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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**).

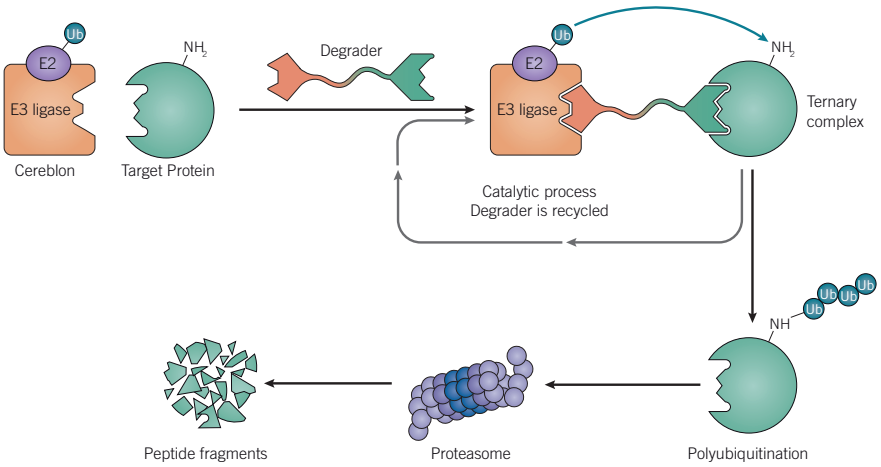

FIGURE 1. Schematic showing the catalytic mode of action of heterobifunctional Degrader molecules. Degraders initiate the formation of a ternary complex between an E3 ubiquitin ligase, in this case cereblon, and a target protein, resulting in polyubiquitination of the target protein and subsequent degradation 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%.

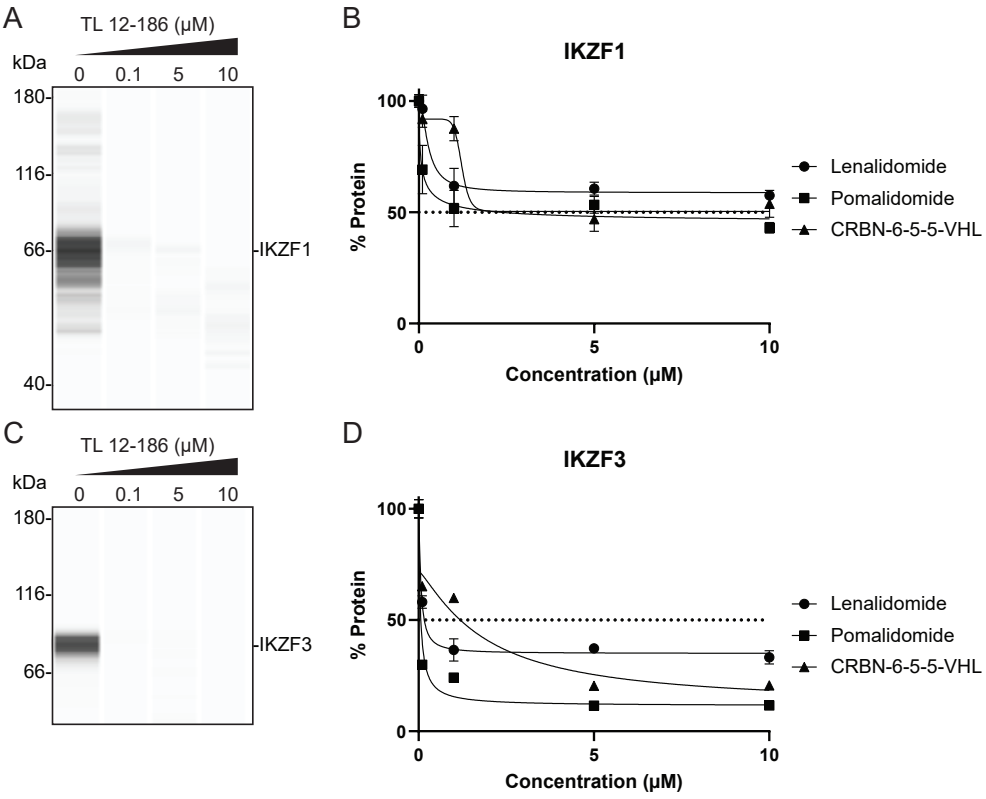

FIGURE 2. Degradation of IKZF1 and IKZF3 by IMiDs and Degraders in RPMI 8266 cells. (**A**) Lane view of IKZF1 degradation by TL 12-186. (**B**) Percent IKZF1 degradation by concentration of degrader or IMiD. The dotted line represents the 50% degradation threshold used to calculate the DC_{50} values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