	1.1	1.2	1.2	1.1	0.8	1.0	1.1
Salermide	0.8	1.1	1.1	0.9	1.2	1.1	1.0	1.1	1.1
MC-1293	1.2	1.2	1.0	1.1	1.1	1.1	1.0	1.1	1.0
Anacardic acid	0.8	1.1	1.0	0.8	1.1	1.1	1.1	1.1	1.0
B2	1.7	1.1	1.3	1.4	1.1	1.1	1.0	1.0	1.0
BIX-01294	2.7	1.1	1.0	1.0	1.1	1.0	1.6	2.0	0.9
Butyrolactone 3	1.1	1.1	1.1	1.2	1.2	1.1	1.0	1.0	1.0
CTPB	1.0	1.1	1.0	1.0	1.0	0.9	1.1	1.0	1.1
Oxamflatin	1.6	1.4	1.0	1.1	1.3	0.9	1.5	1.5	1.0
Sirtinol	1.0	1.0	1.1	0.8	0.9	1.1	1.0	1.1	1.1
Suramin-Na	1.2	1.1	1.1	1.0	1.1	1.0	1.1	1.2	1.1
BML-278	0.9	1.0	1.0	0.6	0.8	0.8	1.0	1.1	1.0
NCH-51	1.6	1.5	0.9	1.2	1.1	0.7	1.3	1.2	1.0
CI-994	2.1	1.1	1.0	1.2	0.8	0.9	1.6	1.1	1.0
NSC-3852	0.7	1.3	1.0	0.6	1.0	0.9	3.4	1.3	1.0
Aminoresveratrol sulfate	0.9	1.2	1.0	0.7	0.8	0.7	0.7	1.2	1.1
BML-281	1.5	1.5	1.2	1.0	1.1	0.9	1.6	1.5	1.0
Triacetylresveratrol	0.8	1.1	0.9	0.7	1.0	0.9	0.3	0.9	1.0
Sinefungin	0.9	1.0	0.9	0.8	1.0	0.8	1.1	1.0	1.0
2,4-Pyridinedicarboxylic Acid Monohydrate	1.0	1.0	1.0	0.9	0.9	0.8	0.9	1.0	1.1
(+)-JQ1	23.5	8.2	1.5	8.2	3.6	1.6	1.5	1.5	1.3
UNC 0646	0.5	0.9	0.9	0.4	0.9	0.9	1.4	17.8	15.0
UNC 0638	2.7	1.0	0.9	0.6	0.9	0.8	11.8	16.2	5.4

Table 1 – Enzo Epigenetics Library compound screen results. *Fold induction values listed for all compounds tested with the Brd2(1), Brd4(1), and G9a assays. Compound concentrations yielding >2 fold induction highlighted in red.*

Fold induction was determined for the three concentrations of each compound tested with the three assay chemistries by comparing the luminescent signal from compound containing wells to those with no compound. A fold induction value of two represents a 2 fold increase in RLU values over the average from wells containing no compound. An increase in luminescence is indicative of compound binding to the target protein-ePL fusion, and an increase in the steady state of the molecule. This can also indicate the potential that the compound has to inhibit the activity of the target protein. Those compounds yielding greater than a 2 fold increase in luminescent signal were carried forward for testing in dose response format.

Dose Response Confirmation

Dose response tests were performed on "Hit" compounds to discern their full inhibitory potential. Serial 1:4 titrations were performed with each compound starting at a concentration of 100 µM. All compounds included in the experiment were tested with the Brd2(1), Brd4(1), and G9A assays in order to examine target selectivity. Fold induction was again determined by comparing RLU values from wells containing compound to the average value from wells containing no compound.

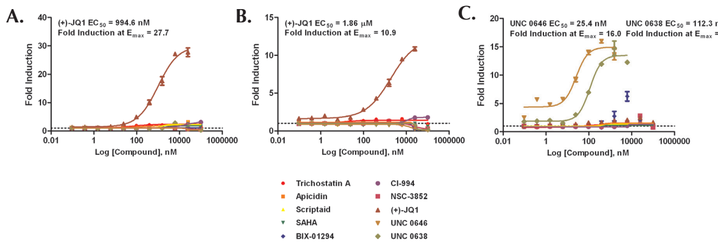

Figure 5 – Dose Response Test Results. *Fold induction values for all compounds, across all concentrations tested with the Brd2(1)* ***(A.)****, Brd4(1)* ***(B.)****, and G9a* ***(C.)*** *assays. Induction curves and EC_{50} values listed for known inhibitory compounds of each target protein. Dotted line indicates fold induction value of 1.*

Screen and dose response test results were validated by the fact that the known bromodomain inhibitor, (+)-JQ1, and G9a methyltransferase inhibitors, UNC 0646 and UNC 0638, demonstrated the expected inhibitory profiles and IC_{50} values[2,3,4]. BIX-01294 also demonstrated a binding affinity to G9a with increasing concentration. This phenomenon was seen to be selective for the G9a, as no appreciable binding was seen with the two bromodomain proteins. This is confirmed in literature references stating that BIX-01294 is an inhibitor of G9a and binds to the substrate peptide groove of the protein[5].

Conclusions

1. The InCELL Hunter eXpress G9a Methyltransferase, and Brd2(1) and Brd4(1) assays provide an accurate, easy-to-use, all in one cell-based format to assess compound binding and potential inhibition of multiple epigenetic proteins.
2. Screening results prove that an increase in luminescent signal will only be seen when compounds interact with the specific intracellular target protein, yielding a low false positive rate.
3. The assay procedure is easily automated in 384-well format using the contact and non-contact dispensing capabilities of the Precision and MultiFlo, respectively.
4. The high-performance luminescence detection system of the Synergy NEO microplate reader affords sensitive detection of the chemiluminescent signal, allowing for use of cell concentrations in the assay as low as 1000 cells/well.
5. The combination of assay chemistry, as well as liquid handling and detection instrumentation, create a simple, yet robust and definitive cell-based solution for the identification of inhibitory compounds of these important epigenetic targets.

[1]Zhang, et al.: A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* 1999 4(2): 67-73. [2]InCELL Hunter™ eXpress Brd2(1) Bromodomain Assay (Product Code: 96-0004E1; Lot Number 12H20SG2) Data Sheet, Oct 22, 2012.
[3]InCELL Hunter™ eXpress Brd4(1) Bromodomain Assay (Product Code: 96-0005E1; Lot Number 12H2202) Data Sheet, Oct 22, 2012. [4]InCELL Hunter™ eXpress G9a Methyltransferase Assay (Product Code: 96-0003E15; Lot Number 12H2107) Data Sheet, Oct 22, 2012. [5]Chang, et al.: Structural basis for G9a-like protein lysine methyltransferase inhibition by BIX-01294. *Nat Struct Mol Biol* 2009 16(3): 312-317.

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