Application Note

Drug Discovery and Development

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Automating a Direct, Cell-Based, Target-Compound Interaction Assay for Methyltransferase and Bromodomain Proteins Using the InCELL Platform

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Introduction

Histone modifications are essential in eukaryotic gene expression and regulation, and are driven by histone writer, eraser, and reader proteins. These histone tail changes are dynamic, and a normal part of a cell's embryonic differentiation from its original totipotent state. However, aberrant histone modifications are linked to numerous disease states[1], including many human cancers. For example, the lysine-specific SET domain, containing G9a methyltransferase writer protein, is over-expressed in various cancers. It is also deficient in CD4+ T helper cells, leading to increased rates of intestinal infection.[2] In another example, the BET bromodomain reader protein Brd4 is implicated in NUT midline carcinoma (NMC) through a fusion of the protein and NUT.[3] Due to these and other findings, methlytransferase and bromodomain proteins are targets for numerous drug discovery projects.

Current biochemical assay technologies examine the enzyme activity of methlytransferase and bromodomain protein inhibition. These assays lack monitoring compound-target engagement in the cellular milieu, which is essential to validate the pharmacology of drug candidates. Additionally, cell-based assays for analyzing inhibitors to epigenetic proteins were previously limited to the detection of specific histone modifications using antibodies. When antibodies are not available, another method is needed. Hence there exists a need for a robust, high-throughput cell-based assay that can identify compound binding to a protein's catalytic domain without the dependency on antibodies. This study describes the antibody-free, cell-based assay platform InCELL Hunter that enables the detection of specificity binding and direct protein engagement of potential small molecule inhibitors to G9a methyltransferase and multiple bromodomain proteins.

Complementary to this study, learn how this platform was used to develop high-throughput cell-based assays for additional Brd4 inhibitors as well as an arginine methyltransferase 3 (PRMT3) inhibitor, as outlined in the Supplemental Data section.

InCELL Hunter cell-based assay principle

InCELL Hunter assays from Eurofins DiscoverX (Fremont, CA) are target-specific assays used to investigate compound-target engagement in cells by detecting changes in protein stability (Figure 1). Such changes in protein stability are measured via a chemiluminescent signal brought about leveraging the industry-validated enzyme fragment complementation (EFC, discoverx.com/efc) technology. This technology provides a quantitative, homogeneous assay format that is rapid, robust, and scalable without the use of antibodies, mass spectrometry, or imaging. The target protein is fused with a small inactive β-galactosidase (β-gal) enzyme fragment called ProLabel (ePL) and expressed in a select cell background. In the absence of a binding compound, the target-ePL fusion reaches a steady state, increasing only when a compound binds the target. The addition of the complementary larger β-gal fragment, enzyme acceptor (EA), facilitates the complementation of ePL and EA, resulting in an active β-gal enzyme. Hydrolysis of an added substrate by the β-gal enzyme generates a chemiluminescent signal, which indicates altered ePL-tagged protein stability. Particularly useful for proteins with a relatively high turnover rate, InCELL Hunter assays have been successfully used to determine compound cellular permeability and potency.

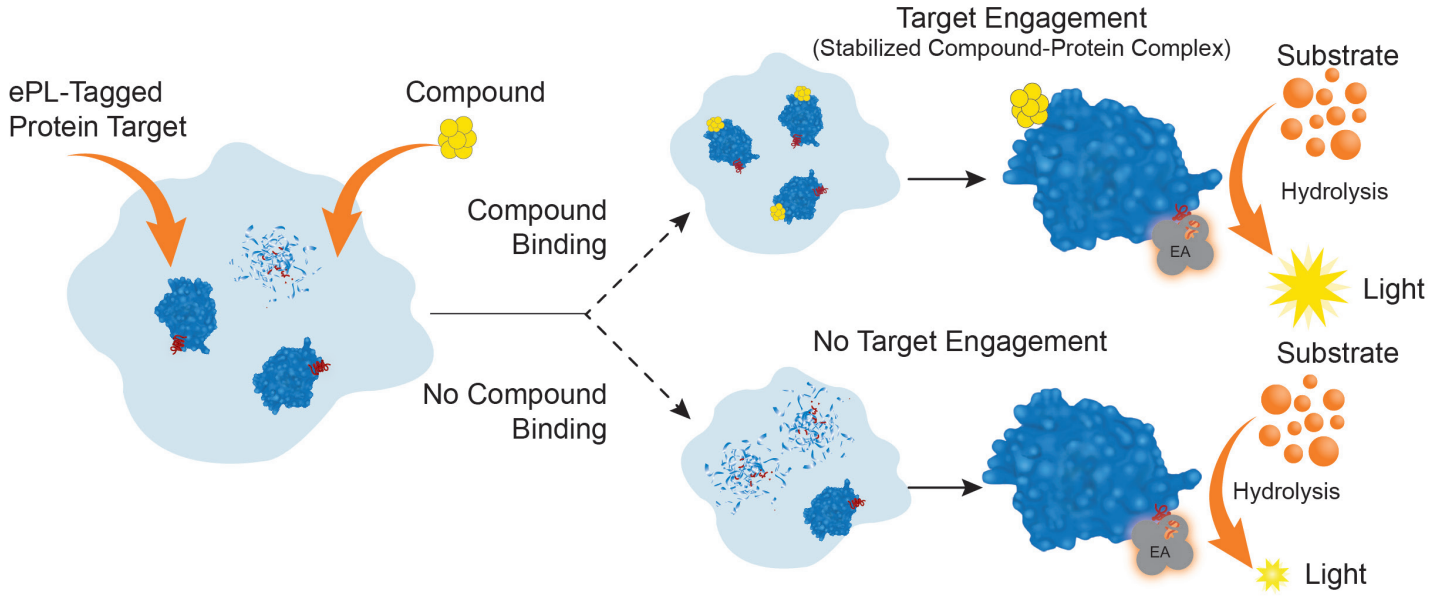

Figure 1. InCELL Hunter assay principle based on Eurofins DiscoverX EFC technology. The intracellular target protein in these assays is fused with a small enzyme donor fragment, ePL, of the β-gal enzyme. Upon addition of a compound that binds the target, protein levels are stabilized or altered in the cell, and this change can be monitored by measuring target protein abundance using chemiluminescent detection. The detection reagents include a chemiluminescent substrate added with a large enzyme acceptor (EA) fragment that naturally complements with the ePL tag on the target protein to create an active β-gal enzyme. The resulting active enzyme hydrolyzes the substrate to generate a chemiluminescent signal. The greater the signal corresponds to greater presence of compound-target engagement in the cell.

Materials and methods

Materials

Assay components

InCELL Hunter eXpress Brd2(1) Bromodomain assay (part number 96-0004E1CP0S), InCELL Hunter eXpress Brd4(1) Bromodomain assay (part number 96-0005E1CP0S), and InCELL Hunter eXpress G9a Methyltransferase assay (part number 96-0003E15CP7S) kits, containing assay-ready InCELL Hunter cells expressing the appropriate epigenetic target protein, Cell Plating (CP) reagent, and Detection reagent, were all used from Eurofins DiscoverX (Fremont, CA). InCELL Hunter HEK 293 Brd2(1) and HEK 293 Brd4(1) cells were included with the Bromodomain assays, while InCELL Hunter A549 G9a cells were included with the G9a Methyltransferase assay.

Compounds

The 43-compound Screen-Well Epigenetics Library, version 1.0 (part number BML-2836-0500) was generously donated by Enzo Life Sciences (Plymouth Meeting, PA). The known bromodomain inhibitor (+)-JQ1 (part number 92-1149) was obtained from Eurofins DiscoverX (Fremont, CA). Sinefungin (part number S8559) and 2,4-Pyridinedicarboxylic acid (2,4-PDCA) monohydrate (part number P63395) were purchased from Sigma-Aldrich Co. (St. Louis, MO). UNC 0646 (part number 4342) and UNC 0638 (part number 4343) were purchased from R&D Systems (Minneapolis, MN).

Instrumentation

The Agilent BioTek Precision microplate pipetting system combines an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. Precision was used to dilute the Epigenetics library and transfer the final 5x concentrations to the 384-well assay plates.

Microplate dispenser

The Agilent BioTek MultiFlo microplate dispenser offers fast, accurate plate dispensing capabilities through its two peristaltic and two syringe pumps, with volumes ranging from 0.5 to 3,000 µL. The MultiFlo was used to dispense all assay components including cells and prepared detection reagent in 384-well format.

Multimode microplate reader

The Agilent BioTek Synergy Neo HTS multimode microplate reader with patented Hybrid Technology 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 using an integration time of 0.2 seconds and gain of 120.

Methods

Automated assay procedures were carried out in 384-well format using high-throughput liquid handling and detection instrumentation. Initial experiments included cell number optimization and postplating optimization of compound incubation times. This was done to maximize the signal-to-background between wells containing compound-bound target cells, and wells with target cells only, as well as a Z'-factor[4] validation. A small, focused library of epigenetic inhibitor small molecule compounds was then screened with each target assay. Finally, dose-response testing was completed using hits from the compound screen and known inhibitor positive control compounds. Experimental results confirm the automated cellular assay's ability to accurately detect the target-specific interaction of test compounds, with a low false positive rate, in a simple, yet robust manner for these important epigenetic targets.

Cell preparation

A 500 µL amount of the appropriate CP reagent was added to 100 µL of assay-ready InCELL Hunter cells at a concentration of 12×10^6 cells/mL to make 600 µL of 2.0×10^6 cells/mL. The cells were further diluted to the appropriate concentration for each experiment by transferring an aliquot of cells to additional CP reagent.

Cell concentration and reaction time optimization

InCELL Hunter HEK 293 Brd2(1) cells were plated into two separate 384-well microplates in concentrations ranging from 16,000 to 0 cells/well. The plates were incubated at 37 °C and 5% CO_2 for either 24 or 48 hours. Following incubation, 10 µM of (+)-JQ1 bromodomain inhibitor (1x) was added to half the wells at each cell concentration, while plating medium was added to the other half. The plates were incubated at 37 °C and 5% CO_2 for 6 hours. Detection reagent was then added to each well, and the plate was incubated at room temperature for 30 minutes. The luminescent signal was then quantified using the Synergy Neo and aforementioned luminescence settings.

Automated InCELL Hunter assay workflow

A 20 µL amount of InCELL Hunter cells, at a concentration of 5×10^4 cells/mL were plated into each microplate well of a 384-well plate, then the plate was incubated at 37 °C and 5% CO_2 for 48 hours. Following incubation, 5 µL of titrated compound was added to each well, and the plate was incubated at 37 °C and 5% CO_2 for six hours. A 30 µL amount of detection reagent was then dispensed into each well, and the plate was incubated a final time at room temperature for 30 minutes. The luminescent signal was then quantified using the Synergy Neo and aforementioned luminescence settings.

Automated assay Z'-factor validation

The automated assay workflow was run using Brd2(1) and Brd4(1) cells and 10 µM and 0 µM concentrations of the bromodomain inhibitor (+)-JQ1 as positive and negative controls in a Z'-factor experiment to measure assay robustness. The automated assay workflow was also run using the G9a cells and 10 µM and 0 µM concentrations of the methyltransferase inhibitor UNC 0638 as positive and negative controls. Forty-eight replicates of each compound concentration were included for each test. The cell concentration and postplating incubation time optimized during the initial experiment were also implemented.

Compound library screen

Forty-eight compounds, including the 43 compound Screen-Well Epigenetics Library, and inhibitors sinefungin, 2,4 PDCA, (+)-JQ1, UNC 0646 and UNC 0638, were each diluted in the appropriate CP reagent from their original 10 mM concentration to final 1x concentrations of 20 µM, 2 µM, and 200 nM. Each compound concentration was tested using the Brd2(1), Brd4(1) and G9a assays following the aforementioned automated workflow. Dose-response tests were performed by using serial 1:4 titrations on each hit compound from the compound library screen starting at a 100 µM 1x concentration. Each hit compound was tested using the Brd2(1), Brd4(1) and G9a assays following the aforementioned automated workflow.

Calculations

Reaction time optimization of the cell concentration per well and postplating incubating time is critical to maximize the signal-to-background ratio (S/B) between wells containing compound-bound target cells, and those containing unbound target cells. Relative luminescent units (RLU) were plotted for wells containing either 10 µM or 0 µM (+)-JQ1 from cells incubated for 24 hours (Figure 2A) or 48 hours (Figure 2B) postplating. S/B values were calculated as follows:

$$\frac{RLU_{(10\ \mu M\ (+)\text{-}JQ1)}}{RLU_{(0\ \mu M\ (+)\text{-}JQ1)}}$$

Calculated S/B values were then plotted for all cell concentrations (Figure 2C) and for 250 to 2,000 cells/well only (Figure 2D). Per Figure 2D, the calculated S/B between wells containing 10 or 0 µM of inhibitor remain steady to slightly increasing for the 24-hour and 48-hour plating times up to 2,000 cells/well.

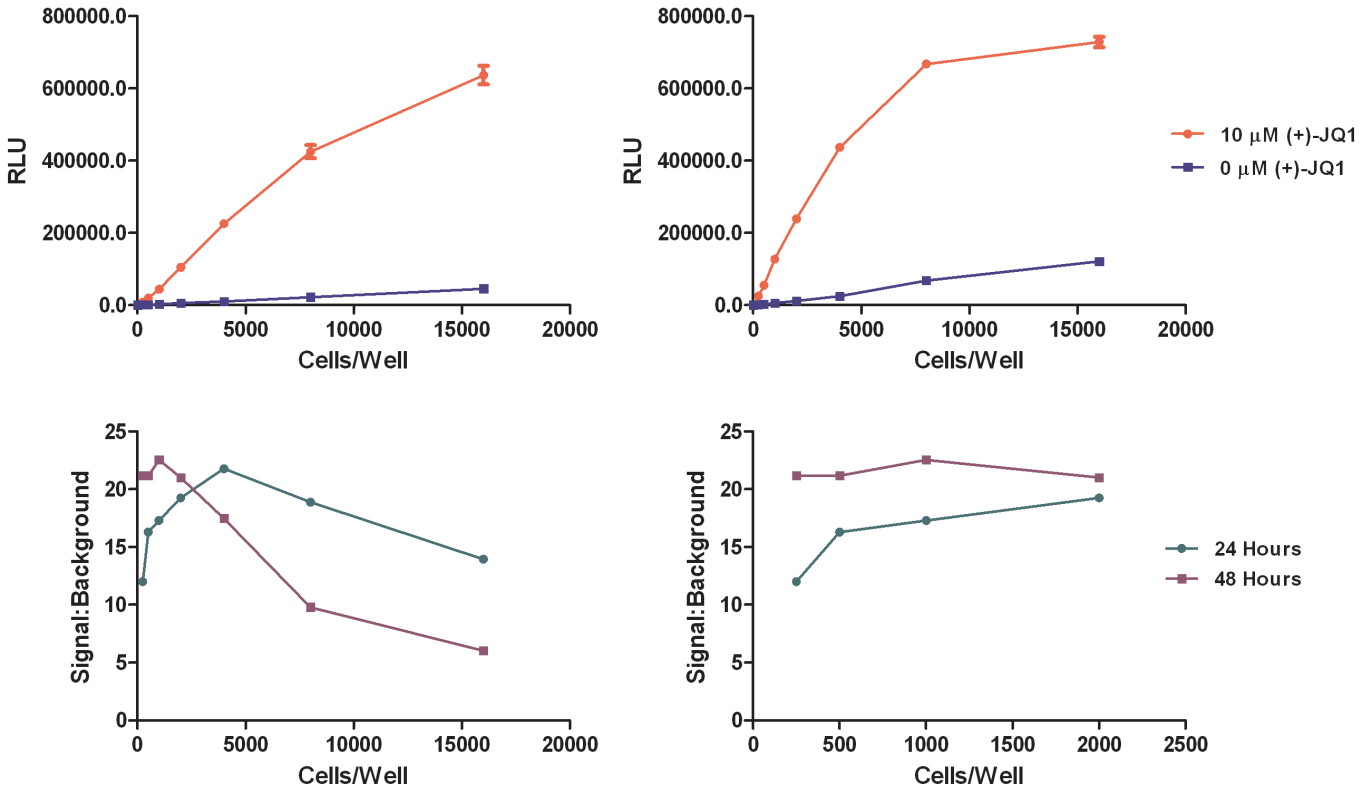

Figure 2. Cell concentration and postplating incubation results. RLU values plotted for wells containing either 10 µM or 0 µM (+)-JQ1 from cells incubated for 24 hours (A) or 48 hours (B) postplating. Calculated S/B values for all cell concentrations tested (C) as well as for 250 to 2,000 cells/well only (D).

Results and discussion

When comparing results from cells incubated for either 24 or 48 hours postplating across the same cell concentrations, S/B data from 48-hour incubated cells were found to be consistently higher than from 24-hour incubated cells, with S/B values greater than 20. Therefore, a cell concentration of 1,000 cells/well, and a 48-hour postplating incubation time was chosen for use in subsequent experiments and across cell strains to ensure a high S/B ratio while also minimizing cell usage.

Automated assay Z'-factor validation

The Z'-factor value takes into account the difference in signal between a positive and negative control, as well as the signal variation amongst replicates. A scale of 0 to 1 was used, with values greater than or equal to 0.5, indicating an excellent assay. Z'-factor values generated with the three automated assays (Figure 3) of 0.82 (Brd2(1)), 0.79 (Brd4(1)), and 0.80 (G9a), each indicates an excellent, robust assay.

Compound library screen

The Screen-Well Epigenetics Library and five known inhibitors were screened using the validated, automated Brd2(1), Brd4(1), and G9a assays to look for potential target protein inhibitors. Fold induction (data not shown) was determined for each of the three compound concentrations with each of 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.

Increased luminescence is indicative of compound binding to the target protein-ePL fusion, and an increase in the molecule's steady state. The degree of luminescence can also indicate a compound's potential to inhibit the target protein's activity. A decrease in fold induction was seen at the highest concentration screened with certain compounds, including BIX-01294, UNC 0646, and UNC 0638. This decrease was likely due to cytotoxic effects from the compound on the cell line incorporated for the particular assay. Compounds yielding greater than a 2-fold luminescent signal increase were further tested to discern their full inhibitory potential. For the Screen-Well Epigenetics Library and screen results, contact DRX_SupportUS@eurofinsUS.com.

Those compounds showing greater than a 2-fold luminescent signal increase in the compound screen were carried forward for dose-response analysis using the validated, automated Brd2(1), Brd4(1) and G9a assays to examine target selectivity. Fold induction values were plotted for all compounds included in the dose-response test, with each assay. Points were excluded from consideration where obvious cytotoxic effects were seen from high compound concentration.

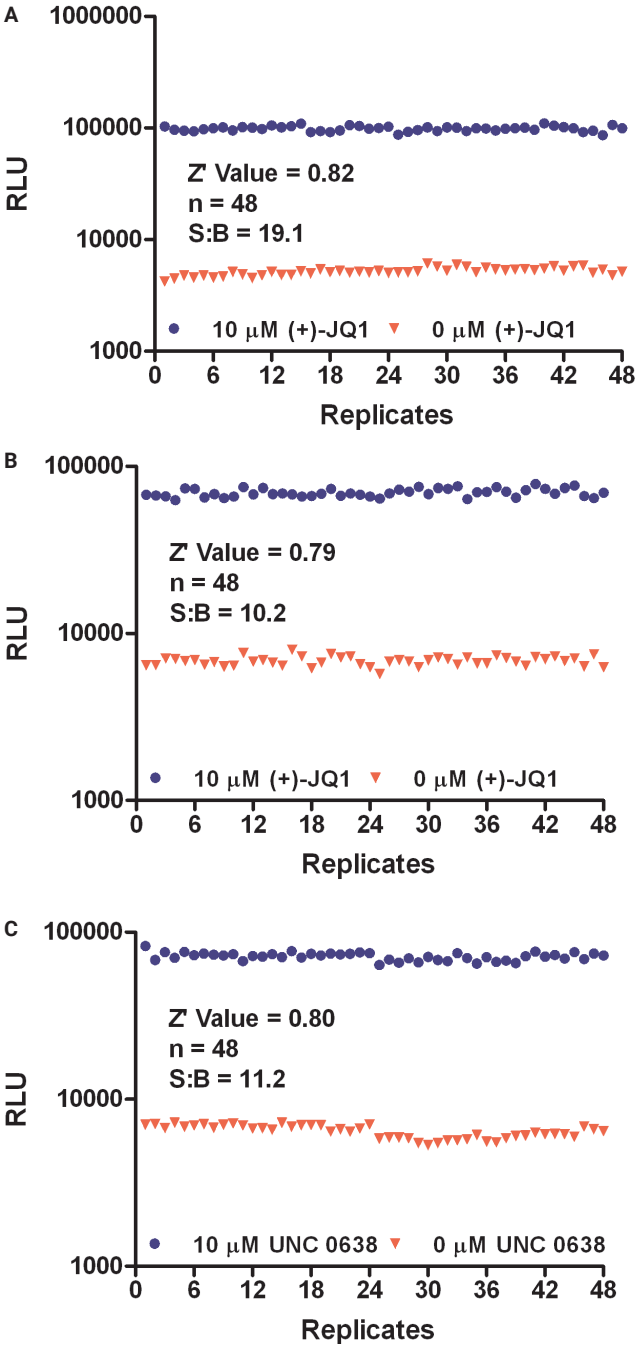

Figure 3. Automated assay Z-factor validation. Z'-factor results for automated (A) Brd2(1), (B) Brd4(1), and (C) G9a assays.

Per Figure 4, the known bromodomain inhibitor, (+)-JQ1, and G9a methyltransferase inhibitors, UNC 0646 and UNC 0638, each demonstrated the expected inhibitory profiles and EC_{50} values[5,6,7], thus validating screen and dose-response test results. Figure 4 also shows that the BIX-01294 compound also demonstrated binding affinity to G9a with increasing concentration. This phenomenon was selective for the G9a assay, and is confirmed in literature references showing that BIX-01294 binds to the substrate peptide groove of the protein.[8] No appreciable BIX-01294 binding was observed with the two bromodomain proteins.

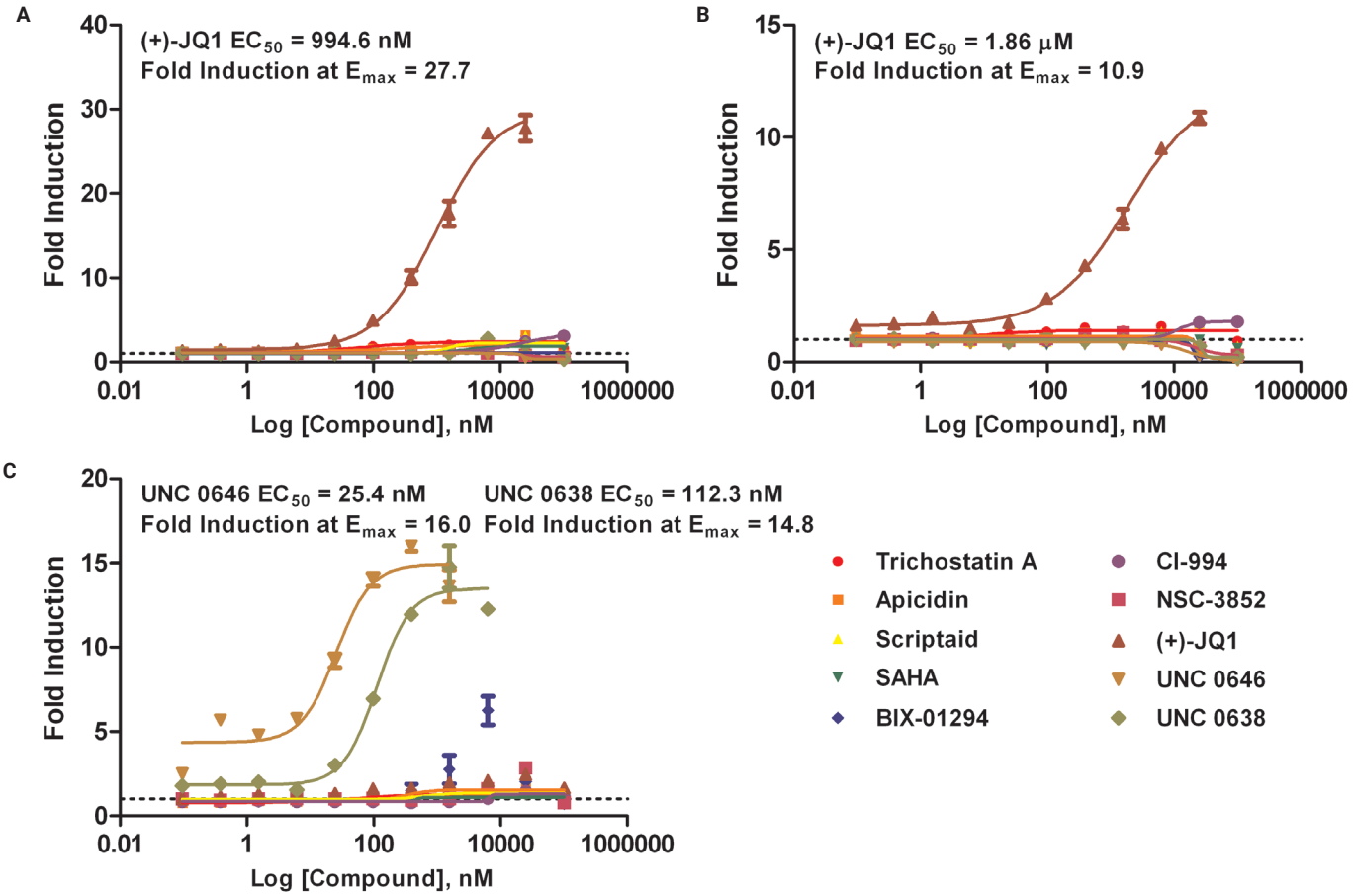

Figure 4. Compound library screening dose-response analysis. Dose-response test results, showing induction curves and EC_{50} values for known inhibitory compounds with (A) Brd2(1), (B) Brd4(1), and (C) G9a assays. Dotted line indicates fold induction value of 1 for reference.

Conclusion

The InCELL Hunter epigenetic G9a methyltransferase, Brd2(1), and Brd4(1) assays provide an accurate, simple, all-in-one cell-based format to assess compound binding to the specific target of interest. The assay procedure is easily automated for high-throughput analysis in 384-well format using the Agilent BioTek Precision microplate pipetting system and the Agilent BioTek MultiFlo microplate dispenser. Additionally, the Agilent BioTek Synergy Neo high-performance luminescence detection affords sensitive chemiluminescent signal detection, allowing use of cell concentrations as low as 1,000 cells per well.

Screening data show that an increased luminescent signal is only seen when compounds interact with the specific intracellular target protein, yielding a low false-positive rate. Finally, the combination of assay chemistry and liquid handling and detection microplate instrumentation create a simple, robust and definitive cell-based solution for inhibitory compound identification of these important epigenetic targets.

Supplemental data

Cell-based protein stabilization assays for the detection of interactions between small-molecule inhibitors and BRD4

Bromodomain protein 4 or BRD4 is a transcriptional and epigenetic regulator that is known to drive the expression of oncogenes in several cancers. BRD4 binds to acetylated histone tails through two bromodomains, BD1 and BD2.

Inhibition of BRD4-histone binding through small molecule inhibitors is found to be a useful future cancer treatment. This paper describes two orthogonal assays, including a miniaturized one based on the InCELL Hunter platform, that were used to evaluate BRD4 stabilization by small molecule inhibitors.[11]

A potent, selective, and cell-active allosteric inhibitor of protein arginine methyltransferase 3 (PRMT3)

PRMT3 catalyzes the mono- and asymmetric demethylation of arginine residues. This enzyme is implicated in several diseases such as oculopharyngeal muscular dystrophy, coronary heart disease, and in tumor growth. This paper describes the first allosteric inhibitor of PRMT3, called SGC707, and its binding with PRMT3 using the InCELL Hunter platform.[12]

InCELL Pulse target engagement assays

The InCELL Pulse assay format allows for easy and rapid measurement of compound-target engagement, particularly for thermally labile proteins. Both InCELL Hunter and InCELL Pulse format types share the same underlying EFC principles, but with some crucial differences that suit them for different targets. Learn more about the InCELL platform and access the available cell lines and kits at discoverx.com/InCELL.

Acknowledgements

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