Application of Plexium's Picowell Screening Technology to the Identification of IKZF2 Molecular Glue Degraders

Erika Green, Amber Senese, Vincent Aguilar, Elliot Imler, Gabrielle Blanco, Alex Campos, Duc Tran, Quinn Spalding, Craig Stumpf, Greg Parker, Peggy Thompson, Lisa Liu, Cindy Huynh, Julie Huynh, Paige Burgin, Kira Ostrander, Michelle Pandolfi, Anastasia Velentza, Edward Hujber, Kenneth Chng, Tomás Duffy, Edgar Gutierrez, Jesse Lu, Mitchell Hull, John Bucci, Guy Oshiro, Daniel Sipes, Farhana Barmare, Animesh Roy, John Maina Ndungu, Andrew Burritt, Simon Bailey, Kevin Freeman-Cook, Michael Hocker

Plexium, Inc., San Diego, CA

Overview

The present disclosure describes the successful application of Plexium's Picowell screening technology to the identification of IKZF2 (Helios) molecular glue degraders (MGDs). This approach involved generation of a large One-Bead-One-Compound cereblon targeted chemical library, in which the beads are encoded with DNA tags, and the compounds are orthogonally attached to the beads through a photocleavable linker. Upon irradiation and release, compounds unencumbered with DNA tags are screened utilizing an ultrahigh density 'picowell' screening platform in phenotypic cellular assays that enabled identification of potential MGDs that promote depletion of IKZF2. The technology enables the rapid generation and screening of 100,000's of compounds, with dense coverage of chemical space, in a cost-effective and efficient manner and is applicable to numerous proteins of interest (POIs) and a variety assay types.

Introduction

Targeted Protein Degradation (TPD) is an emerging strategy that holds great promise in enabling modulation of targets that have previously been considered to be 'unligandable' due to the absence of a well-defined binding site on the protein of interest (POI). One modality within the TPD umbrella involves utilizing a small molecule to enhance the interaction of an E3 ligase complex[1] with a 'neosubstrate' POI to enable ubiquitination of the POI and initiate 26S proteasome mediated degradation. Such molecules are often referred to as 'Molecular Glue Degraders' (MGDs).

Identification of chemical starting points for MGD discovery programs is challenging.[2] Multiple literature reports suggest that MGs can have very steep structure activity relationships (SAR).[3] In many cases, small (single atom) changes in molecular structure convert a non-degrader to a degrader. Because of this steep SAR, screening a traditional HTS collection of analogs, in which large collections of relatively diverse compounds are screened, may fail to identify MGD hits. Due to the steep SAR around MGDs, screening of diverse chemical libraries that densely cover chemical space may be advantageous.

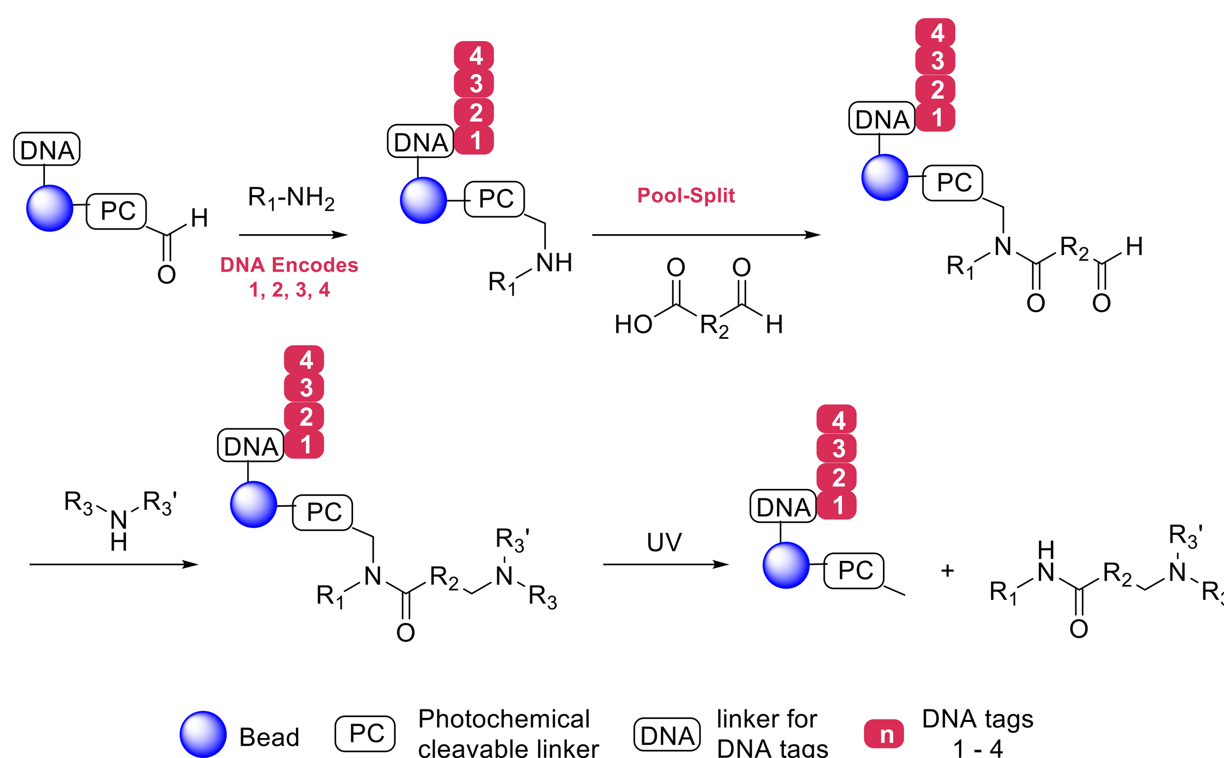
Plexium's approach to the identification of MGDs is to utilize its proprietary Picowell Technology platform to efficiently generate and screen large compound libraries that densely cover chemical space around known ligands of E3 ligases. Libraries consisting of 1000's to 100,000's of compounds are generated by diversification of these ligands and the compounds then screened in cellular assays to identify potential MGDs that promote depletion of a protein of interest.

The application of this technology to the identification of MGDs of IKZF2, a zinc finger transcription factor that promotes immune suppression, by screening a cereblon targeted library, agnostic to the POI (IKZF2), is described.

Methods

Library generation: A One-Bead-One-Compound DNA encoded (OBOC-DNA) cereblon targeted library (28.6k compounds) was generated by derivatizing known cereblon binding ligands with two additional points of diversity. Building blocks were selected to optimize physicochemical property distributions, to ensure drug-likeness, and to maximize coverage of available chemical space from both diversity and density perspectives. The library was generated by solid-phase synthesis on Tentagel resin beads (split-and-pool methodology) to produce a full combinatorial array of compounds (Scheme 1). Beads were derivatized with linkers to separately enable DNA-tag encoding of beads, and synthesis of screening compounds via a photochemically cleavable linker. After synthesis and DNA tagging, photochemical release yields compounds unencumbered by DNA tags.

Picowell IKZF2 phenotypic assay: Library beads were loaded into proprietary picowell microfluidic screening devices (56k wells per device). HEK293T IKZF2-GFP reporter cells were seeded into the wells and compounds photochemically released. After 24 h treatment, high-content imaging and analysis (proprietary technology) enabled direct measurement of IKZF2 depletion. Beads were 'picked' from device wells, the DNA-tags sequenced, and unique DNA-tag sequences used to deconvolute the chemical structure of each putative hit (Figure 1).



Scheme 1: Synthesis route and DNA tag encoding scheme of the library.

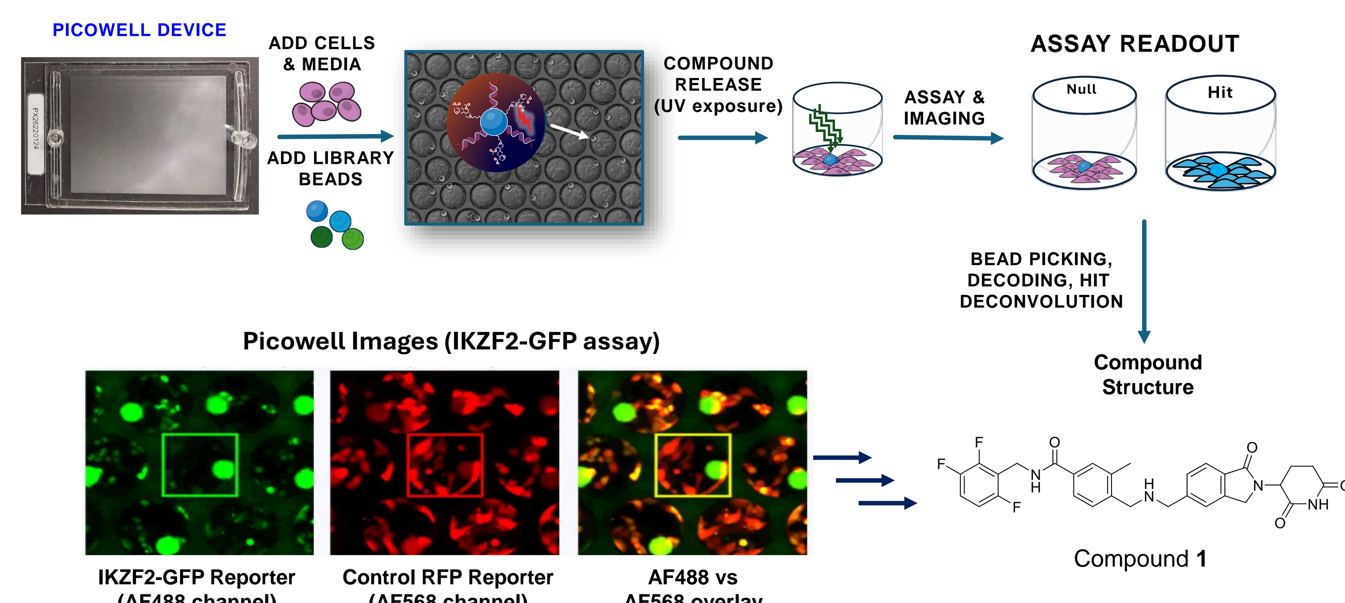


Figure 1: Picowell screening workflow

Validation Assays

Cereblon Target Engagement (CRBN TE): Ligand binding to cereblon was measured using the NanoBRET Target Engagement Assay (Promega).

IKZF2 degradation and selectivity: IKZF2/1 fluorescent reporter and HiBiT-tagged GSPT1 protein levels were measured after 24 h treatment with single or increasing concentrations of compounds.

Rescue experiment: Jurkat cells were treated with compound ±bortezomib for 6 h prior to analysis on the Simple Western system (Protein Simple/Biotechne)

Proteomics analysis: Unbiased global proteomics was performed using data independent acquisition with parallel accumulation serial fragmentation technology. Using three replicates per condition, Jurkat cells were treated with either 1 uM compound or with DMSO alone for 24 h. Following cell lysis and protein digestion with trypsin overnight, samples were analyzed by LC-MS/MS on a timsTOF HT (Bruker Daltonics) mass spectrometer connected to an Evosep One system using the 30SPD method. Raw data processing was performed using Dia-NN 1.8.2 and an in-house developed pipeline was used for downstream statistical analysis with R Bioconductor.

Results

The screening & hit confirmation workflow is shown in Figure 2a. Compounds were selected for hit confirmation based upon statistical representation of the compound frequencies observed within the hit set and representation of the building block combinations incorporated in the library (Figures 2b & 2c).

Initial hit resynthesis utilized spatially encoded solid-phase synthesis (SPOS) to generate solutions of 'crude' products. The concentration of each compound was estimated by LCMS analysis (ELS detection) and compared to standard curves constructed from purified representative compounds.[4]

Hits showing >50% degradation of IKZF2 in a single concentration assay (46 cmpds, 25% confirmation rate, Figure 3a) were then confirmed by dose response. A small subset of confirmed hits (8 compounds) were selected for powder form resynthesis. Good correlation was generally observed between data (DC_{50}, D_{max}) generated from 'crude' vs. purified compounds (Figures 3b & 3c, and Figure 4). Degradation data for one compound did not reconfirm, possibly indicating that an impurity in the unpurified material was causing the observed depletion of IKZF2.

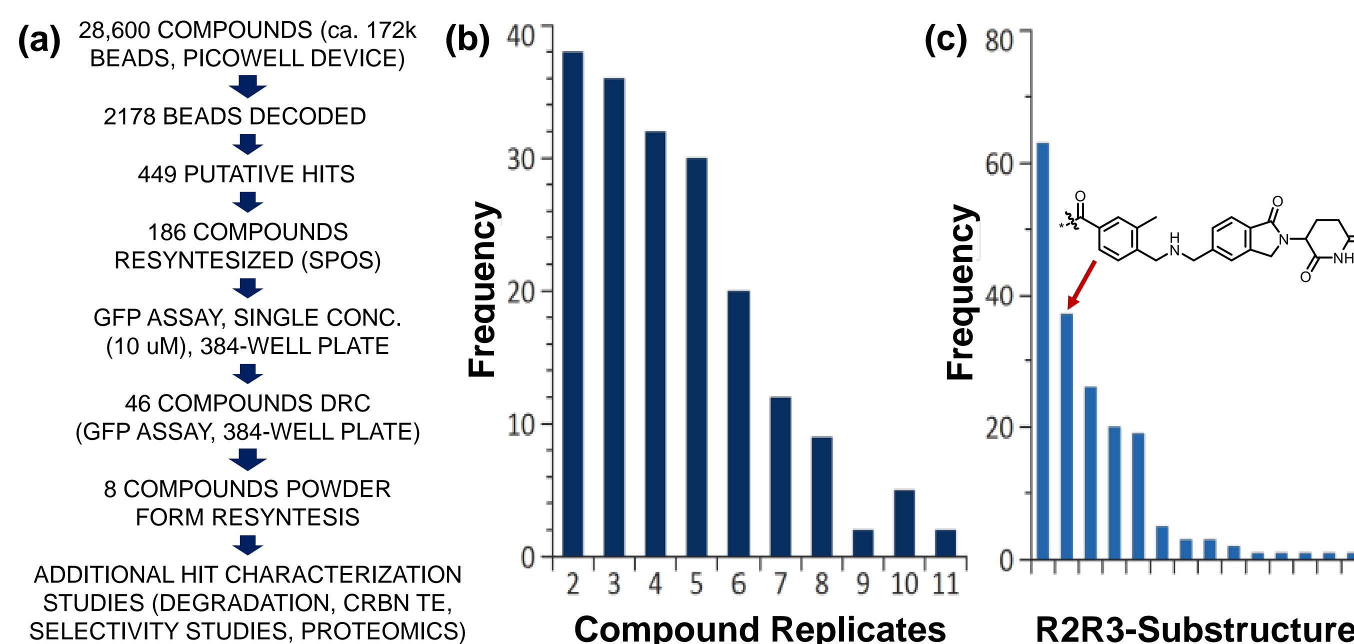


Figure 2: (a) Screening workflow **(b)** Number of times compound identified as a hit **(c)** Frequency of R2R3-combination present in hit set.

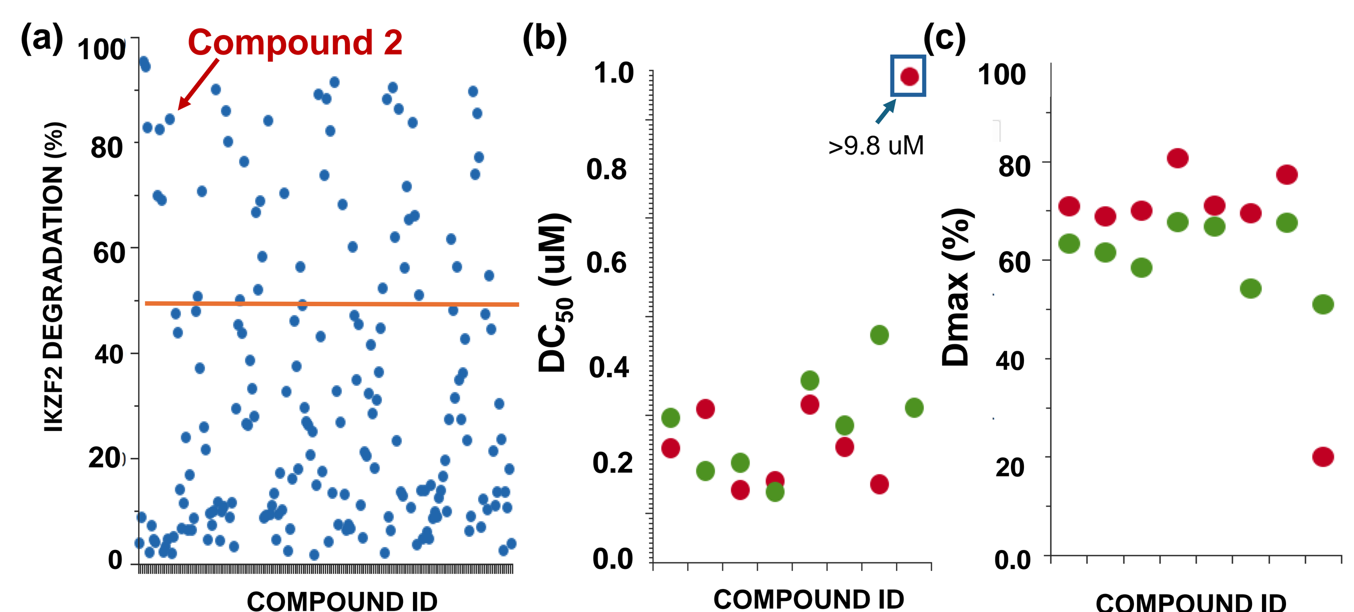
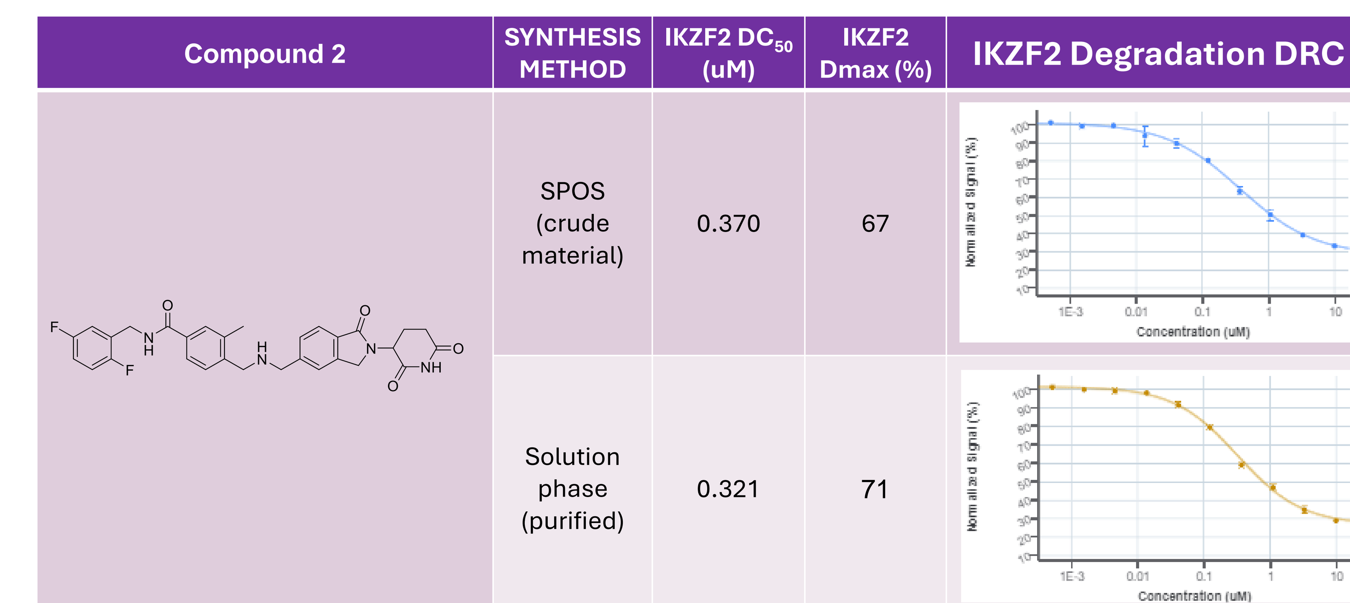


Figure 3: **(a)** Hit confirmation. Single conc. (10 uM) IKZF2-GFP depletion assay (SPOS compound resynthesis). **(b)** IKZF2-GFP assay DC_{50} (uM) SPOS resynth. (●) & pure powder form (●) compounds **(c)** IKZF2-GFP assay Dmax (%) SPOS resynth. (●) & pure powder form (●) compounds.



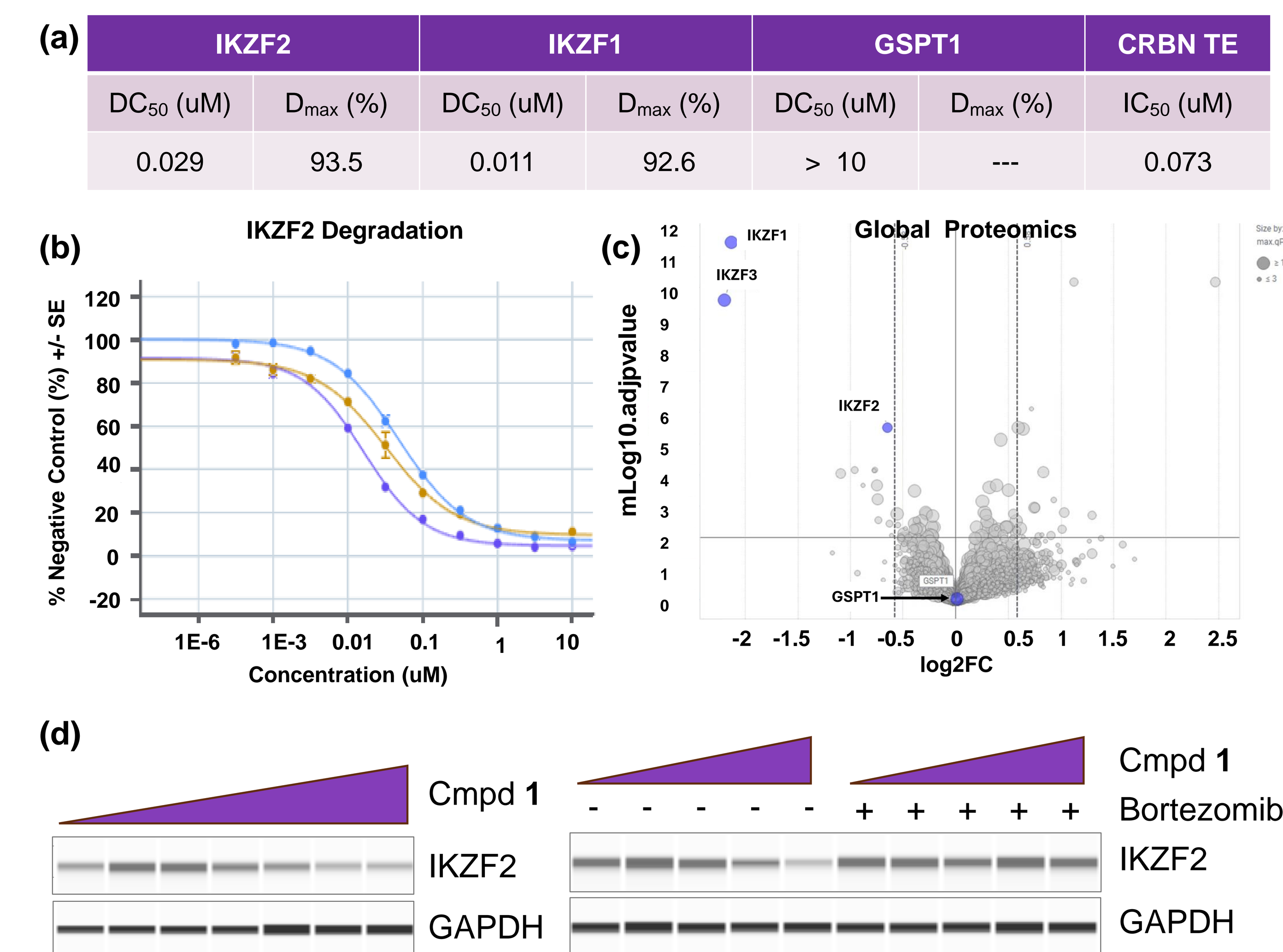
Compound 2	SYNTHESIS METHOD	IKZF2 DC_{50} (uM)	IKZF2 Dmax (%)	IKZF2 Degradation DRC
	SPOS (crude material)	0.370	67	
	Solution phase (purified)	0.321	71	

Figure 4: IKZF2-GFP dose response curves of crude (SPOS) resynthesized & purified powder forms of compound **2.**

The picowell screen workflow identified numerous compounds that deplete IKZF2. Data for compound **1** exemplifies the results obtained for many of the hits identified (Figure 5). The compound is a potent non-selective degrader of IKZF2 and IKZF1, does not significantly degrade GSPT1, and is a potent cereblon binder.

Degradation of IKZF2 is inhibited in the presence of the Bortezomib, a proteasome inhibitor, indicating degradation is proteasome mediated.

Global proteomics analysis provided additional evidence for non-specific degradation of IKZF1/2/3, and that **1** does not degrade GSPT1.



(a)

IKZF2		IKZF1		GSPT1		CRBN TE
DC_{50} (uM)	D_{max} (%)	DC_{50} (uM)	D_{max} (%)	DC_{50} (uM)	D_{max} (%)	IC_{50} (uM)
0.029	93.5	0.011	92.6	> 10	---	0.073

Figure 5: **(a)** Selectivity of **1** towards degradation of IKZF2, IKZF1, & GSPT1, and cereblon target engagement. **(b)** IKZF2-GFP 293T DRC 24 h from three independent experiments **(c)** Global proteomics analysis of **1**. **(d)** IKZF2 depletion as determined by western blot (24 h, Left). Degradation of IKZF2 by **1** is inhibited in the presence of a proteasome inhibitor (6 h, Bortezomib, Right).

Conclusions

Application of Plexium's Picowell screening technology platform successfully identified compounds that mediated depletion of IKZF2.

Screening hits identified for IKZF2 were determined to bind to cereblon; degradation was independently confirmed in an IKZF2-GFP assay and by global proteomics. Loss of IKZF2 protein was rescued by co-treating with a proteasome inhibitor confirming dependence on the ubiquitin proteasome system. Collectively, these results demonstrate that Plexium's ultrahigh density screening platform enables the discovery of MGDs of IKZF2.

The technology has been shown to be a useful tool for the identification of MGDs and enables the rapid generation and screening of 100,000's of compounds, with dense coverage of chemical space, in a cost-effective and efficient manner.

The platform technology is compatible with various assay methodologies and may be applied to hit finding activities for many challenging targets.

References

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3) Słabicki M, Kozicka Z, Petzold G, Li YD, Manojkumar M, Bunker RD, Donovan KA, Sievers QL, Koeppel J, Suchyta D, Sperling AS, Fink EC, Gasser JA, Wang LR, Corsello SM, Sellar RS, Jan M, Gillingham D, Scholl C, Fröhling S, Golub TR, Fischer ES, Thomä NH, Ebert BL. The CDK inhibitor CR8 acts as a molecular glue degrader that depletes cyclin K. *Nature* **2020**, 585(7824), 293 – 297, DOI: 10.1038/s41586-020-2374-x.
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