Real-time Monitoring of PROTAC and Molecular Glue Targeted Degradation in Living Cells

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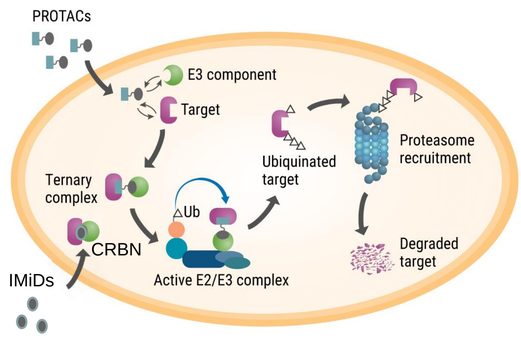
1. Introduction

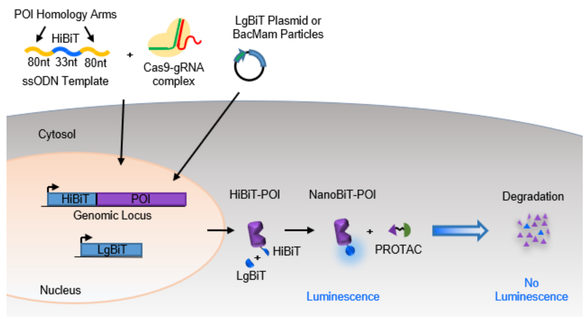
The emergence of targeted protein degradation as a broad, new therapeutic modality has greatly expanded the opportunities for treatments of many diseases. Currently, small molecule degrader compounds fall under two major categories; molecular glues and heterobifunctional PROTACs. The two types of compounds both facilitate and induce a ternary complex consisting of an E3 ligase-degrader-target protein, bringing into proximity the machinery proteins required to ubiquitinate and ultimately degrade the target protein. Significant challenges exist in characterization and rank-ordering of degradation compounds in live cells given the differences in dynamics of protein loss and recovery among compounds. Currently, the availability of technologies to interrogate real-time protein degradation is severely lacking. Here, we present a live-cell, luminescence-based technology platform with these capabilities. We use CRISPR/Cas9 endogenous tagging of target proteins with the small peptide, HiBiT, which has high affinity for and can complement with the LgBiT protein to produce NanoBiT luminescence. This allows for sensitive detection of endogenous protein levels in living cells and can also serve as a BRET energy donor to study protein:protein or protein:small molecule interactions. We demonstrate the power of this technology in continuous 24-hour monitoring of endogenous target protein levels, and the ability to quantify key degradation parameters for compound ranking including rate, Dmax, and Dmax50. We further show the ability to measure the mechanistic steps important for the degradation including kinetics of PROTAC- or molecular glue-induced ternary complex formation and target ubiquitination. These studies facilitate discernment of individual parameters required for successful degradation, ultimately enabling chemical design strategies for optimization and rank ordering of therapeutic degradation compounds.

2. Targeted Protein Degraders: PROTACs and Molecular Glues

Hijacking the UPS with PROTACs and Molecular Glues

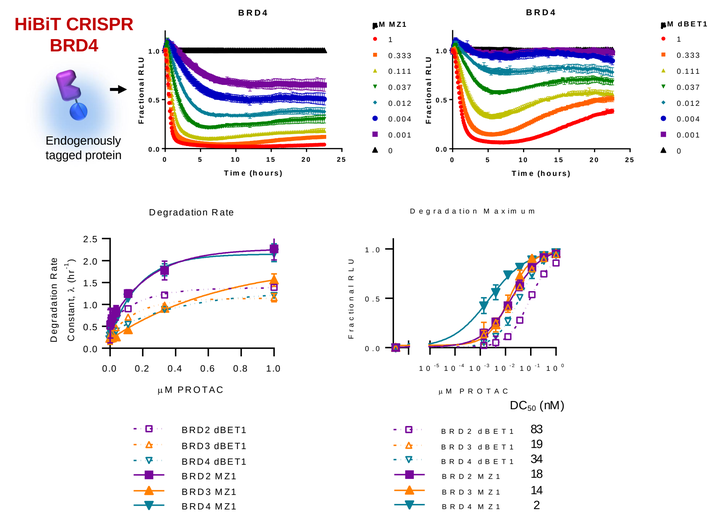


HiBiT CRISPR Tagging Strategy and Experimental Approach



3. PROTAC-Induced Live Cell Degradation Kinetics

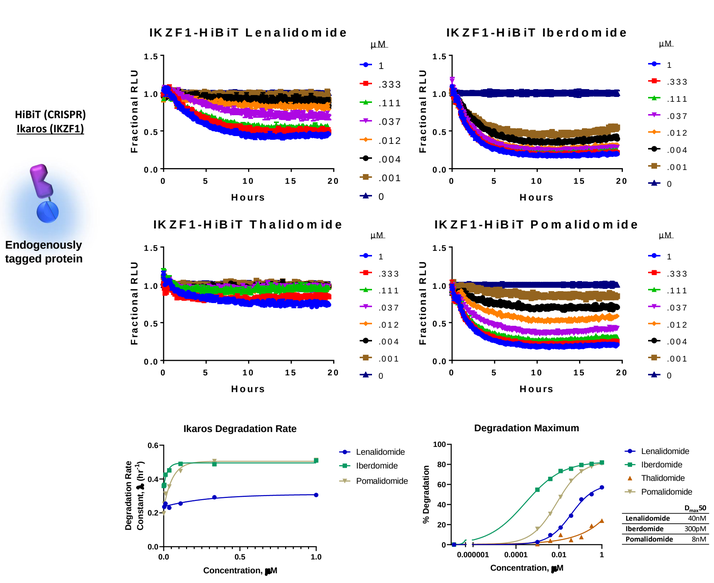
HiBiT Endogenous Tagging and Kinetic Degradation of BRD4



- BET family members were endogenously tagged using CRISRP/CAS9 with 11AA HiBiT fusion tag
- Complete cellular degradation profiles determined with continual luminescent reads on GloMax Discover
- Degradation rate, Dmax, and $Dmax_{50}$ determined to rank compounds and BET family members

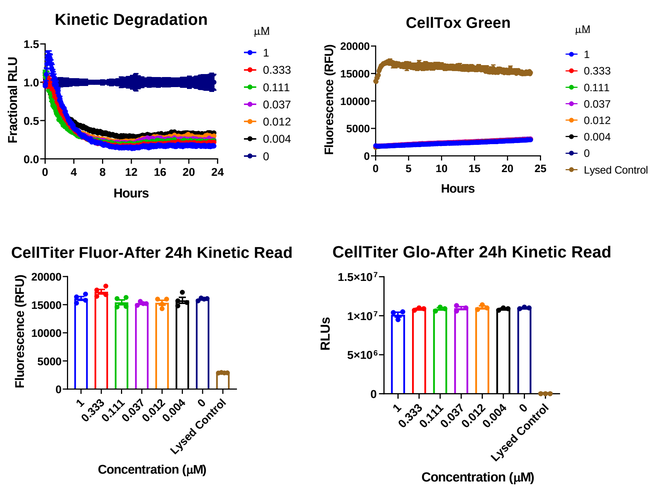
4. Molecular Glue-Induced Live Cell Degradation Kinetics

HiBiT Endogenous Tagging and Kinetic Degradation of Ikaros



- Observe degradation of molecular glue targets and can rank compounds by degradation rate, Dmax, and $Dmax_{50}$
- Iberdomide shows most potent degradation
- Can use for counter-screening PROTACs that have a glue handle

5. Multiplexing Ikaros degradation by Iberdomide with cell viability



- Confirm loss of signal (degradation) is not due to impacts on viability
- Kinetics can help distinguish target degradation that may later lead to toxicity
- Different viability assays to measure different stages of toxicity:

Kinetic Multiplex:

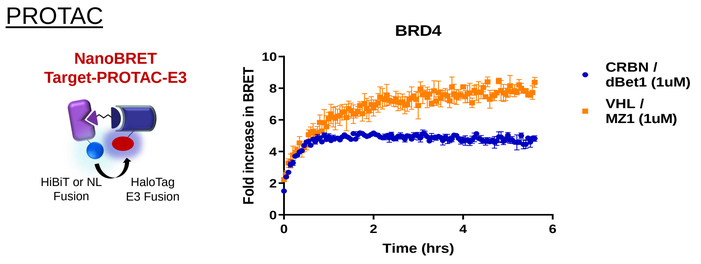
- CellTox-Green: membrane integrity

Endpoint Mulitplex:

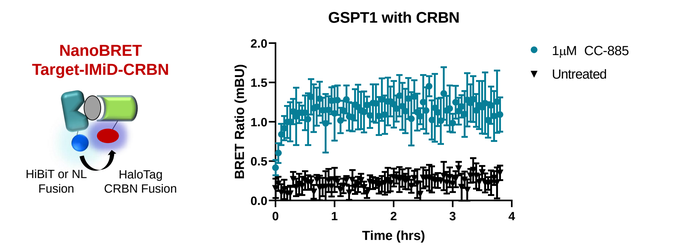
- CellTiter-Fluor: active cellular proteases
- CellTiter-Glo: cellular ATP levels

6. Live Cell NanoBRET Ternary Complex Kinetics with PROTACs and Glues

PROTAC

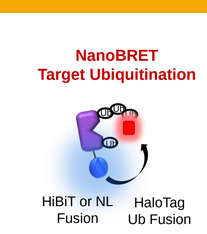


Molecular Glue

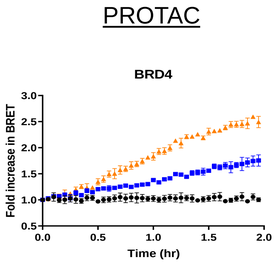


- Monitor ternary complex formation and degradation simultaneously in a single NanoBRET assay
- Use endogenous HiBiT-fused target protein, or ectopic expression
- Use of MG132 can increase signal window
- Kinetic analysis allows for understanding of cellular ternary complex stability

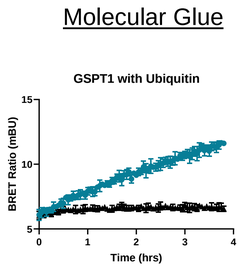
7. Live Cell NanoBRET Ubiquitination Kinetics with PROTACs and Glues



PROTAC



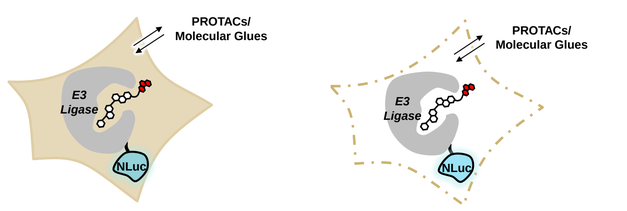
Molecular Glue



- Monitor target ubiquitination kinetics induced by PROTACs or molecular glues.
- Use endogenous HiBiT-fused target protein, or ectopic expression
- Differential target ubiquitination observed with compounds of different potency and E3 recruitment
- Ubiquitination levels correlate with degradation rate

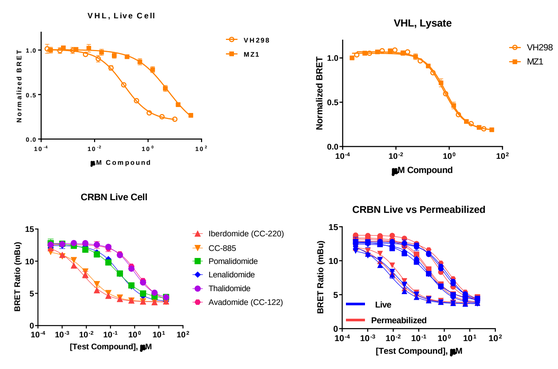
8. NanoBRET E3 Ligase Binding Affinity and Permeability of PROTACs and Glues

A. Live Cells **B. Permeabilized Cells**



PROTAC

Molecular Glue



- Run NanoBRET Target Engagement in live or permeabilized cells (A & B), enabling assessment of compound affinity and permeability.
- MZ1 binds to VHL with the same affinity (lysate) but has reduced permeability (live cell) compared to monovalent VH298.
- Panel of molecular glues shows range in binding affinities to CRBN, and all are readily cell-permeable (live vs permeabilized).

9. Conclusions

Differentiating cellular technologies to study key processes in PROTAC and Molecular Glue-mediated degradation for more rapid profiling of compounds

HiBiT and NanoLuc technology:

- Live cell kinetic degradation
- Amenable for use with CRISPR to study endogenous proteins
- Allows for quantitation of key degradation parameters

NanoBRET technology:

- Monitoring dynamic pathway interactions and signaling mechanisms in live cells
- Useful for assessment of PROTAC cellular permeability
- Follow induced interactions with E3 ligase components and target ubiquitination
- Provides mechanistic understanding of degradation kinetics

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