PROTAC Drug Discovery : - Lessons from a CRO

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Introduction

PROteolysis-**TA**rgeting **C**himeras (PROTAC) is a new drug discovery approach that utilises the intracellular ubiquitin-proteasome system to induce targeted protein degradation. A chimeric small molecule is designed with two "warheads", one binds to the protein target of interest and the second binds to the E3 ligase. The two are joined by a flexible linker that brings the two into close proximity. The E3 ligase ubiquitinates the protein targeting it for degradation.

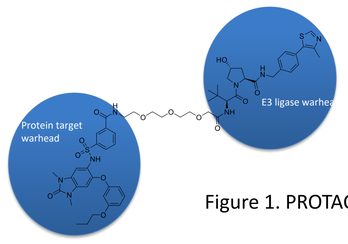

Figure 1. PROTAC molecule dTRIM24

This approach is receiving much attention in the field of targeted therapies particularly in oncology with the added potential of specifically eliminating targets from cells that have previously proved "undruggable".
TRIM24 is a multi-domain protein that has been characterised as a co-regulator of transcription. High levels of TRIM24 have been associated with oncogenesis in a wide range of cancer types. TRIM24 is also an E3-ubiquitin ligase that negatively regulates p53 by directly targeting p53 for ubiquitination. Aberrant expression of TRIM24 may promote tumour development and progression by co-activating oestrogen receptor functions and/or by negatively regulating p53 activity.

We have conducted studies with a number of PROTAC target molecules using the Wes™ system (ProteinSimple) for quantitation. Key to the quantitation is a good quality antibody and a suitable loading control, typically a protein that is not too highly expressed.

Methodology

Cells were plated between $4x10^5$ and $1.2x\ 10^6$ in 6 well dishes in growth media (DMEM, high glucose with pyruvate, glutamine and 10% FCS) and allowed to adhere overnight prior to treatment with PROTAC small molecules.
We have used HEK293T, HCT116 and MCF7 cells.
We have also used MV4-11 cells which are a suspension cell line seeded at $2x10^6$ in RPMI supplemented with 20% FCS and glutamine. Suspension cells were treated on the same day as plating in 6 well dishes.
For TRIM24, we have tested dTRIM24 (Tocris). The compound was dissolved in DMSO and then diluted in media with 10% DMSO at 100x concentration when added to the dish containing cells. The final concentration of DMSO was 0.1%. PROTAC compounds were incubated with cells for the desired incubation time, typically 4 or 24hrs.
Following compound treatment, cells were washed x 1 with cold PBS and then lysed in ice cold RIPA or NP-40 lysis buffer containing protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche). Lysates were cleared by centrifugation at 13,000rpm for 5mins at 4°C.
The amount of protein in each sample was measured using the BCA assay. A range of protein concentrations between 0.1 and 2.0mg/ml were used to optimise target detection and antibody dilution. TRIM24 primary antibody (Proteintech) was used at a dilution of 1 in 50 or 1 in 100. The vinculin antibody (CST) was also used at a 1 in 50 dilution.

Samples were prepared for running on the ProteinSimple Simple Western™ system by diluting to an appropriate concentration in the sample buffer and then boiling for 5mins. The Wes™ is an automated Western Blotting system that includes internal standards in each sample and combines sample separation with quantitation. The system is capillary based and data is presented as an electropherogram (peak trace) or as a "traditional lane view" as a virtual-blot image.

Cell line Selection and Positive control

There are a number of factors to consider when selecting a cell line for measurement of target knockdown. Good expression level of the target is required, but a high level of expression can make it difficult for the effect of the PROTAC molecule to be detected. Several cell lines may need to be compared for expression. We have found that a cell lysate concentration of 0.1- 0.5 mg/ml with a chemiluminescence signal of around 20,000 is ideal for measuring PROTAC.

HEK293T cells and HCT116 cells gave a similar peak profile and showed high level of TRIM24 protein (Mwt 158kDa). At high protein concentrations a number of peaks were detected but these were irrelevant at lower protein concentrations. Optimal protein concentration was between 0.1 to 0.25mg/ml. Varying the amount of antibody also reduced the background, whilst still detecting the TRIM24.

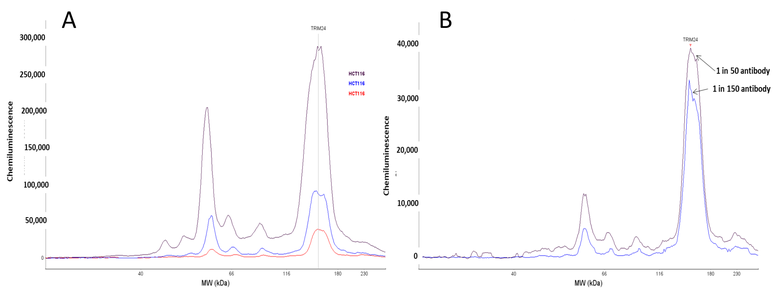

Figure 2. A) Peak signal traces for protein titration 2, 0.5 and 0.125mg/ml of HCT116 and B) 0.125mg/ml HCT116 lysate and 1 in 50 or 1 in 150 dilution of TRIM24 antibody

Having optimised the protein and antibody concentrations, the next step is to demonstrate knockdown with a positive control PROTAC molecule. The E3 ligase complement varies between different cell types and so it may be important to test the positive control in more than one cell background. PROTAC's often hook, hence the need to find the optimal incubation time and concentration to see degradation.

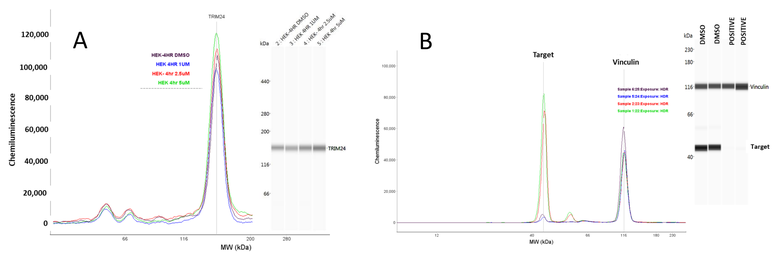

Figure 3. A) Effect of dTRIM24 measured at 3 concentrations after 4 hours. B) Effect of a PROTAC positive control on undisclosed target protein in HEK293T cells

Loading Control Optimisation

When optimising loading control antibodies we look for:

- Good separation between the target protein and the loading control protein
- Chemiluminescence values in a similar range
- No cross reactivity between the loading control and the primary antibody

The majority of loading controls e.g. tubulin or actin require very dilute antibodies to bring the chemiluminescence signal into the target range. This affects antibody saturation and will not accurately reflect small changes in protein concentration. Vinculin is a cytoskeletal protein associated with cell-cell and cell-matrix junctions with a molecular weight of 117kDa. We have found it to be less highly expressed than others e.g. tubulin. It therefore represents one of our standard loading controls for PROTAC. The vinculin antibody is used at a dilution of 1 in 50 and is therefore not saturated by the amount of vinculin present in the sample whereas the tubulin antibody has to be diluted 1 in 10,000 to achieve the same level of chemiluminescence.

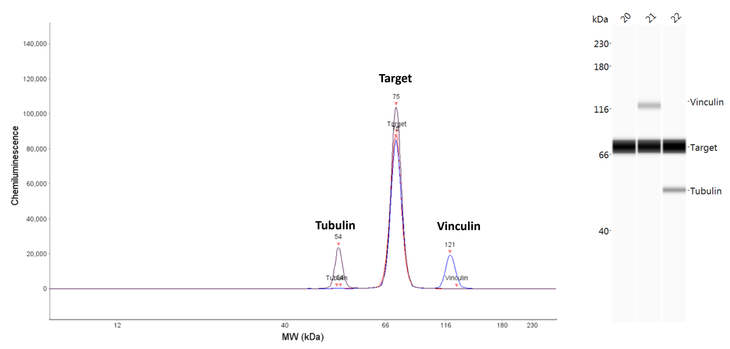

Figure 4 . Peak trace and Western blot "lane view" showing multiplexing of detection of target protein with either tubulin or vinculin loading control

PROTAC Screening

An undisclosed target was optimised as described in the earlier sections and a screen of PROTAC compounds was performed. Compounds were initially screened at three concentrations and at 4 and 24 hour time points. It is important to look for degradation at different concentrations as PROTAC effects can "hook" when the concentration of the PROTAC is higher than the DC_{50}.
It is also important to look at multiple time points as in some cases the cell compensates for the PROTAC effect by increasing target protein synthesis. When screening and looking for small changes(20-30%) in protein degradation, normalising to a good loading control is of key importance.

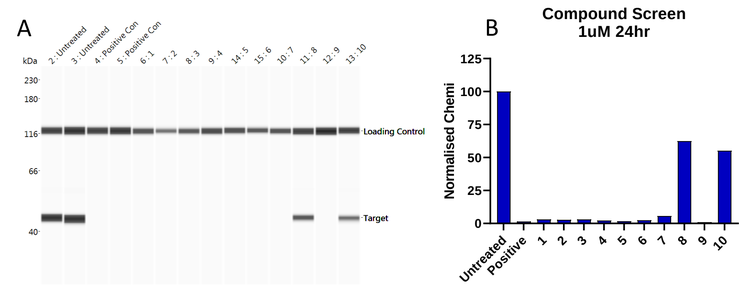

Figure 5. A) Simple Western™ of 10 PROTAC molecules multiplexed with vinculin as a loading control. B) Bar graph of target protein normalised to the vinculin loading control and expressed as a percentage relative to the untreated cells

PROTAC molecules are designed to complex with a specific E3 ligase. To date the most popular are VHL and Cereblon but there are ~600 E3 ligases in the genome. Different cell lines express a different complement of E3 ligases so it is essential to test PROTACs in more than one cell line.

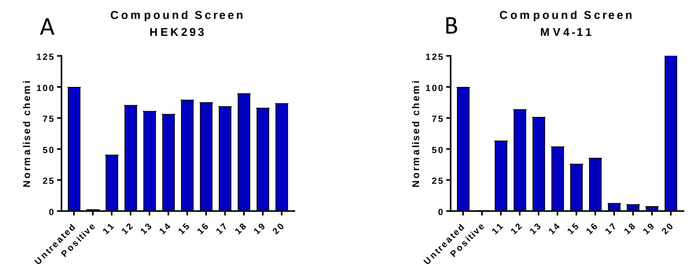

Figure 6. A) Effect of 10 PROTAC molecules on a target protein expressed in HEK293T cells measured by Simple Western™ B) Effect of the same 10 PROTAC molecules on target protein expressed in MV4-11 cells measured by Simple Western™

Active PROTAC molecules are screened as a concentration response to determine the concentration that achieves 50% degradation(DC_{50}).

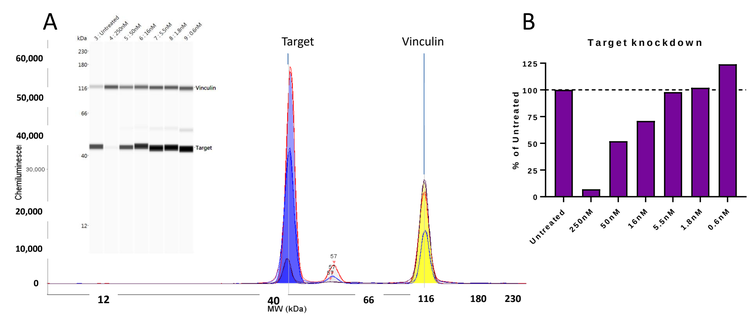

Figure 7. A) Concentration response to a PROTAC molecule shown in "lane view" and peak trace on Simple Western™ B) Bar graph of concentration response target knockdown normalised to loading control and expressed as % of untreated cells

Summary and Conclusion

PROTAC is receiving a lot of attention as a new approach to targeted therapy. It offers a number of advantages over traditional occupancy-based drugs and has the potential to be employed against so called "undruggable" targets.
As a small dynamic CRO receptive to the needs of the drug discovery community, we have established a screening strategy and methodology for accurately measuring protein degradation utilising the Simple Western™ system (ProteinSimple).
Key factors for accurately quantifying protein degradation include: -

- A cell line with target expression at a relatively low level
- A high quality primary antibody
- A loading control that is not overly-expressed and can be multiplexed with the target antibody
- A well-defined methodology for PROTAC drug treatment and sample preparation.

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