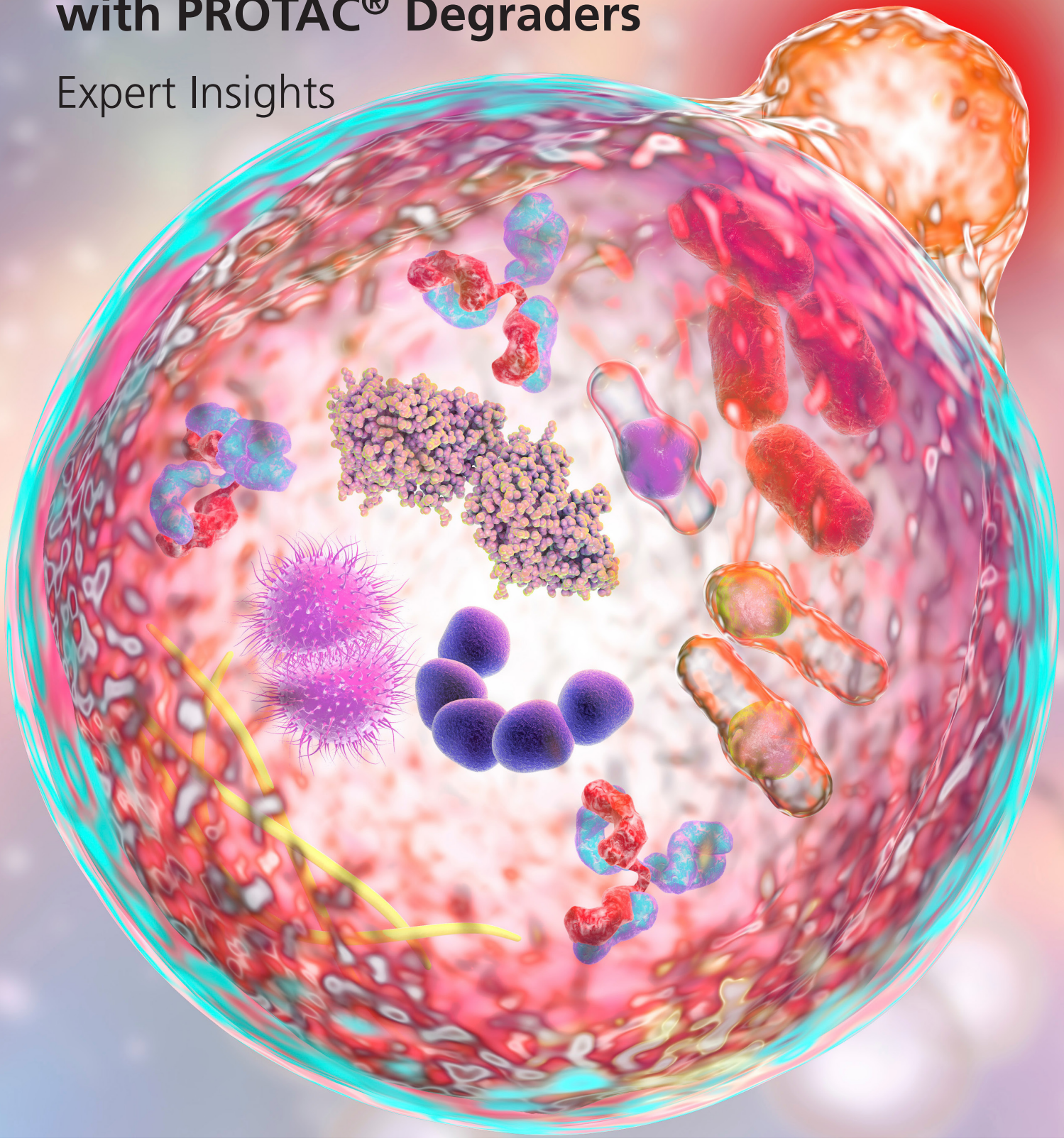
Exploring the Benefits of Targeted Protein Degradation with PROTAC® Degraders

Expert Insights



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Introduction

Dear Readers,

Protein homeostasis requires a balance between protein production and removal. To achieve this, cells rely on two main pathways: the ubiquitin-proteasome system (UPS) and autophagy-lysosomal degradation. UPS utilizes ubiquitin, a small 76 amino acid protein, to mark proteins for proteasomal degradation via a series of complimentary ubiquitin ligases (E1, E2, and E3). Autophagy works by sequestering proteins into double-membrane vesicles and delivering them to the lysosome for breakdown. Chemical biology tools are being developed to manipulate these native cellular processes and achieve targeted and tunable protein degradation - a concept called targeted protein degradation (TPD). TPD offers an alternative to conventional small molecule approaches and enables the broadening of the druggable space. This exciting concept has generated a great deal of interest in both the pharmaceutical industry and academia due to its potential drug discovery capabilities and has led to many compounds entering clinical trials.

There are two main classes of small-molecule protein degraders: monovalent (e.g., small-molecule hydrophobic tags and selective estrogen receptor degraders) and bivalent (e.g., proteolysis targeting chimeras [PROTACs]), which offer potential development opportunities for a new therapeutic modality.

This expert insight begins with a digest of a review article that outlines techniques and strategies for developing PROTAC degraders. It explains how PROTAC degraders work, provides resources and methodologies for their rational design and systematic optimization, and suggests approaches to developing them, specifically for researchers who may be new to the field.

Next, a whitepaper outlines the validation of targets for TPD using TAG degradation technology (dTAG), which provides a generalized strategy for degrading any intracellular protein of interest, without the need for a pre-existing ligand or PROTAC. This strategy can be particularly useful for exploring and validating new targets in degrader development programs due to its broad applicability.

An application note highlights the profiling of PROTAC-induced cereblon neosubstrate degradation using the automated immunoassay platform Simple Western™. Cereblon is an E3 ligase very commonly harnessed for PROTAC Degrader development programs.

Finally, in a publication spotlight on leading peer-reviewed research, learn how fully automated Simple Western™ instruments enable cutting-edge TPD research.

Through the methods and applications presented in this expert insight, we hope to educate scientists on new technologies and techniques for TPD. For more information, we encourage you to visit Bio-Techne to learn and explore more options for enhancing your research.

Róisín Murtagh
Editor at *Wiley Analytical Science*

Targeted Protein Degradation: Design Considerations for PROTAC Development

Adapted from Tran N.L. et al 2022

Proteolysis-targeted chimeras (PROTACs) are becoming a useful research tool and a novel strategy for developing therapeutic candidates. A potent selective PROTAC can help elucidate disease biology and target validation. Here, we provide resources and points to consider during the initial design assessment and validation of PROTACS.

Introduction

Proteolysis-targeted chimeras (PROTACs) comprise a major class of engineered small molecules that induce the degradation of novel substrates. PROTACs hijack the ubiquitin-proteosome system (UPS) by redirecting an E3-ligase to degrade a protein of interest (POI) that is not normally degraded by the UPS. A PROTAC is comprised of a POI binding ligand conjugated to a linker and a ligand that recruits an E3-ligase and supports access and modification of recruited POI, thereby increasing its degradation by the UPS.

Getting Started

Identification of a ligand for the selected POI is the first requirement for the development of a PROTAC. A search of compiled ligand information and recent research used to develop and characterize small molecule degraders can benefit from multiple public databases. DrugBank and ChEMBL compile the largest datasets of drug-related information. BindingDB compiles ligand-protein interaction data from additional databases such as patents.

An overview of the design, development, and validation of PROTACs is depicted in Figure 1.

Figure 1

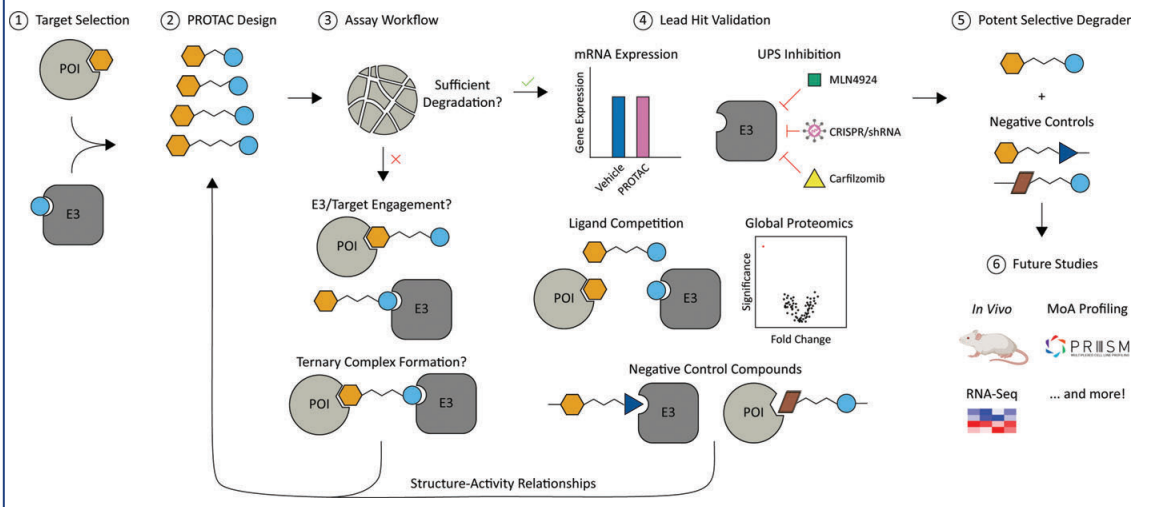

Fig. 1. Schematic of project workflow for the development of a typical PROTAC candidate.

Figure 2

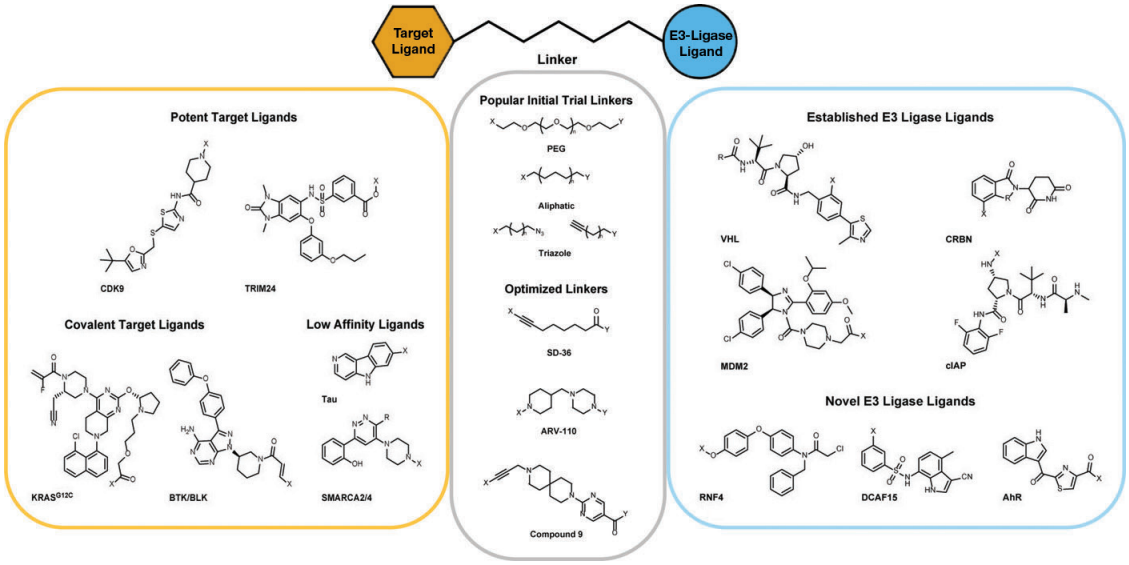

Fig. 2. Examples of the three components of PROTACS. The left panel shows examples of ligands that can recruit a target protein. Their binding ability for the target protein ranges from potent target ligands to low-affinity ligands. The middle panel shows examples of common linkers used in the construction of PROTACs. The right panel shows a variety of ligands for different E3 ligases.

The first step involves the selection of the target protein and its ligand and secondly, a binding molecule for an E3 ligase. In step 2, linkers are selected and several potential PROTAC molecules containing the POI ligand linked to the binder of an E3 ligase are engineered. Step 3 shows a typical assay workflow: It includes assays to assess the efficiency of degradation, E3/target engagement, and formation of ternary complexes. Iterative synthesis and testing of related PROTACs for this target protein can lead to increased efficiency of degradation. In step 4, lead hit validation involves the assessment of mRNA expression of target protein, UPS inhibition, ligand competition, as well as the fold change of target protein in global proteomics and reduced PROTAC lead by negative control compounds. In step 5, the specificity of the selected potent lead degrader is assessed with negative controls in competition assays. Further characterization of the selected PROTAC includes *in vivo* experiments, mechanism of action (MoA), and mRNA studies.

PROTAC Designs

PROTACs consist of three design elements: a ligand that selectively binds the target protein, a ligand that recruits the E3 protein, and a linker. Examples of each component are shown in Figure 2. In contrast to drug design, the ligand that recruits the target protein can bind transiently and can be a silent binder, i.e., it does not inhibit or stimulate the activity of the target protein. Ligands that bind with high affinity and are potent inhibitors also may work for PROTACs and may support efficient degradation. However, the role of degradation in the inhibition of a target protein by a PROTAC containing a high affinity binder of the target protein will need to be confirmed. Ligand binding to the target protein can be measured by a fluorescence polarization assay, isothermal titration calorimetry, and/or cellular thermal shift assay.

Several potent selective ligands for recruitment of E3 ligases (e.g., Von Hippel–Lindau protein E3 ligase complex (CRL2VHL), RNF4, Cereblon E3 ligase complex (CRL4CRBN)), have been used in developing PROTACs. Interestingly, the activity of a PROTAC on a specific target protein may be improved by switching the ligand to recruit a different E3-ligase.

The composition, flexibility, solubility, total polar surface area, and length of the linker can affect ternary complex formation and cell permeability. Thus, a panel of PROTACs for a specific target protein and E3-ligase combination should be investigated with different linkers for their efficiency of degradation activity.

Assay Workflow

After the construction of the initial PROTAC candidates, measuring target degradation can identify the active degraders. Verifying the mechanism of action (see Lead Compound Validation) of active degraders is the next step. If no active degraders were identified, then subsequent assays to identify the inefficient steps such as cell permeability, target engagement, and ternary complex formation can inform the iterative redesign and testing process.

Degradation

The most common assay for assessing the efficiency of degradation is quantification of the target protein levels by the western blot. Other methods such as automated capillary-based immunoassays (Simple Western, Bio-Techne) have also been employed that offer higher throughput, better reproducibility and quantitation. This quantitative western-like method can be used to compare the degradation efficiency of different PROTAC constructs. Assays should be performed at both a short (between 4 and 8 hr) and a long (12 to 24 hr) time point. After elucidating an optimal time point, researchers can perform a dose-response curve to identify the concentration yielding the max degradation concentration (DC_{max}) and the concentration that yields 50% degradation (DC_{50}).

Other strategies involve tagging the target protein with a luminescent or fluorescent marker and tracking the loss of signal over time.

E3-ligase and Target Engagement

When sufficient target protein is available, cell-free biochemical assays can provide direct evidence of target binding and E3-ligase attachment. For example, fluorescence polarization is commonly used for screening and measuring binding affinities of PROTACs and monovalent constructs.

Cell membrane permeability can affect degrader activity *in vivo.* Assays to measure cell membrane permeability include Parallel Artificial Membrane Permeability Assays (PAMPA), Caco-2 cell platforms, and competition assays using a well-characterized degrader or a fluorescent tracer. Binary protein-ligand interactions can be measured in cellular competition platforms using permeabilized cells.

Ternary Complex Formation

Assessment of the efficiency of ternary complex formation aids in confirming the MoA of the PROTAC and determining whether optimization of this step can increase its effectiveness. Several assays such as co-immunoprecipitations, BRET assays, or affinity pull-downs followed by western blots or automated capillary-based immunoassay (e.g., Simple Western, Bio-Techne) can assess ternary complex formation. Additional assays include time-resolved Förster resonance energy transfer, surface plasmon resonance, size exclusion chromatography, and luminescence proximity strategy such as NanoBiT.

Measurement of ternary complex formation in cells uses inhibitors of UPS such as carfilzomib, bortezomib, MG132, or the NAE inhibitor MLN4924 to inhibit the degradation of the target protein.

Lead Compound Validation

The MoA and selectivity of the potent candidate PROTAC should be established before investigating efficacy in biological systems. PROTAC treatment of cells should not affect the mRNA levels of the target protein. Competition assays with ligands for parental E3 ligase or target protein should reduce ternary complex formation and degradation of the target protein. Negative control compounds that fail to recruit E3 ligase or target protein due to altered stereochemistry or steric bulk can help validate target degradation via E3-ligase.

Global proteomics can confirm lower target protein levels and identify its downregulated substrates, thereby elucidating the pathways affected by the target protein.

Future Considerations

The same PROTAC molecule has yielded different degradation profiles in different cell lines that did not correlate with E3-ligase levels. Thus, some cellular studies of the PROTAC should be performed in cells from the target tissue. Furthermore, *in vivo* experiments may require additional optimization of the distribution profile, pharmacokinetic, and pharmacodynamic properties of the PROTAC for the development of a therapeutic entity. The databases and resources described here provide a starting point but are not exhaustive.

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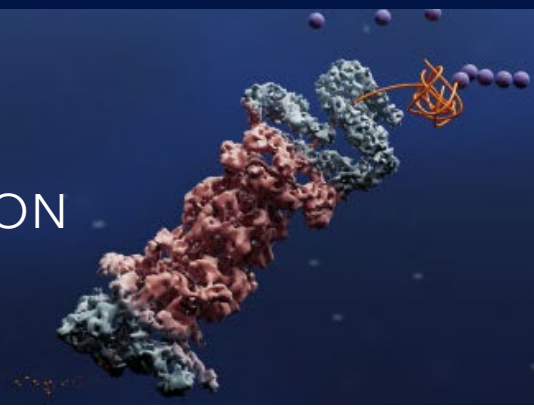
WHITE PAPER

VALIDATING TARGETS FOR TARGETED PROTEIN DEGRADATION USING dTAG – A COMPREHENSIVE WORKFLOW SOLUTION



INTRODUCTION

Degraders and the field of Targeted Protein Degradation offer a mechanistically differentiated way to modulate target proteins using small molecules. While a small molecule inhibitor will block or modulate a specific protein domain or function (for example an enzymatic role), a Degrader will knockdown the entire protein, removing all possible functions. Inhibition therefore does not always phenocopy degradation and careful assessment of potential targets for new Degrader development programs is vital. Target validation is therefore an important stage of the Degrader development project workflow.

TAG degradation technology offers a generalizable strategy to degrade, in principle, any intracellular protein of interest (POI). Its key benefit is that it does not rely on the pre-existence of a ligand or PROTAC® for the POI. The broad applicability that it offers makes this a useful strategy for exploration and validation of targets, particularly in the context of new Degrader development programs.

The dTAG degradation mechanism is illustrated in **FIGURE 1**. The protein of interest is expressed as a fusion protein with a dTAG domain (identity: $FKBP12^{F36V}$), either via transgene expression or CRISPR-mediated locus-specific knock-in (KI). The dTAG degrader molecule recruits the fusion protein for ubiquitination by an E3 ligase, which targets it for destruction by the proteasome. Several dTAG Degraders and matched pair negative controls are available, that recruit different E3 ligases e.g. CRBN: dTAG-13 (Tocris Cat. No. 6605) and dTAG-7 (Tocris Cat. No. 6912) or VHL: $dTAG^{V}$-1 (Tocris Cat. No. 6914). A comparison of dTAG methodology compared to more traditional genetic knock-down/knock-out strategies is given in **TABLE 1**. Key advantages of the dTAG platform include the ability to tune the extent of protein knockdown by varying the Degrader dose, and the more rapid onset of action (kinetics) for studying 'fast biology'. In addition, dTAG Degraders can be washed out of cell culture media, reversing their effect.

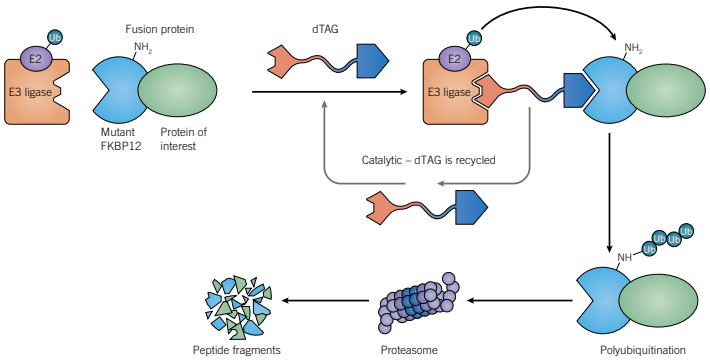


FIGURE 1. Schematic showing the mode of action of dTAG Degraders. A protein of interest is expressed as a fusion with a "TAG" protein. For the dTAG system the protein of interest is tagged with F36V single-point mutated FKBP12. The dTAG Degrader initiates the formation of a ternary complex between an E3 ubiquitin ligase and the fusion protein which results in polyubiquitination of the target protein, its recognition by the proteasome and subsequent degradation of the entire protein. dTAG molecules act catalytically, repeatedly engaging and directing the ubiquitination of target molecules.

	DOSE TUNEABILITY	EFFICACY	REVERSIBILITY	KINETICS	SELECTIVITY
TAG Degradation Platform (dTAG/aTAG)	***	****	****	***	****
Gene knockout e.g. CRISPR/Cas9	*	****	*	*	****
Gene knockdown e.g. RNAi	*	***	*	*	**

TABLE 1. Comparison between dTAG Degradation and genetic knock-down/knock-out strategies.

dTAG WORKFLOW

A typical dTAG workflow is outlined in **FIGURE 2**. The first step is to generate the POI·dTAG domain fusion, for example by CRISPR-mediated knock-in at the gene of interest (GOI). The POI-dTAG fusion protein will be expressed at the level of endogenous, unmodified POI. In contrast, lentivirus-mediated gene engineering will result in a non-physiological overexpression of the fusion protein. Our Gene Engineering Services can provide custom cell lines with both *N*-terminal and *C*-terminal dTAG knock-in, knock-out of the wild type GOI, as well as multiplex knock-out or knock-in of additional genes.

The next step is to perform dTAG Degrader treatments with the engineered cell line. The response to dTAG Degrader treatment will be target and cell line specific, so it is recommended to first optimize the treatment regime. The key variables to explore are:

THE E3 LIGASE RECRUITED BY THE dTAG DEGRADER

Tocris offers dTAG Degraders recruiting two different E3 ligases, CRBN and VHL. Some targets are preferentially degraded by one E3 ligase over another, so trying both options is recommended. CRBN-recruiting Degraders: dTAG-13 (Tocris Cat. No. 6605) and dTAG-7 (Tocris Cat. No. 6912). VHL-recruiting Degrader: dTAG^{V}-1 (Tocris Cat. No. 6914).

OPTIMAL DOSE

We recommend performing an initial dose-response experiment with treatments between 5-500 nM and a 0 nM negative control.

INCUBATION TIME

A 24-hour incubation time with the dTAG Degraders is usually appropriate, but shorter or longer incubation times may provide a different level of response.

Finally, it is good scientific practice to perform negative control experiments. Matched-pair negative controls are available for both dTAG-13 (dTAG-13-NEG, Tocris Cat. No. 6916) and dTAG^{V}-1 (dTAGV-1-NEG, Tocris Cat. No. 6915). These control compounds have the same chemotype as the active Degrader with a small modification to remove E3 ligase binding capability.

Profiling the knock-down response to dTAG Degrader treatment is important prior to assessing downstream pharmacology. There will be target-specific variation in response to dTAG Degrader treatment in addition to potential variation between *N*-term versus *C*-term KI response. Gaining a meaningful understanding of the knock-down response involves running dose-response experiments with a serial dilution series of dTAG Degraders. To do so, an analytical method is needed with two critical features: (1) accurate quantification of protein abundance, and (2) throughput that is amenable to analyse replicate samples at each concentration. When it comes to meeting these criteria, the traditional Western blot is severely limited as it has a relatively low throughput, and it is only semi-quantitative at best. Instead, Simple Western™ assays are fully automated immunoassay platforms that generate reproducibly quantifiable data, and they have the high throughput necessary to comprehensively profile the knock-down response. Following sample preparation, up to 96 samples may be analysed overnight in a single run without user intervention. Furthermore, Simple Western is an open platform and Western blot antibodies may be used to detect target proteins following validation. This enables detection of target-specific variation to dTAG Degrader treatment in addition to monitoring the variation between *N*-term and *C*-term KI response.

Here we show two case studies illustrating the Bio-Techne dTAG workflow. We present data to demonstrate the value of testing both CRBN- and VHL-recruiting dTAG Degraders for each POI target as well as the use of negative control Degraders. We also show the utility of using a FKBP12 antibody for detecting POI·dTAG fusion molecules, allowing for a more simplified procedure when probing multiple targets.

FIGURE 2. TAG Degradation Platform workflow. Bio-Techne offers products and services across the TAG Degradation Platform Workflow including Custom knock-in TAG Cell Lines, dTAG and aTAG Degraders, as well as Simple Western instruments and validated antibodies for western blotting to measure target knockdown.

MATERIALS AND METHODS

CELL LINE GENERATION

Cells were transfected with Cas9 Nuclease ribonucleoprotein (RNP) complexes targeting the GOI and single stranded DNA template containing dTAG ($FKBP12^{F36V}$) and the edits of interest surrounded by flanking arms matching the genomic region of interest. After confirmation of the knock-in event at the population level with junction PCR, cells were cloned and expanded for characterization. Clones containing dTAG-GOI fusion were verified for a homozygous KI via PCR of the edited region and Sanger Sequencing (FIGURE 3).

dTAG TREATMENTS

Cells were cultured in a 6-well plate with RPMI-1640 Media (Available from R&D Systems Cat. No. M30150) supplemented with 10% Premium grade FBS (available from R&D Systems Cat. No. S11150) and 1% Penicillin-Streptomycin (available from R&D Systems Cat. No. B21210), reaching 50% confluency after 3 days. 24 hours after stimulation to induce target protein expression, dTAG Degraders were added as follows to generate 8 samples per KI:

dTAG-13 (Tocris Cat. No. 6605): 5 nM, 50 nM and 500 nM

$dTAG^V$-1 (Tocris Cat. No. 6914): 5 nM, 50 nM and 500 nM

dTAG-13-NEG (Tocris Cat. No. 6916): 500 nM

$dTAG^V$-1-NEG (Tocris Cat. No. 6915): 500 nM

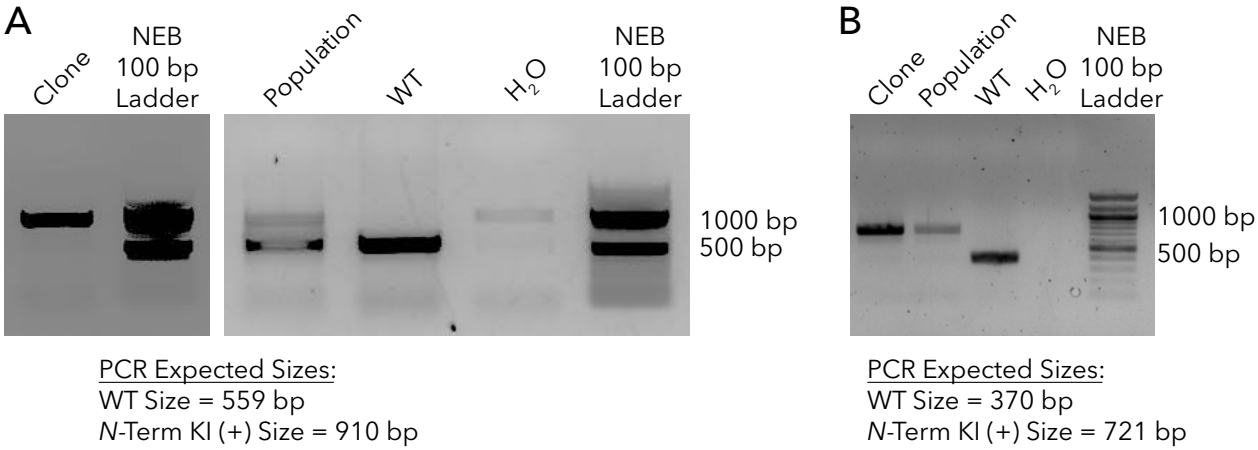
Wild-type samples with the same stimulation were also grown as a control. Cells were harvested after an additional 24 hours of growth. One well of a 6-well plate was used for each sample. Cells were harvested from the plates, washed once with PBS, then flash frozen in liquid nitrogen. Cell pellets were held at -80 °C until time of lysis. Cells were lysed using Lysis Buffer 16 (R&D Systems Cat. No. 895935) with 1 x protease inhibitor (PPI). Cold lysis was performed as follows. 100 µL ice cold Lysis Buffer 16 with 1 x PPI was added to the cell pellets. Cells were vortexed for 10 seconds, then incubated on ice for 15 minutes. Tubes were vortexed again and incubated for an additional 15 minutes on ice.

SIMPLE WESTERN

The final experimental conditions for Simple Western analysis are shown in TABLE 2. All experiments were performed according to manufacturer's instructions on Peggy Sue, a high-throughput Simple Western instrument that can analyze up to 96 samples in one automated run. The samples were diluted with 0.1X sample buffer to the concentration listed in TABLE 2 prior to analysis.

TARGET	ANTIBODY	VENDOR	AB. DIL.	LYSATE CONC. (mg/mL)
1	1	Novus Biologicals	1:33	0.25
2	2	Cell Signaling Technologies	1:10	0.25
FKBP12.6	MAB4174	R&D Systems	1:10	0.8

TABLE 2. Experimental conditions for Simple Western analysis.



PCR Expected Sizes:
WT Size = 559 bp
N-Term KI (+) Size = 910 bp

PCR Expected Sizes:
WT Size = 370 bp
N-Term KI (+) Size = 721 bp

FIGURE 3. Gel electrophoresis images from PCRs performed on genomic extractions from the clones used in the experiment. The clones derived from single cells isolated from the transfected populations were tested via a PCR reaction for homozygous KI of the dTag on the *N*-terminus of Gene 1 (**A**) and Gene 2 (**B**). For gene 1, a homozygous KI results in a single PCR band at 910 bp as shown in (**A**), whereas the WT does not contain the KI of interest and results in a single band at 559 bp. For Gene 2, a homozygous KI results in a single PCR band at 721 bp as shown in (**B**), whereas the WT does not contain the KI of interest and results in a single band shown at 370 bp. Gel bands were extracted and sent for Sanger sequencing to confirm correct KI sequence of the dTAG (data not shown).

RESULTS

TARGET 1 – *N*-TERM KNOCK-IN COMPARING dTAG DEGRADER TREATMENTS

A cell line expressing an *N*-term knock-in of the FKBP12^{F36V} domain with Target 1 was generated according to the methods described. The sensitivity of this fusion protein to dTAG Degrader treatment was evaluated by performing exploratory dose-response experiments and subsequently analyzed in triplicate on Simple Western with an anti-Target 1 antibody. The results are shown in **FIGURE 4**. No loading control is shown since in each case equal amounts of cell lysate concentrations were loaded. Treatment with the CRBN-recruiting dTAG Degrader, dTAG-13 resulted in dose-dependent degradation of Target 1, with maximum observed degradation at the highest dose tested (500 nM, **FIGURE 4A**). Treatment with the corresponding negative control (dTAG-13-NEG) appears, in this case, to have reduced the target protein level slightly compared with the 5 nM dTAG-13 treatment but not compared with the optimum dose of dTAG-13 tested (500 nM). A sample of wild-type (WT) cell line was also tested for comparison, demonstrating the anticipated shift in MW (12 kDa) between WT Target 1 and the dTAG·Target 1 fusion (data not shown).

FIGURE 4B provides the corresponding dataset where treatments were performed with the VHL-recruiting dTAG Degrader, dTAG^{V}-1. The corresponding negative control in this case was dTAG^{V}-1-NEG. As with dTAG-13 treatment, dose-dependent degradation was observed with maximum observed degradation at the highest dose tested (500 nM). The quantified data (**FIGURE 4**, right panel) reveals a difference in sensitivity for Target 1 between the two dTAG Degraders tested. In this case, dTAG^{V}-1 would be the preferred Degrader to use for subsequent experiments because Target 1 appears to be more sensitive to knock-down following treatment with dTAG^{V}-1 compared to dTAG-13.

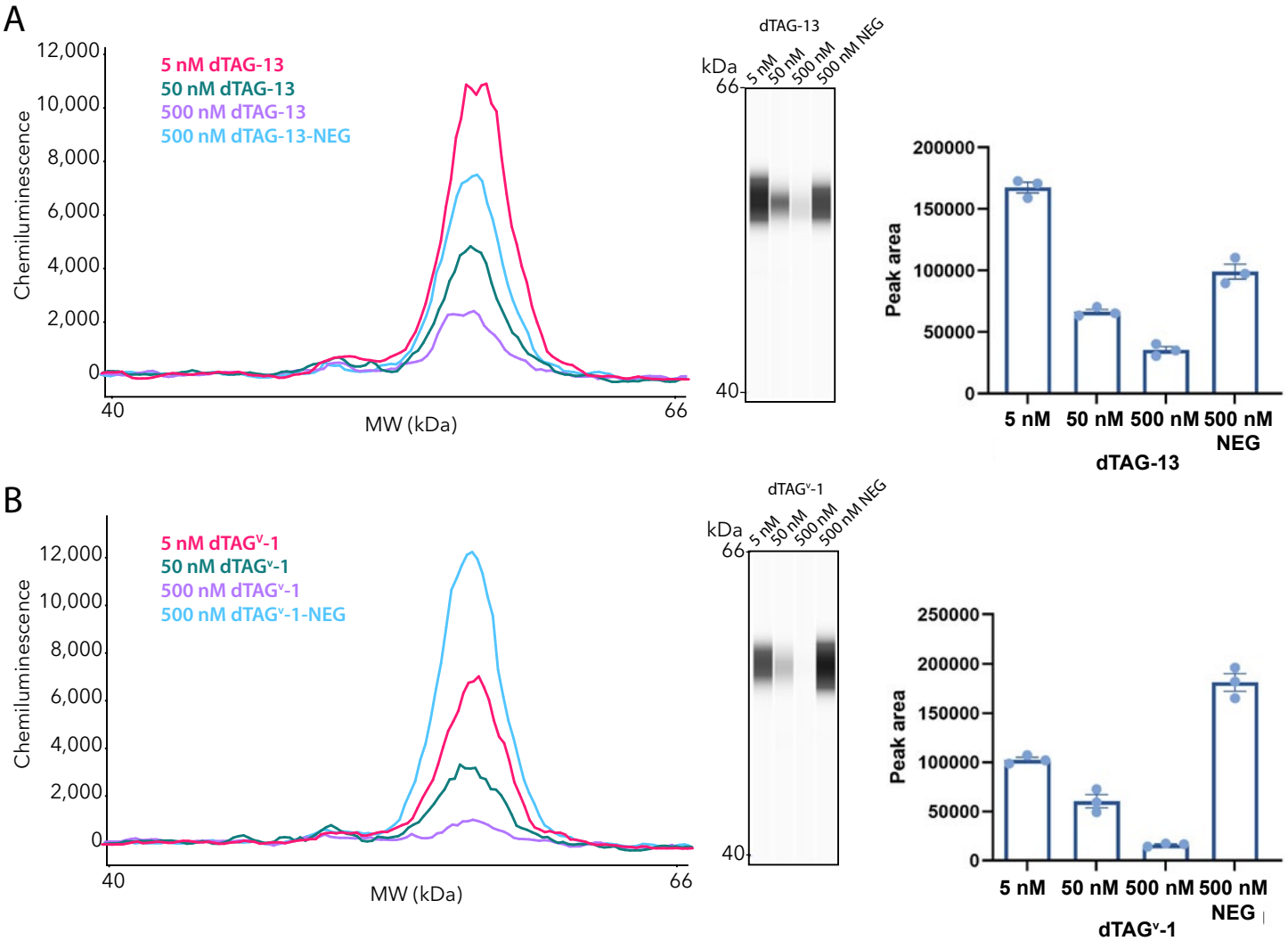


FIGURE 4. Simple Western data from a dTAG·Target 1 KI cell line following treatment with either dTAG-13 (A) or dTAG^{V}-1 (B) and detection with an anti-Target 1 antibody. The following matched pair negative control degraders were also used as controls: dTAG-13-NEG (**A**) and dTAG^{V}-1-NEG (**B**). Degrader treatments are color-coded as indicated by the key top left. Left panel: electropherogram data, Middle panel: lane-view data, Right panel: graphs showing quantitative data collected by integrating the peaks in the electropherograms. Bars show average of 3 measurements; individual data points and error bars are shown.

TARGET 2 – *N*-TERM VERSUS *C*-TERM KI AND COMPARISON OF dTAG DEGRADER TREATMENTS

Cell lines expressing both *N*-term and *C*-term knock-in of the FKBP12^{F36V} domain with Target 2 were generated according to the methods described. The sensitivity of this fusion protein to dTAG Degrader treatment was evaluated by performing exploratory dose-response experiments and subsequently analyzed in triplicate on Simple Western with an anti-Target 2 antibody. The results for the *N*-term KI are shown in FIGURE 5. Degradation of the *C*-term KI for this target was unsuccessful in this case (data not shown), which highlights the importance of testing both *N*- and C-term knock ins for each target of interest. No loading control is shown since in each case equal amounts of cell lysate concentrations were loaded. Treatment with the CRBN-recruiting dTAG Degrader, dTAG-13 resulted in dose-dependent degradation of Target 2, with maximum observed degradation at the highest dose tested (500 nM, FIGURE 5A). Treatment with the corresponding negative control (dTAG-13-NEG) did not result in significant knock-down of the target. A sample of wild-type (WT) cell line was also tested for comparison, demonstrating the anticipated shift in MW (12 kDa) between WT Target 2 and the dTAG·Target 2 fusion (data not shown).

FIGURE 5B provides the corresponding dataset where treatments were performed with the VHL-recruiting dTAG Degrader, dTAG^{V}-1. The corresponding negative control in this case was dTAG^{V}-1-NEG. As with dTAG-13 treatment, dose-dependent degradation was observed with maximum observed degradation at the highest dose tested (500 nM). The quantified data (FIGURE 5, right panel) shows similar sensitivity to knock down with the two dTAG Degraders tested, with slightly enhanced sensitivity to dTAG^{V}-1 treatment.

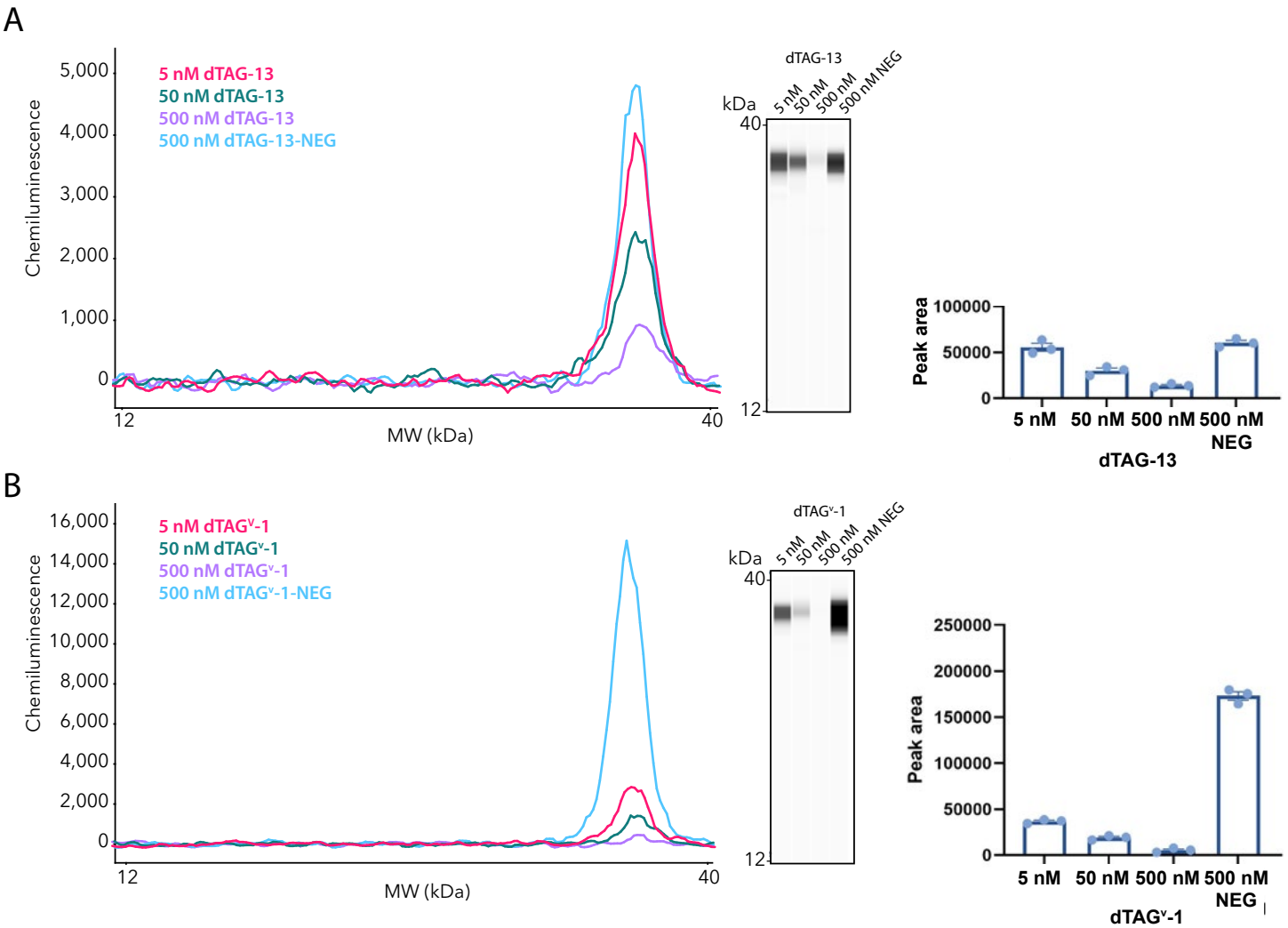


FIGURE 5. Simple Western data from a dTAG·Target 2 KI cell line following treatment with either dTAG-13 (A) or dTAG^{V}-1 (B) and detection with an anti-Target 2 antibody. The following matched pair negative control degraders were also used as controls: dTAG-13-NEG (A) and dTAG^{V}-1-NEG (B). Degrader treatments are color-coded as indicated by the key top left. Left panel: electropherogram data, Middle panel: lane-view data, Right panel: graphs showing quantitative data collected by integrating the peaks in the electropherograms. Bars show average of 3 measurements; individual data points and error bars are shown.

DETECTING DEGRADATION WITH A 'dTAG' ANTIBODY

Antibody validation is a pre-requisite for successful western blotting. In the examples shown previously, the antibody used has reactivity for the target protein, necessitating a different antibody and separate validation for each target protein. A more streamlined approach would be to use an antibody that recognized the 'dTAG' domain, FKBP12^{F36V}. Data in **FIGURE 6** was generated using the same samples used to generate the data in **FIGURE 5B**, run with an anti-FKBP12 antibody (MAB4174, R&D Systems) instead of an anti-Target 2 antibody. The electropherogram data (**FIGURE 6**, left panel) demonstrates that this antibody has reactivity both for WT FKBP12 and the single point mutant used for the dTAG-domain, FKBP12^{F36V}. This dual reactivity can be useful since the WT FKBP12 peak serves as an internal control. The quantified data (**FIGURE 6**, right panel) aligns with the data generated using an anti-Target 2 antibody (**FIGURE 5B**, right panel), as expected, demonstrating that either antibody could be used in this case to detect degradation.

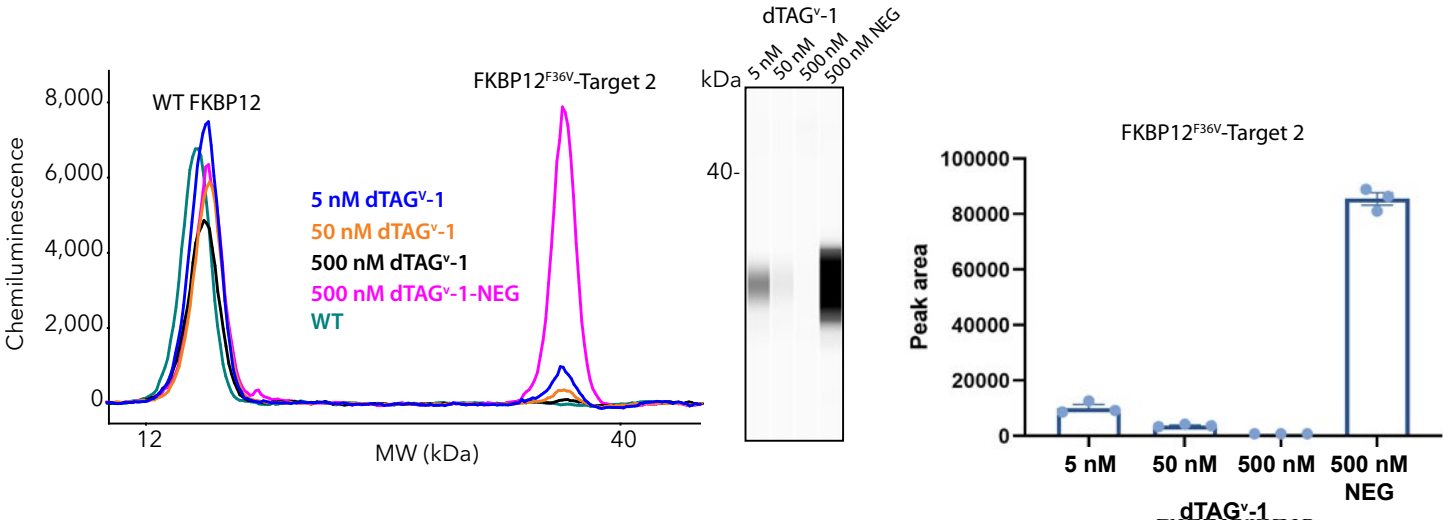


FIGURE 6. Simple Western data from a dTAG·Target 2 KI cell line following treatment with dTAG^{V}-1 and detection with an anti-FKBP12 antibody. dTAG^{V}-1-NEG was used as a negative control. Degrader treatments are color-coded as indicated by the key in the left panel. Left panel: electropherogram data showing both the peak corresponding to WT FKBP12 and the peak corresponding to the FKBP12^{F36V}·Target 2 fusion protein. Middle panel: lane-view data showing the FKBP12^{F36V}·Target 2 band only, Right panel: quantitative data collected by integrating the peaks in the electropherogram. Bars show average of 3 measurements; individual data points and error bars are shown.

CONCLUSIONS

We present data to highlight a full dTAG workflow solution, from custom KI cell lines for a protein of interest, to different dTAG Degraders available for treatment and subsequent quantitative characterization using an automated high throughput western blotting platform, Simple Western. We also show preliminary data to demonstrate that in some cases it is possible to detect dTAG degradation using an antibody that recognizes the TAG domain ($FKBP12^{F36V}$), rather than that individual proteins of interest. Initial work should be performed, however, to ensure that the anti-FKBP12 antibody detects the "dTAG" domain when fused with a given POI, to ensure that the anti-FKBP12 targeted epitope has not been blocked during the generation of the dTAG·POI fusion. Finally, we highlight best practise for the use of the dTAG degradation platform, including trying both *N*- and *C*-term KI, testing both CRBN- and VHL-recruiting dTAG Degraders to select the most effective compound for a given target, and the use of negative control dTAG Degraders.

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APPLICATION NOTE

AUTOMATED PROFILING OF PROTAC®-INDUCED CEREBLON NEOSUBSTRATE DEGRADATION USING SIMPLE WESTERN

EXPANDING THE DRUGGABLE PROTEOME

Many targets implicated in diseases are refractory to knockdown by traditional therapeutics like antibodies and small molecules. PROTAC® (PROteolysis TArgeting Chimeras) Degraders (hereinafter 'Degraders') are heterobifunctional small molecules that harness the ubiquitin-proteasome system to selectively degrade target proteins within cells. They represent an exciting new modality, repurposing small molecule chemical tools to achieve selective degradation (knockdown) of target proteins. Moreover, they have the potential to expand the 'druggable proteome', since they can be used to degrade proteins that, although bound, are not effectively inhibited by small molecules.

Degraders are modular in design and consist of three covalently linked components, an E3 ubiquitin ligase ligand, a chemical linker, and a 'warhead ligand' for a target protein of interest. Their mechanism of action induces the formation of a ternary complex between the E3 ligase, Degrader, and target protein. This effectively hijacks the E3 ligase to direct ubiquitination of a chosen target protein. Polyubiquitinated target proteins are then degraded into peptide fragments via the 26S proteasome (**FIGURE 1**).

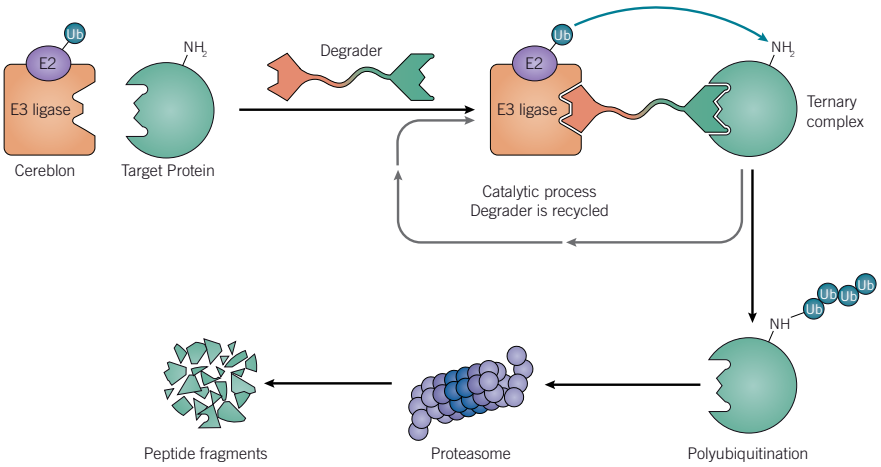

FIGURE 1. Schematic showing the catalytic mode of action of heterobifunctional Degrader molecules. Degraders initiate the formation of a ternary complex between an E3 ubiquitin ligase, in this case cereblon, and a target protein, resulting in polyubiquitination of the target protein and subsequent degradation by the proteasome. Adapted from Tinworth et al. *Med. Chem. Comm.*, 2016; **7**: 2206-2216.

Cereblon (CRBN, R&D Systems Cat. No. E3-650-025) is an E3 ligase very commonly harnessed for PROTAC® Degrader development programs. It can be effectively recruited by the 'IMiD' class of small molecules, exemplified by thalidomide, lenalidomide, and pomalidomide. The pharmacology of the IMiDs themselves is complex and it is well known that these compounds can act as 'molecular glues', inducing the formation of ternary complexes between cereblon and 'neosubstrate' proteins that are subsequently ubiquitinated and degraded via the proteasome.[1-4] This neosubstrate recruitment and degradation can still occur when the IMiD small molecules are converted into Degraders by attachment of a linker moiety and warhead ligand.

HIGH THROUGHPUT SIMPLE WESTERNS FOR RAPID AND QUANTITATIVE CHARACTERIZATION OF DEGRADATION CONSTANT (DC_{50}) VALUES

To understand the effectiveness of specific Degraders, it is important to profile Degrader neosubstrate knockdown by measuring degradation constant (DC_{50}) values, or the concentration of Degrader that induces 50% degradation of the target protein. To characterize the efficacy of Degrader molecules, researchers often run dose-response curves by way of traditional Western blotting methods. But the lengthy, manual workflow and resulting low reproducibility make it an unreliable approach for the determination DC_{50} values. Instead, the ideal solution would be highly reproducible, allow for easy quantitation, and have a short time to results. The automated immunoassay platform known as Simple Western™ is just that, making it ideal for studying Degraders. Simple Western assays can generate up to 96 data points in a single overnight run, all in an automated fashion, resulting in highly reproducible and quantifiable data. This enables Simple Western to generate quantitative DC_{50} curves with triplicate data points overnight -- something that would take days, if not weeks, with traditional Western blotting. Here, we present data showing the power of using automated Simple Western platforms to screen panels of Degrader and IMiD compounds in order to quantify degradation activity. In this study, we demonstrate the time savings achieved by automating these large screens as well as Simple Western's ability to accurately quantify DC_{50} and D_{max} values for specific Degraders and IMiDs.

MATERIALS AND METHODS

The reagents used in this study are listed in **TABLE 1**.

REAGENT	VENDOR	PART NUMBER
12-230 kDa Peggy Sue Separation Module	ProteinSimple	SM-S001
Anti-Rabbit Detection Module for Jess, Wes, Peggy Sue or Sally Sue	ProteinSimple	DM-001
CRBN-6-5-5-VHL	Tocris	6948
TL 12-186	Tocris	6524
Pomalidomide	Tocris	6302
Lenalidomide	Tocris	6305
Ikaros/IKZF1 Antibody	Novus Biologicals	NBP2-38242
Aiolos/IKZF3 Antibody	Novus Biologicals	NBP2-16938

TABLE 1. Reagents used in this study. Both antibodies were diluted 1:50 (saturating) in Antibody Diluent 2.

The cell line used in this study was RPMI 8266 and the neosubstrates (degraded proteins of interest) analyzed were IKZF1 (Ikaros) and IKZF3 (Aiolos). The IMiDs tested were pomalidomide, lenalidomide, and the Degraders tested were CRBN-6-5-5-VHL and TL 12-186. Samples were treated with IMiDs or Degraders for 24 hours to determine dose-response at the following concentrations: 0 µM (DMSO only), 0.1 µM, 1 µM, 5 µM and 10 µM. Each treatment was performed in triplicate to determine reproducibility. The lysates were prepared for Simple Western analysis using 5X Master Mix under reducing conditions for 5 mins at 95 °C at concentrations of 1.0 mg/mL for the IKZF1 target and 0.1 mg/mL for the IKZF3 target. All samples were analyzed on Peggy Sue™, a Simple Western instrument from ProteinSimple.

PROFILING CEREBLON NEOSUBSTRATE DEGRADATION WITH SIMPLE WESTERN

TL 12-186 is a tool Degrader consisting of pomalidomide and a promiscuous kinase-inhibitor warhead ligand, which is used to evaluate the degradable kinome[5]. In agreement with published data,[5] we observed pronounced degradation of IKZF1 and IKZF3 by TL 12-186 (**FIGURE 2**, panels A and C), with a maximum level of degradation (D_{max}) of 88.47% and 98.83% respectively (**TABLE 2**). While Simple Western assays generate electropherograms as the default data view, Simple Western data may also be viewed in a virtual lane view, which resembles the results generated by a traditional Western blot, as shown in **FIGURE 2**, panels A and C.

CRBN-6-5-5-VHL is a Degrader that targets cereblon itself for degradation by recruiting another E3 ligase, VHL to affect the ubiquitination. Cereblon is recruited here by the warhead ligand, pomalidomide. In MM1S cells, this Degrader does not induce degradation of IKZF1 at concentrations up to 10 µM with a 16 hour treatment.[6] However, in the RPMI 8266 cells used here, we observed some IKZF1 degradation at higher concentrations (**FIGURE 2B**). This illustrates cell-line variations in neosubstrate degradation, which is not unprecedented.[2] CRBN-6-5-5-VHL has a DC_{50} value of 1.5 nM for its primary target, cereblon, so at the relevant concentrations for standard use, IKZF1 would not be considered an off-target. CRBN-6-5-5-VHL induced pronounced degradation of IKZF3 (**FIGURE 2D**), with a D_{max} of 79.35% (**TABLE 2**). Finally, the IMiDs, pomalidomide and lenalidomide, induced degradation of IKZF1 and IKZF3 (**FIGURE 2**), in agreement with previous studies.[2-4,7] Published results suggest that IKZF1 and IKF3 are more sensitive to pomalidomide than lenalidomide[4], in agreement with our data (**FIGURE 2**, **TABLE 2**).

	IKZF1		IKZF3	
Degrader/IMiD	DC_{50}	D_{max}	DC_{50}	D_{max}
TL 12-186	<0.1 µM	88.47%	<0.1 µM	98.83%
CRBN-6-5-5-VHL	2.11 µM	53.10%	1.18 µM	79.35%
Lenalidomide	N/A	42.47%	0.17 µM	66.77%
Pomalidomide	2.32 µM	56.97%	0.07 µM	88.42%

TABLE 2. DC_{50} and D_{max} values of Degraders and IMiDs acting on IKZF1 and IKZF3 in RPMI 8266 cells. No DC_{50} was calculated for lenalidomide because its D_{max} was less than 50%.

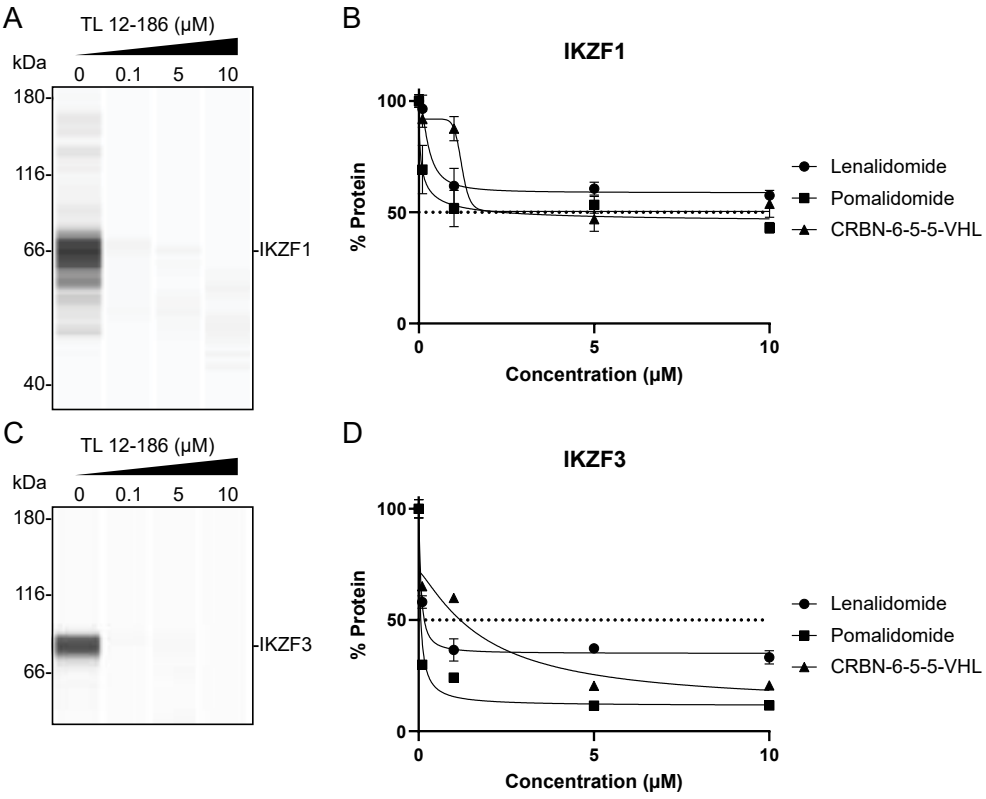

FIGURE 2. Degradation of IKZF1 and IKZF3 by IMiDs and Degraders in RPMI 8266 cells. (**A**) Lane view of IKZF1 degradation by TL 12-186. (**B**) Percent IKZF1 degradation by concentration of degrader or IMiD. The dotted line represents the 50% degradation threshold used to calculate the DC_{50} values shown in **TABLE 2**. (**C**) Lane view of IKZF3 degradation by TL 12-186. (**D**) Percent IKZF3 degradation by concentration of Degrader or IMiD. The dotted line represents the 50% degradation threshold used to calculate the DC_{50} values shown in **TABLE 2**.

SIMPLE WESTERN AUTOMATES DEGRADER R&D

Using the automated Simple Western platform, we screened degradation of the cereblon neosubstrates IKZF1 and IKZF3 with cereblon-recruiting Degraders and IMiDs. To generate the data shown here, more than 120 samples were run on Simple Western, and that is excluding the additional experiments needed to determine the optimal saturating antibody concentrations. While running 120 samples by traditional Western blot would be an immense undertaking, Simple Western can generate up to 96 data points in a single run. Thus, it is feasible to generate the data shown here in only two overnight runs, all in an automated fashion. Furthermore, Simple Western's highly quantifiable data allows for accurate determination of DC_{50} and D_{max} values, as opposed to the only semi-quantitative abilities of traditional Western blot. For these reasons, Simple Western is the ideal solution for studying and developing targeted protein degradation strategies.

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2. The myeloma drug lenalidomide promotes the cereblon-dependent destruction of Ikaros proteins, G. Lu, R. Middleton, H. Sun, M. Naniong, C. Ott, C. Mitsiades, K. Wong, J. Bradner and W. Kaelin, *Science*, 2014; **343**:305–9.

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7. Rate of CRL4(CRBN) substrate Ikaros and Aiolos degradation underlies differential activity of lenalidomide and pomalidomide in multiple myeloma cells by regulation of c-Myc and IRF4, C. Bjorklund, L. Lu, J. Kang, P. Hagner, C. Havens, M. Amatangelo, M. Wang, Y. Ren, S. Couto, M. Breider, Y. Ning, A. Gandhi, T. Daniel, R. Chopra, A. Klippel and A. Thakurta, *Blood Cancer Journal*, 2015; **5**:e354

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LEARN HOW FULLY AUTOMATED WESTERNS ENABLE CUTTING-EDGE TARGETED PROTEIN DEGRADATION RESEARCH

WHY YOUR RESEARCH MATTERS TO US

Targeted Protein Degradation (TPD) with heterobifunctional small molecule compounds (e.g., PROTAC®) is a novel approach to knockdown target proteins within cells. Degraders consist of binding moieties for an E3 ubiquitin ligase and a target protein joined by a linker, and selectively remove proteins via the ubiquitin-proteasome system (UPS). Degraders have recently emerged as an attractive mechanism to explore previously "undruggable" targets. Advances in our understanding of how heterobifunctional Degraders achieve potency and selectivity will help to design more efficient degraders.

To characterize the efficacy of Degrader molecules, researchers generally run dose response curves by way of traditional SDS-PAGE Western blotting methods. But the lengthy, manual workflow and resulting low reproducibility make it an unreliable approach for the determination of DC50 values. Instead, the ideal solution would be highly reproducible, allow for easy quantitation and have a short time to results. Simple Westerns are just that–they let you separate and analyze proteins by size (or charge) from 2 kDa to 440 kDa in just 3 hours. You'll get quantitative results, reproducibility that's spot on, and use less sample in the process.

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All experiments in Figure 1 were performed by the Simple Western applications science team. All molecules utilized in these experiments were synthesized and supplied by Tocris, a Bio-Techne brand. Further details are available at: www.tocris.com/tpd

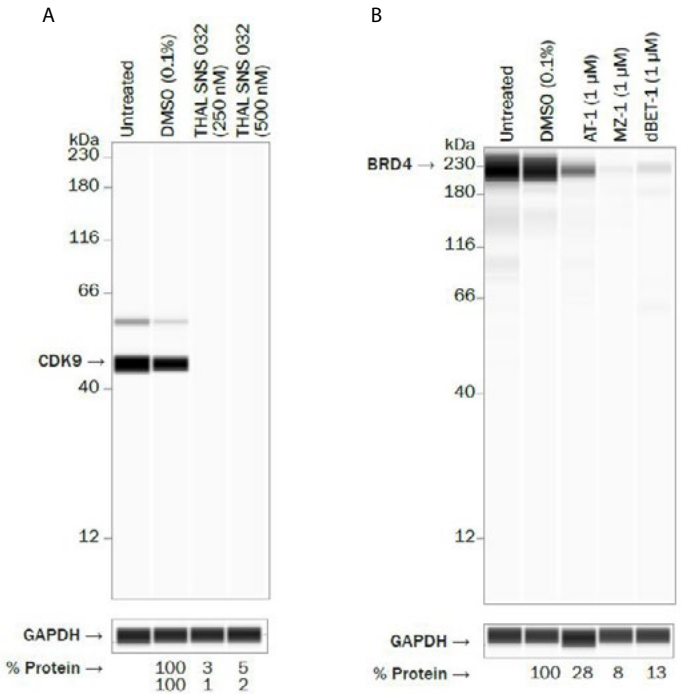

FIGURE 1. Knockdown of CDK9 and BRD4 with Degraders and analysis by Simple Western Assay on WesTM. (A) Knockdown of both CDK9 isoforms after THAL SNS 032 treatment (Tocris Cat. No. 6532) of MOLT-4 cells (4 h incubation). (B) Knockdown of BRD4 long isoform after treatment of HeLa cells with AT-1 (Tocris Cat. No. 6356, 1 µM), MZ-1 (Tocris Cat. No. 6154, 1 µM) or dBET-1 (Tocris Cat. No. 6327, 1 µM). Protein quantification (relative to DMSO-only control) is shown beneath the corresponding lane.

SIMPLE WESTERN ASSAYS IN THE HANDS OF YOUR PEERS

BAF COMPLEX VULNERABILITIES IN CANCER DEMONSTRATED VIA STRUCTURE-BASED PROTAC DESIGN

Boehringer Ingelheim and University of Dundee researchers develop PROTAC® Degraders of the BAF ATPase subunits SMARCA2 and SMARCA4. Protein degradation levels of SMARCA2 and SMARCA4 were assessed using Simple Western.

TARGETS ANALYZED BY SIMPLE WESTERN:
SMARCA2 and SMARCA4
SAMPLE TYPE: Human acute myeloid leukemia MV-4-11 cells
EXPERIMENTAL DESIGN: Dose response curves

Read more at:

W Farnaby, M Koegl, M Roy….A Ciulli,
Nat Chem Biol, 2019; 15: 672–680.

DELINEATING THE ROLE OF COOPERATIVITY IN THE DESIGN OF POTENT PROTACS FOR BTK

Pfizer researchers addressed efficient PROTAC® activity and if positive cooperativity is necessary for potent protein degradation. They show that within a Bruton's tyrosine kinase (BTK)/cereblon PROTAC® system, potent knockdown correlates with alleviation of steric clashes in the absence of thermodynamic cooperativity. Protein degradation levels of BTK and controls were assessed using Simple Western.

TARGETS ANALYZED BY SIMPLE WESTERN:
BTK, Vinculin and Actin
SAMPLE TYPE: Ramos cell line, Rat lung and spleen
EXPERIMENTAL DESIGN: Dose response curves, competition, confirmation of mass spectrometry results, efficacy and specificity in vivo.

Read more at:

A Zorba, C Nguyen, Y Xu…M Calabrese. *PNAS*, 2018;
115:E7285-E7292.

ADDITIONAL HIGHLIGHTED TPD PUBLICATIONS USING SIMPLE WESTERN

1. CHEMICALLY INDUCED DEGRADATION OF THE ONCOGENIC TRANSCRIPTION FACTOR BCL6.
N Kerres, S Steurer, S Schlager…M Koegl.
Cell Reports, 2017; 20:2860–2875.
Targets Analyzed by Simple Western: BCL6 and GAPDH.
Sample Type: Human diffuse large B-cell lymphoma (DLBCL) cells lines

2. HIGHLY SELECTIVE PTK2 PROTEOLYSIS TARGETING CHIMERAS TO PROBE FOCAL ADHESION KINASE SCAFFOLDING FUNCTIONS.
J Popow, H Arnhof, G Bader…P Ettmayer.,
J Med Chem, 2019; 62(5):2508-2520.
Targets Analyzed by Simple Western: PTK2 and GAPDH.
Sample Type: Human hepatocellular carcinoma and pulmonary adenocarcinoma cell lines

3. ITERATIVE DESIGN AND OPTIMIZATION OF INITIALLY INACTIVE PROTEOLYSIS TARGETING CHIMERAS (PROTACS) IDENTIFY VZ185 AS A POTENT, FAST, AND SELECTIVE VON HIPPEL–LINDAU (VHL) BASED DUAL DEGRADER PROBE OF BRD9 AND BRD7.
V Zoppi, S Hughes, C Maniaci, A Testa, T Gmaschitz…A Ciulli,
J Med Chem, 2019 62(2): 699–726.
Targets Analyzed by Simple Western: BRD9 and GAPDH.
Sample Type: HeLa and Hek293 cells

4. SHAPE-BASED VIRTUAL SCREEN FOR THE DISCOVERY OF NOVEL CDK8 INHIBITOR CHEMOTYPES.
L He, Y Zhu, Q Fan, D Miao, S Zhang, X Liu, C Zhang.,
Bioorg Med Chem Lett, 2019 29(4):549-555.
Targets Analyzed by Simple Western: p-Stat1 and Stat1
Sample Type: Shape-Based Virtual Screen for the Discovery of Novel CDK8 Inhibitor Chemotypes.

5. ANDROGEN RECEPTOR DEGRADERS OVERCOME COMMON RESISTANCE MECHANISMS DEVELOPED DURING PROSTATE CANCER TREATMENT.
S Kregel, C Wang, X Han, L Xiao, E Fernandez-Salas…A Chinnaiyan.,
Neoplasia, 2020 22(2):111-119.
Targets Analyzed by Simple Western: Total Androgen Receptor (AR).
Sample Type: CRISPR engineered LNCaP-derived cells

6. DEGRADATION OF POLYCOMB REPRESSIVE COMPLEX 2 WITH AN EED-TARGETED BIVALENT CHEMICAL DEGRADER.
F Potjewyd, A Turner, J Beri, J Rectenwald, J Norris-Drouin…L James.,
Cell Chem Biol, 2020 27(1):47-56.
Targets Analyzed by Simple Western: EED and EZH2.
Sample Type: HeLa cells

7. EXTENDED PHARMACODYNAMIC RESPONSES OBSERVED UPON PROTAC-MEDIATED DEGRADATION OF RIPK2
A Mares, AH Miah, IED Smith, M Rackham, AR Thawani,
J Cryan … G Watt, J Denyer, P Scott-Stevens, John D. Harling
Communications Biology, 2020 3:140
Targets Analyzed by Simple Western: RIPK2, cIAP1, cIAP2, XIAP, β-actin and vinculin
Sample Type: THP-1 cells

8. SELECTIVE CDK6 DEGRADATION MEDIATED BY CEREBLON, VHL, AND NOVEL IAP RECRUITING PROTACS
NA Anderson, J Cryan, A Ahmed, H Dai, GA McGonagle,
C Rozier, AB Benowitz,
Bioorganic & Medicinal Chemistry Letters, 2020.
Targets Analyzed by Simple Western: CDK4, CDK6 and Vinculin
Sample Type: Jurkat T cell line

TPD RESEARCH PRODUCT GUIDE

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