APPLICATION NOTE

Quantifying the Degradation of RBM39 in Neuroblastoma Cells Treated with Molecular Glue Degraders Using HTRF Technology

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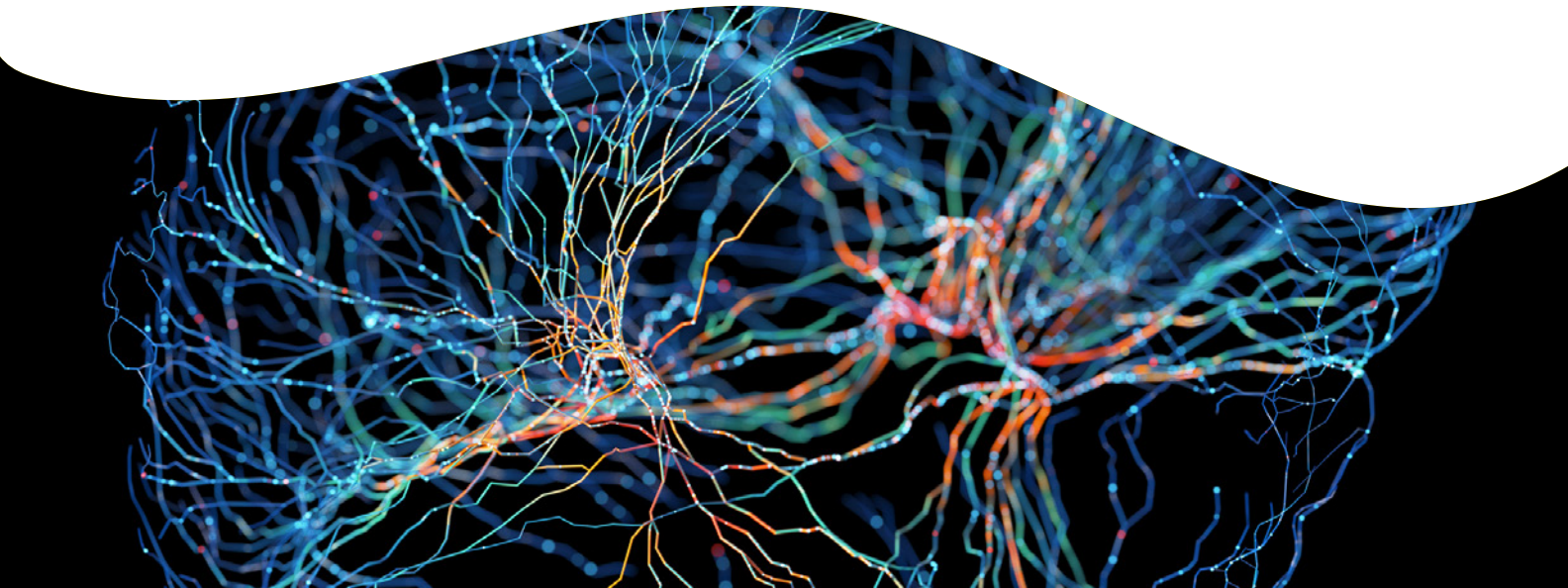
Introduction

RNA-binding motif protein 39 (RBM39) is a serine/arginine-rich RNA-binding protein initially identified from a patient with liver cirrhosis that progressed to hepatocarcinoma. RBM39 co-localizes with core spliceosome subunits in the nucleus and is involved in transcriptional co-activation and alternative RNA splicing.[1] RBM39 is upregulated in most cancers and can be used as an immunological or prognostic biomarker of disease progression.[2] The functional inhibition of RBM39 is lethal to several cancers including lung, breast, and colorectal cancers, making it an excellent target for cancer therapy.[3, 4, 5] The aryl sulfonamide drug, indisulam exhibits anti-cancer activity by acting as a molecular glue degrader, recruiting RBM39 to the CUL4-DCAF15 E3 ubiquitin ligase for polyubiquitylation and subsequent proteasomal degradation.[6] Recently, a library of 2000 small molecule cytotoxic/cytostatic compounds was screened for antiproliferative activity in cancer cells to find additional molecular glue degraders, resulting in the identification of dCeMM1-4.[7] As illustrated in Figure 1, dCeMM1, a sulfonamide, functions to selectively degrade RBM39 by gluing to the CUL4-DCAF15 ubiquitin ligase.[8] The dCeMM2-4 molecules target cyclin K for degradation by binding the CDK12 to the DDB1-CUL4-RBX1 ubiquitin ligase complex.[9] While dCeMM2 and dCeMM3 are novel and structurally similar, dCeMM4 shares structural characteristics with other known cyclin K degraders (Figure 1).[10]



This case study highlights the use of (Homogeneous Time-Resolved Fluorescence) HTRF technology (Revvity) to evaluate RBM39 degradation in neuroblastoma (SH-SY5Y) cancer cells treated with Indilsulam and dCeMM1-4 molecular glue degraders, while simultaneously assessing cell viability using the CellTiter-Blue® Cell Viability Assay (Promega). The HTRF results from cells treated with indisulam and dCeMM1 were validated using Western blot. HTRF was also used to determine the effect of pre-treating SH-SY5Y cells with a potent and selective proteasome inhibitor, Epoxomicin, prior to treatment with indisulam.

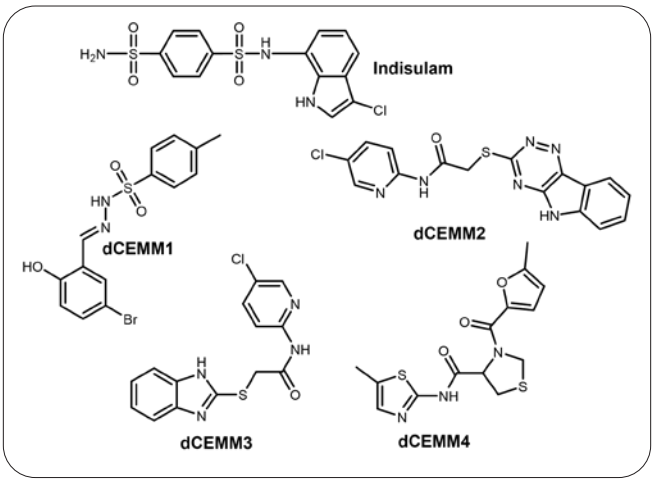


Figure 1: Chemical structure of molecular glue degraders indisulam and dCeMM1-4 compounds used in this case study.

Methods

SH-SY5Y Cell Culture, assessment of cell viability using CellTiter-Blue Assay and Lysis

SH-SY5Y cells were cultured and maintained in DMEM high glucose medium supplemented with GlutaMAX™, pyruvate (Gibco #31966047), and heat-inactivated FBS (BioSell #Abbvie). SH-SY5Y cells were passaged 4 times and harvested using trypsin-EDTA (Gibco #25300054). The viability of the SH-SY5Y cells culture was determined to be 98.8% prior to plating and compound treatment. After treatment, a cell density of 50,000 cells/well was transferred to separate microplates to either assess cellular viability using the CellTiter-Blue Assay following the instructions for use found in the corresponding Technical Bulletin (TB317) on the Promega website or to measure RBM39 levels using HTRF. In preparation for HTRF analysis, SH-SY5Y cells were lysed using the lysis buffer included in the HTRF Human Total RBM39 Detection Kit (Revvity #64RBM39TPEG) following the protocol listed in the respective Technical Data Sheet (TDS). An overview illustrating the preparative workflow for this study is provided in Figure 2. The CellTiter-Blue and HTRF results were read on a PheraStar® FSX Plate Reader (BMG Labtech) and the data was analyzed using Microsoft® Excel® and GraphPad Prism software.

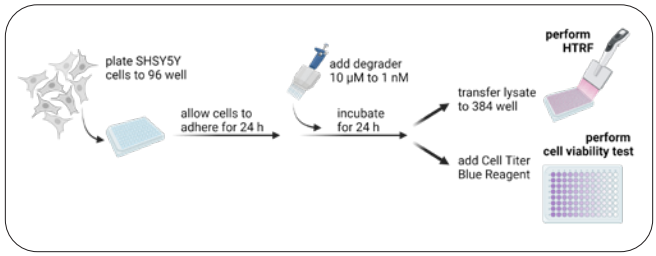


Figure 2: Workflow for assessing the viability of SH-SY5Y cells and RBM39 levels in SH-SY5Y cell lysates after treatment with molecular glue degrader compounds. Image created with software from BioRender.com.

HTRF

HTRF® is a no-wash technology. It combines standard FRET technology with time-resolved measurement of fluorescence, eliminating short-lived background fluorescence. For a sandwich assay, two antibodies that recognize a protein of interest are used, with one antibody coupled to a donor, and the other to an acceptor. If the two antibodies recognize the analyte, the donor will emit fluorescence upon excitation and the energy will be transferred to the nearby acceptor, giving specific acceptor fluorescence. The donor fluorescence is also measured and a ratio with the acceptor fluorescence is applied. An Illustration of the Total RBM39 HTRF assay kit (Revvity #64RBM39TPEG) used in this study is provided in Figure 3.

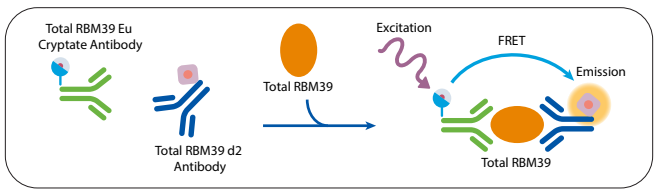


Figure 3: Illustration of HTRF Human Total RBM39 Detection Kit.

Western blot

After treatment with molecular glue degraders or DMSO, the cell culture medium was removed from the microplate wells and the cells were washed with 1X PBS (Gibco #14190-094). PhosphoSafe Extraction Reagent (Millipore/VWR #71296-25) supplemented with cOmplete™ Protease Inhibitors (Roche #11697498001) was added to the cells and the plate was stored at -20 °C until further processing could be completed. The frozen SH-SY5Y cell lysates were thawed, diluted 4:1 with 4x Laemmli buffer (Biorad #1610747) and incubated at 95 °C for 5 minutes. Samples and molecular weight marker (Thermo Fisher Scientific #26616) were loaded into a 4-20% SDS -PAGE gel (Biorad #5671095) and the gel was run according to the manufacturer's specifications. The resulting protein bands were transferred to a nitrocellulose membrane using a Trans-Blot Turbo Midi system and respective transfer packs (Biorad #1704159). The membrane was blocked with Skim Milk Powder (Sigma # 70166-500mg) dissolved in 1X TBST (Biorad #1706435). The blots were incubated in primary antibodies (1:1000 anti-vinculin, rabbit - Abcam #ab129002 or anti-RBM39, rabbit – Merck #HPA001591-100UL in milk) overnight at 4 °C with shaking. The blots were then washed 3x in TBST for 10 minutes. The secondary HRP-conjugated antibody was then added to the blots (1:10,000 in milk) and incubated at room temperature for 1 hour. The blots were washed again in TBST as previously described and incubated with Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific #345480). The resulting chemiluminescent signal was visualized using a ChemiDoc Imaging System (Biorad).

Results

The degradation of RBM39 was measured in neuroblastoma cells treated with indisulam and dCeMM1 using HTRF technology

SH-SY5Y cells were treated with varying concentrations of indisulam and dCeMM1-4, dissolved in DMSO. The Total RBM39 HTRF Kit was used to determine the percentage of RBM39 present at each molecular glue degrader concentration relative to the negative control (DMSO only). Both indisulam and dCeMM1 stimulated RBM39 degradation in SH-SY5Y cells (Figure 4A). Indisulam induced a more potent degradation of RBM39 (DC50 55 nM), compared to the effect of dCeMM1 (DC50 0.8 µM). RBM39 was not degraded in SH-SY5Y cells treated with Cyclin K-specific molecular glue degraders (dCeMM2, -3 & -4). As expected, the CellTiter-Blue Assay results shown in Figure 4B indicate that cell viability is slightly reduced in indisulam-treated SH-SY5Y cells. Treating cells with the dCeMM1-4 compounds resulted in little to no change in viability.

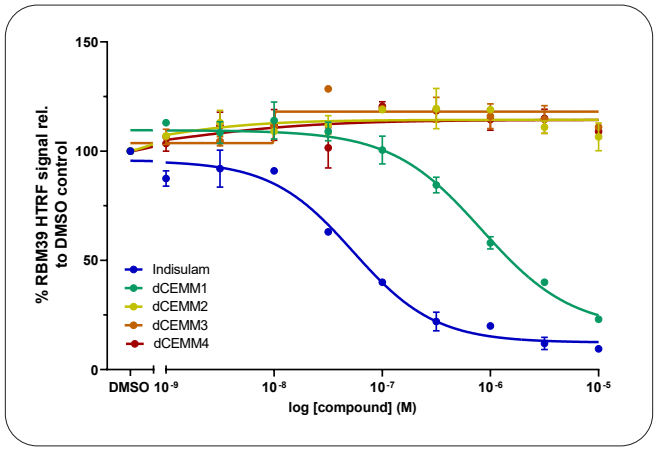


Figure 4A: Concentration-response curves for SH-SY5Y cells treated with indisulam and dCeMM1-4 molecular glue degraders for 24 hours.

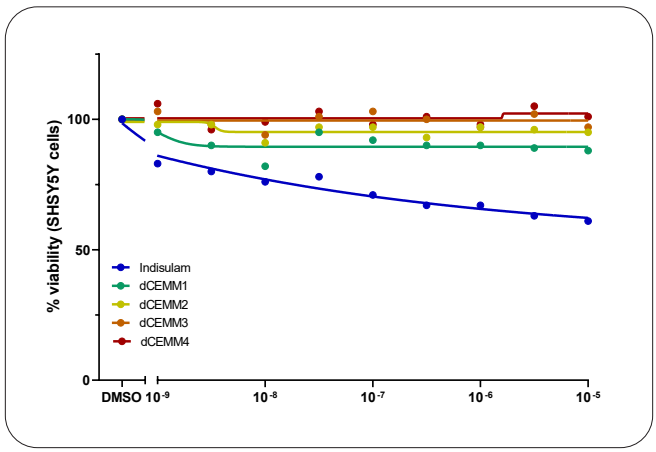


Figure 4B: Percent SH-SY5Y cell viability measured using CellTiter Blue Assay

Validation of HTRF results – Quantification of RBM39 degradation using Western blot

Western blot was used to probe the levels of RBM39 in lysates generated from SH-SY5Y cells treated with a range of RBM39-specific molecular glue degrader (indisulam and dCeMM1) concentrations, resulting in a half-log dose response. The blots in Figure 5A show the RBM39 levels at each of the indisulam and dCEMM1 concentrations tested. These concentrations are the same as the concentrations used in Figure 4A, Figure 6 & Figure 7. The cytoskeleton

protein Vinculin was used as a control to ensure that the decrease in RBM39 over the dosing range was not attributed to a decrease in the total protein levels in SH-SY5Y cells. Densitometry was used to quantify the intensity of each RBM39 band in the blot, which was compared to the intensity of the RBM39 band in the DMSO-only control and normalized to the Vinculin concentration, giving the percentage of RBM39 remaining after treatment (Figure 5B). The normalized results demonstrated that both compounds induced the dose-dependent degradation of RBM39. The results showed that ~20% of RBM39 remained in cells dosed with 3 µM indisulam and ~10% of RBM39 remained in cells dosed with 10 µM dCeMM1, which correlates to the HTRF data provided in Figure 4A. The band directly below the RBM39 band (black arrow) in the RBM39 blot is a ~55 kDa non-specific band that cross-reacts with the RBM39 primary antibody and is clearly unaffected by indisulam or dCeMM1 treatment.

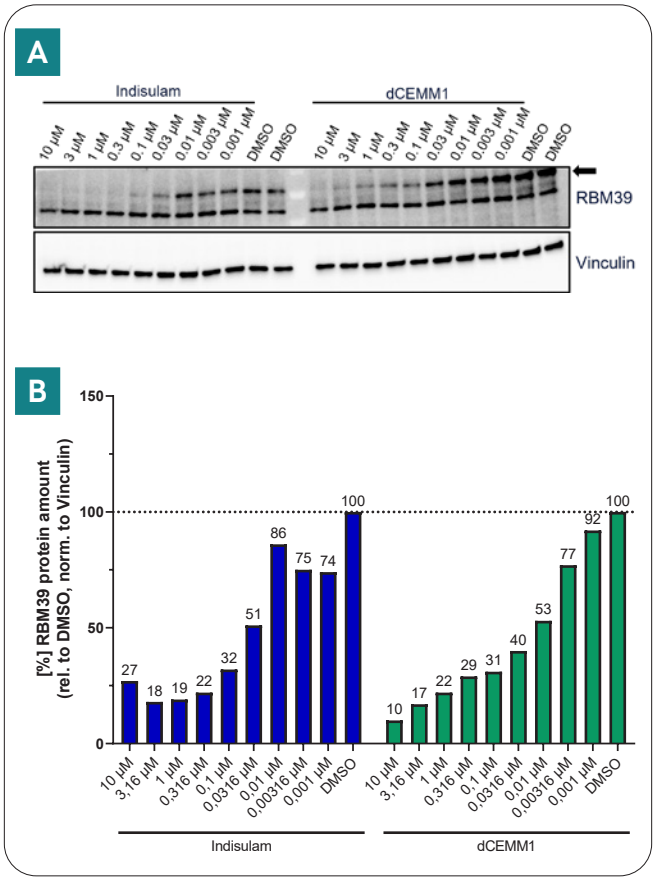


Figure 5: A. Western blot of RBM39 from SH-SY5Y cells treated with 10 µM – 0.001 µM indisulam and dCeMM1 molecular glue degraders for 24 hours. B. Densitometry of RBM39 bands shown in Figure 4A.

Time course of RBM39 degradation in SH-SY5Y cells treated with indisulam

A time-course experiment was used to determine the optimal treatment time for dosing SH-SY5Y cells with indisulam resulting in maximum degradation of RBM39. A half-log dose response curve was set up by treating SH-SY5Y cells with 10-9 to 10-5 M of the molecular glue degrader, indisulam, or DMSO for 1, 2, 6, 16, 24, and 48 hours prior to lysis and the RBM39 levels were quantified using the Total RBM39 HTRF Kit. As shown in Figure 6, there was a dramatic decrease in the remaining RBM39 concentration between 1 and 2 hours. Maximum RBM39 degradation was observed in cells treated with indisulam for ≥6 hours compared to the DMSO-only control. This shows that HTRF technology can be used to determine important treatment parameters related to molecular glue degraders in cancer cell culture.

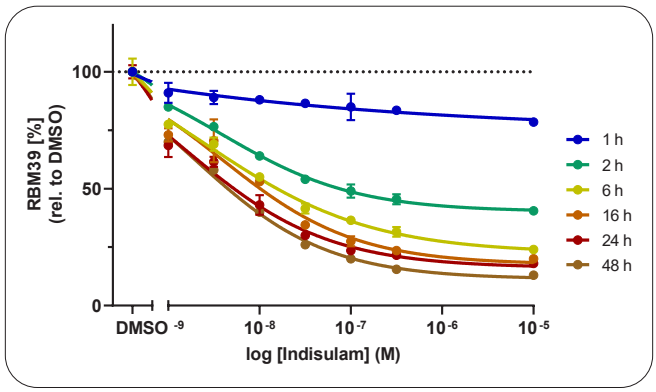


Figure 6: Time course and concentration-response of indisulam-mediated degradation of RBM39 in SH-SY5Y cells quantified using a Total RBM39 HTRF Kit.

The functional inhibition of the proteasome stabilizes RBM39 levels in indisulam-treated cells

Epoxomicin, the epoxyketone-containing proteasome-specific inhibitor11 was used to block RBM39 degradation in indisulam-treated SH-SY5Y cells measured using HTRF. Figure 7 shows the canonical degradation curve of RBM39 when SH-SY5Y cells are pre-treated with DMSO prior to dosing with a range of indisulam concentrations for 24 hours (blue line). RBM39 degradation was not observed in cells pre-treated for 1 hour with epoxomicin prior to the 24-hour indisulam incubation (red line), due to the functional inhibition of the cellular proteolytic machinery needed to degrade RBM39. This result shows that HTRF can be used to monitor the degradation of molecular glue degrader targets in cells and potentially elucidate the mechanism of action of these unique classes of molecules by utilizing small-molecule enzyme inhibitors, such as epoxomicin.

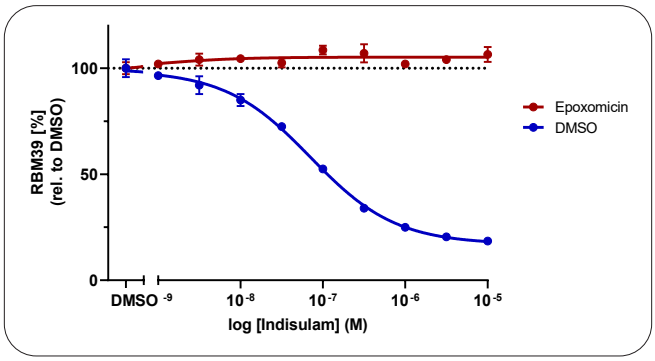


Figure 7: The inhibition of RBM39 degradation was observed in SH-SY5Y cells pre-treated for 1 hour with the proteasome-specific inhibitor epoxomicin (red line) vs. DMSO (blue line) prior to a 24-hour incubation in indisulam. The remaining RBM39 levels at each of the listed indisulam concentrations were measured using the HTRF Human Total RBM39 Detection Kit.

Summary

HTRF is a FRET-based technology that can quantify the level of biomolecular targets in lysate prepared from tissue or cell culture. In this case, HTRF was used to measure the degradation RBM39, a target for the molecular glue degraders indisulam and dCeMM1 in SH-SY5Y (neuroblastoma) cells. The results of this study suggest that HTRF can be used to screen compound libraries for molecules that selectively degrade intracellular targets in cell culture. The integrity of the HTRF results related to the indisulam and dCeMM1-mediated degradation of RBM39 was validated using Western blot. HTRF also provided important information regarding the optimal dosing range and treatment time for maximum degradation of a target molecule within cells. Additionally, the mechanism of action of molecular glue degraders may be potentially elucidated by using HTRF to measure the concentration of a target molecule in cells that are pre-treated with an enzyme-specific inhibitor that influences the degradation of target (e.g., the proteasome-specific inhibitor, epoxomicin, stabilizes RBM39 in indisulam-treated cells). Furthermore, RBM39 stabilization following indisulam treatment could potentially serve as a marker for validating neddylation deficient cell lines.

References

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