Monitoring Target Engagement in Drug Discovery: Application of Wes to the Cellular Thermal Shift Assay

Matias Casás-Selves, Ph.D., Ontario Institute for Cancer Research, Drug Discovery Program, Toronto, ON, Canada
Daniela Ventro, Ph.D. and Susan Harrison Uy, Ph.D., ProteinSimple, San Jose, CA, USA

Introduction

In drug discovery, confirmation of in-cell target engagement is a critical component of the drug development process. Confirming that a drug candidate engages its proposed target in the cell and determining the concentration at which it exerts the desired effect(s) fulfill fundamental criteria for translation to activity and efficacy in its target tissue. Thermal shift assays (TSA) are regularly used by industry and academia to uncover or confirm interactions using purified proteins. Recently, this type of assay has been adapted to a cellular format and is called the Cellular Thermal Shift Assay (CETSA®)[1]. Importantly, by upgrading from a biochemical to a cellular system, biological relevance is preserved. Thus, protein localization, post-translational modifications and other biomolecular crosstalk and interactions remain intact during drug–target engagement analysis[1,2].

CETSA: The Why and How

CETSA provides researchers with the ability to measure drug–target engagement directly in the cell[2]. Conventionally used phenotypic assays that rely on a functional readout such as changes in metabolite levels, the phosphorylation status of downstream targets or cell viability as a measure of response to a drug cannot be precisely attributed to this interaction. As a direct evaluation of the drug–target relationship, CETSA adds a layer of specificity to which the functional drug response can be attributed.

In brief, CETSA initially consists of determining the temperature required for the denaturation of the protein of interest. Once determined, constant heat stress conditions are implemented over a defined period to test a concentration gradient of a putative ligand for its target-binding efficacy. Unbound proteins will denature and precipitate, whereas ligand-bound ones will stabilize and remain in solution[2]. The increasingly stabilized protein can be detected in the soluble fraction but requires a quantitative Western blotting approach for researchers to make meaningful conclusions.

In this application note, quantitative and reproducible CETSA data generated with ProteinSimple's Wes™ instrument (CETSA-Wes) are presented. This assay verifies drug–target binding, and given its quantitative nature, the half maximal inhibitory concentration (IC_{50}) is also calculated.

CETSA and the Case for Wes

Dr. Matias Casás Selves is a Research Scientist within the Drug Discovery Program at the Ontario Institute for Cancer Research (OICR). Using the myelomonocytic leukemia cell line MV4-11, their research group set out to determine the in-cell engagement of their proprietary compound, Compound 1, to their protein of interest, Protein X.

The CETSA protocol requires the denaturation temperature of a target protein be determined first[2]. After subjecting MV4-11 cells to a temperature gradient ranging from 37 °C to 72 °C followed by protein expression analysis by traditional Western blot, it was discovered that most of Protein X was denatured and precipitated from solution at 52 °C

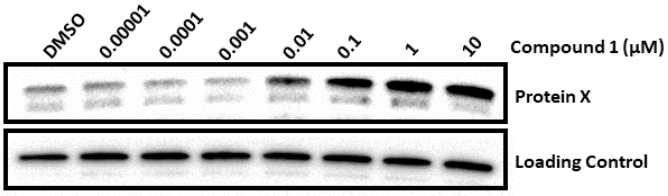

FIGURE 1. Conventional Western immunoblot detecting increased Protein X expression and stability in MV4-11 cells in response to a concentration gradient of Compound 1. The experiment was performed at 52 °C. DMSO, vehicle control.

(results not shown). Next, under heat-stressed conditions (52 °C), increasing concentrations of Compound 1 (0.00001–10 μM) were added to 2.5 x 10^6 MV4-11 cells per treatment for 1 hour, where DMSO was the vehicle control. To measure Compound 1 engagement with Protein X, cell lysates were run using the conventional Western blotting technique to detect changes in Protein X expression and stability (**Figure 1**). Compound 1 induced a major stability shift in Protein X under heat stress between 0.001 μM and 0.01 μM (**Figure 1**). Although these results strongly suggest that Compound 1 engages its target in the low nanomolar range, accurate IC_{50} values cannot be calculated given the semiquantitative nature of the conventional Western blotting method.

How Wes Works

Wes is a fully automated, capillary-based Simple Western system (ProteinSimple, PN 004-600) that solves many of the challenges that come with traditional Westerns. Everything from sample separation to data analysis is completely automated. The sample lysate, stacking and separation matrices are vacuum-loaded into a capillary, voltage is applied to separate proteins, and then proprietary UV-induced capture chemistry immobilizes the protein to the capillary wall. The uncaptured material is washed away, and the primary antibody enters the capillary to bind to its target specifically. Next steps include an HRP-labeled secondary and luminol/peroxide that generate a quantitative chemiluminescent signal (**Figure 2**). Separation requires just 5 μL of the denatured sample, which contains the lysate, 1X Fluorescent Standard, DTT and Sample Buffer for each data point. Wes is a single-

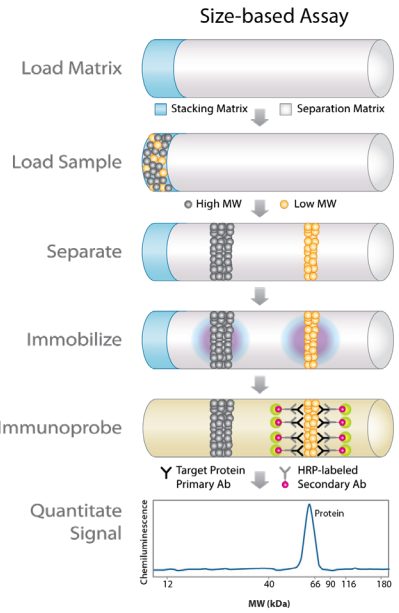

FIGURE 2. A standard Simple Western assay using Wes.

cycle instrument that generates quantifiable results on up to 25 data points in just three hours with only 30 minutes of hands-on time. Visualization and data analysis takes place by way of Compass for Simple Western software in either the electropherogram or traditional lane view.

Using Wes To Monitor Protein Stability During Drug Discovery

To accurately calculate an IC_{50} value for Compound 1, CETSA results obtained from the traditional Western technique (**Figure 1**) were reproduced using Wes in an assay we introduce as CETSA-Wes. This repeat experiment was performed using the same leukemia cell line (MV4-11), number of cells per treatment (2.5 x 10^6) and fixed temperature (52 °C) and time (1 hour), but included an expanded concentration gradient of Compound 1 to generate an isothermal dose-response curve for the calculation of the IC_{50} (**Figure 3 A, B**). Using the CETSA-Wes setup, a similar stability shift in Protein X in the presence of increasing concentrations of Compound 1 could be detected (**Figure 3A**). Importantly, Wes allows for multiplexing if the molecular weight of the target proteins are sufficiently spread out, enabling both Protein X and

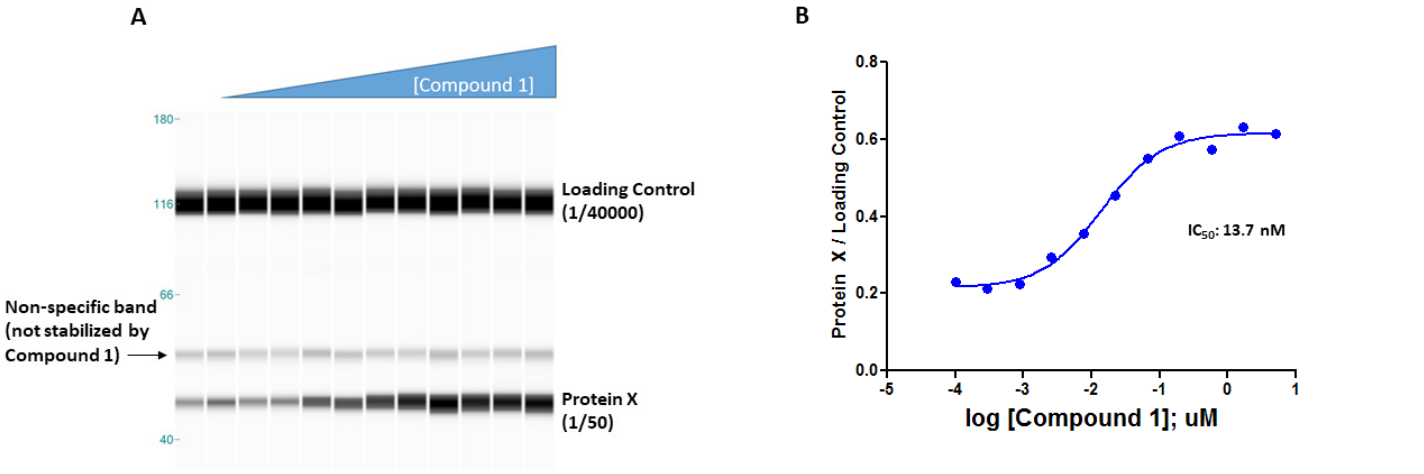

FIGURE 3. Repeat results using CETSA-Wes showing an increase in Protein X expression and stability with increasing concentrations of Compound 1 (**A**). Compound 1 dose-response curve generated from normalization of Protein X signal to loading control, IC_{50} = 13.7 nM (**B**).

the loading control to be detected in one single capillary. This provided for an accurate method of normalizing the signal detected for Protein X to the loading control, and thus, the calculation of an IC_{50} for Compound C, which was determined to be 13.7 nM or 0.0137 µM using Compass for Simple Western software (**Figure 3B**).

CETSA-Wes is Quantitative and Reproducible

With the quantitative performance of Wes for CETSA confirmed, data reproducibility using this approach was validated next. The experiment shown in **Figure 3** was repeated using identical conditions but in technical duplicates. Previously performed orthogonal assays revealed Compound 2 to be an inactive analog of Compound 1, and it was included to demonstrate the assay's ability to also differentiate between molecules of varying potency (**Figure 4)**. The dose-response curve for Compound 1 was reproduced with a similarly induced stability shift in Protein X under heat stress (52 °C), and a refined IC_{50} value was calculated to be 15.2 nM or 0.0152 µM ± 0.092 (**Figure 4**). Importantly, Protein X is not affected by the inactive analog Compound 2, confirming the ability of CETSA-Wes to specifically distinguish and accurately measure the sought-after, in-cell, drug–target engagement.

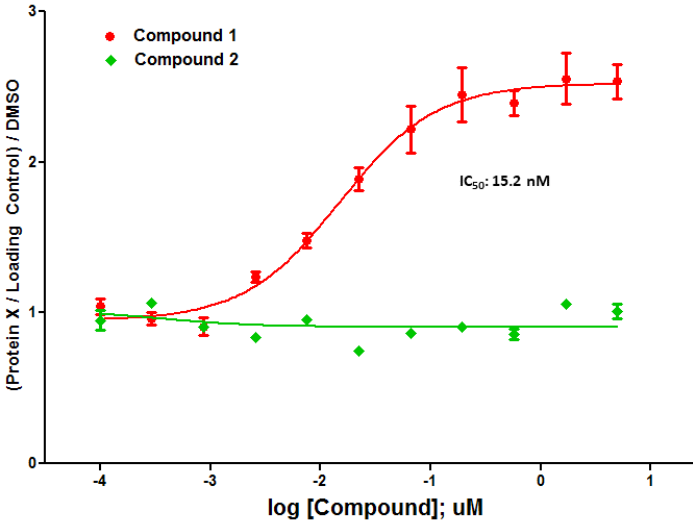

FIGURE 4. Dose-response curve comparing Compound 1 target engagement with Protein X versus the inactive Compound 2. Compound 1, IC_{50} = 15.2 nM ± 0.092; Compound 2, IC_{50} = N/A.

Conclusion

The development of effective novel therapeutics is often limited by the inability to monitor direct drug binding to the protein target inside cells. TSA is classically used to measure interactions between purified proteins and phenotypic activity-based assays for the monitoring of a broad cell response to a drug. Neither provide a means for directly studying drug–target engagement within a cell nor can these methods attribute the cell response to a specific drug–target interaction. CETSA-Wes is a novel approach that overcomes this challenge with the ability to prioritize compounds within a structure-activity relationship (SAR) optimization effort. In conclusion, we present CETSA-Wes as an improved target engagement assay that can accelerate compound optimization in SAR cascades.

References

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2. The cellular thermal shift assay for evaluating drug target interactions in cells, R Jafari, H Almqvist, H Axelsson, M Ignatushchenko, T Lundbäck, P Nordlund, D Martinez Molina, *Nature Protocols*, 2014; 9:2100-22.

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