Monitoring Functional Mechanisms of Protein Degradation in Living Cells

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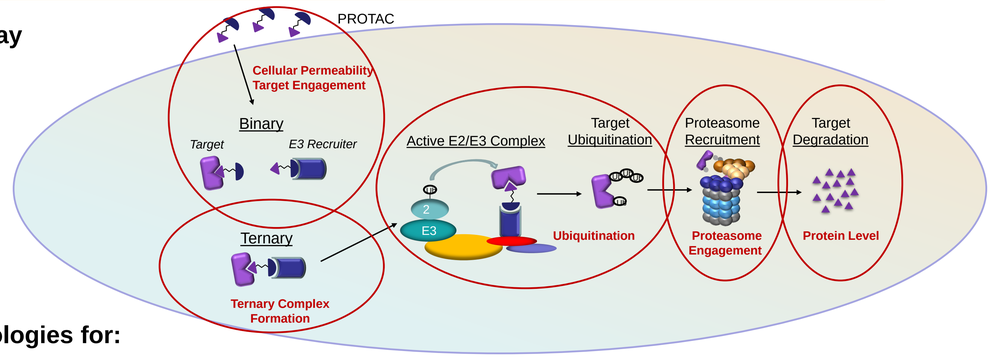
1. Introduction

A new generation of hetero-bifunctional small molecules, termed PROTACs, holds significant therapeutic potential by inducing degradation of target proteins. Characterizing and optimizing PROTACs for degradation efficacy represents a significant challenge, particularly in understanding the individual processes and potential failure points that control whether degradation will result. Currently, the availability of live cell assays to interrogate the multiple steps that are required to achieve degradation by PROTACs is severely lacking.

Here, we present a live-cell, luminescence-based technology platform that enables characterization of PROTAC compound mechanism of action using either ectopic or endogenous target expression formats. We employ 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. Using this combinatorial approach, we demonstrate the ability to measure permeability effects and binding affinities of PROTAC compounds to both target and E3 ligase, as well as monitor the kinetics of the subsequent ternary complex (target:PROTAC:E3 ligase) formation, target ubiquitination and recruitment to the proteasome in live cells. We further show the power of this technology in extended kinetic monitoring of endogenous target protein levels and the ability to quantify key degradation parameters including rate, Dmax, and DC_{50}s. These studies facilitate discernment of individual parameters required for successful degradation, ultimately enabling chemical design strategies for optimization and rank ordering of therapeutic PROTAC compounds.

Key Cellular Assays and Technologies for Monitoring PROTAC Activity

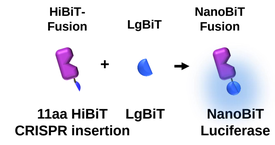
Degradation Pathway



Assays and Technologies for:

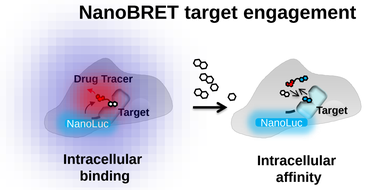
Protein Level

HiBiT binary complementation



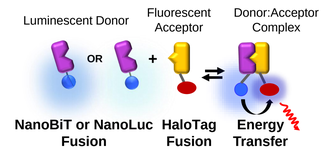
Permeability and Binding

NanoBRET target engagement



E3, Ubiquitin, and Proteasome

NanoBRET protein:protein interactions



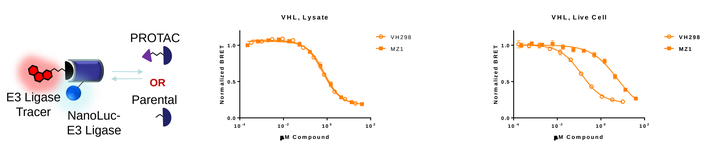
2. Protein Level and Degradation Profiles

HiBiT Endogenous Tagging and Kinetic Degradation

- 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 DC_{50} determined

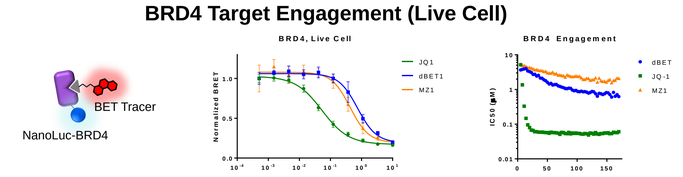
3. Cellular Permeability and Target Binding

E3 NanoBRET Target Engagement (Lytic and Live Cell)



Compound	Target	IC_{50} (nM, Lysate)	IC_{50} (nM, Live Cell)
VH298	VHL	633	127
MZ1	VHL	755	5600

BRD4 Target Engagement (Live Cell)



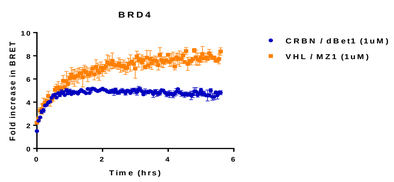
Compound	Target	IC_{50} (nM, Live Cell)
MZ1	BRD4	432
dBET1	BRD4	747
JQ1	BRD4	54

- Compare binding affinities in lytic or live cell mode to understand cell permeability
- NanoBRET target engagement of both E3 component and BET family members indicate reduced permeability of BET family PROTACs

4. E3 Ternary Complex Formation

E3 NanoBRET Ternary Complex Assay

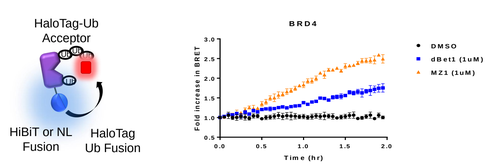
Live Cell Kinetic Analysis Using Vivazine Substrate



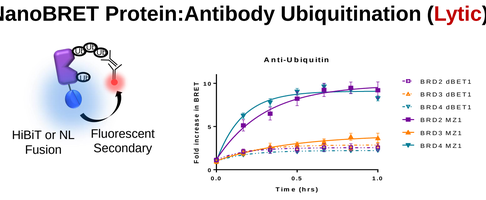
- Monitor ternary complex formation and degradation simultaneously in a single NanoBRET assay
- Use of MG132 can increase signal window
- Kinetic analysis allows for understanding of cellular ternary complex stability

5. Live Cell and Lytic Ubiquitination

NanoBRET Protein:Protein Ubiquitination (Live Cell)



NanoBRET Protein:Antibody Ubiquitination (Lytic)

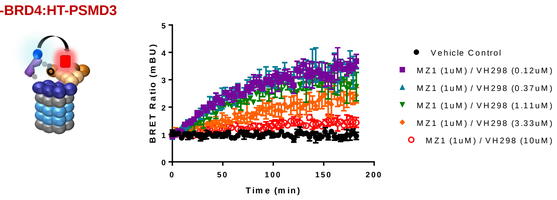


- Monitor live cell ubiquitination of any given target
- The different E3 ternary complexes recruited by dBET1 and MZ1 show differential ubiquitination on respective targets

6. Proteasomal Recruitment

NanoBRET Proteasome Assay

NL-BRD4:HT-PSMD3



- Detect increase of trafficking of BRD4 to proteasome in the presence of PROTAC
- Parental compounds can be used as controls
- It is not recommended to use proteasome inhibitors for the assay
- Can simultaneously monitor loss of target (NL-BRD4) in proteasome assay

7. Conclusions

Differentiating cellular technologies to study key processes in PROTAC-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, Ubiquitin, and subsequent trafficking to proteasome

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