Monitoring dynamics of protein degradation in living cells

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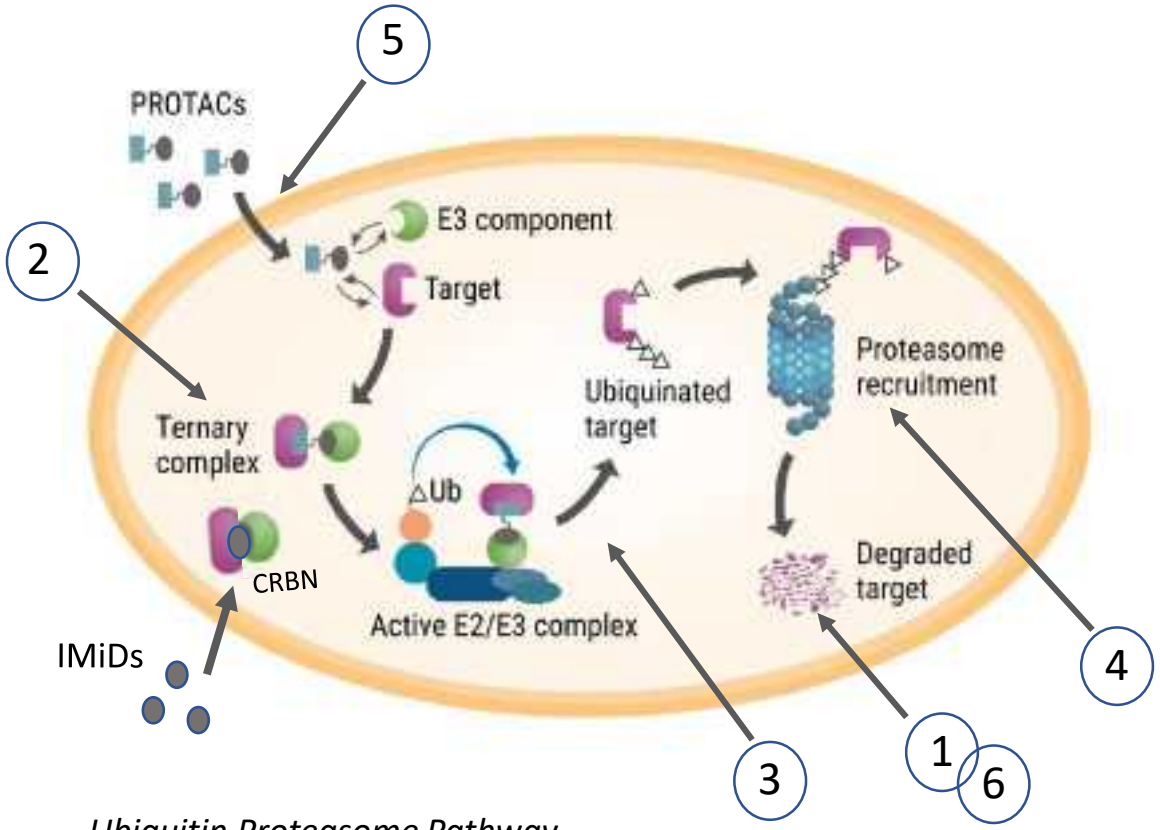
European Protein Degradation Congress; May 2019

Targeted protein degradation mediated by PROTAC and IMiDs



Ubiquitin Proteasome Pathway

Protein degradation is a dynamic and complex process

Can we understand the key steps for efficient targeted degradation?

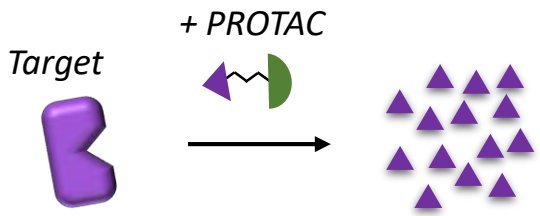
1. Is the target protein degraded
2. Formation of ternary complex
3. Efficiency of protein ubiquitination
4. Recruitment to the proteasome
5. Permeability of PROTACs
6. Phenotypic consequence of degradation

Need for kinetic and mechanistic characterization of degradation compounds in cellular environment

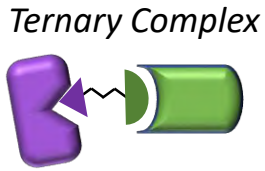
Modular technology program for live cell kinetic monitoring of protein degradation

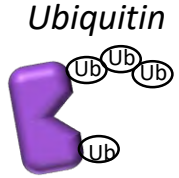
- Comprehensive program to address all key steps of ubiquitin-proteasome pathway
  - Assess kinetics and efficiency of degradation of endogenous target protein
  - Understand kinetics and efficacy of ternary complex formation and protein ubiquitination
  - Understand how PROTAC degradation efficacy correlates to specific mechanisms
  - Determine what steps in the pathway to optimize
  - Provide tools to understand degradation phenotype

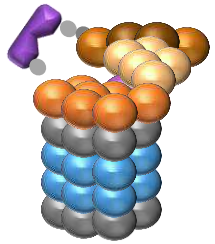
Endogenous target protein degradation



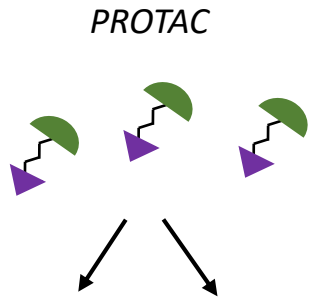
Degradation compound induced interactions





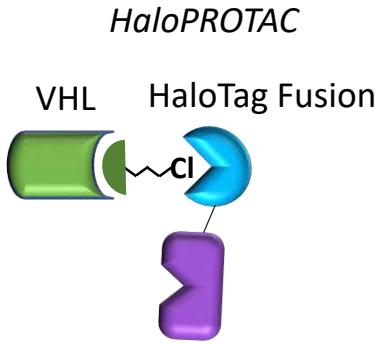


Compound permeability/binding



Binary Complexes

Target protein degradation phenotype

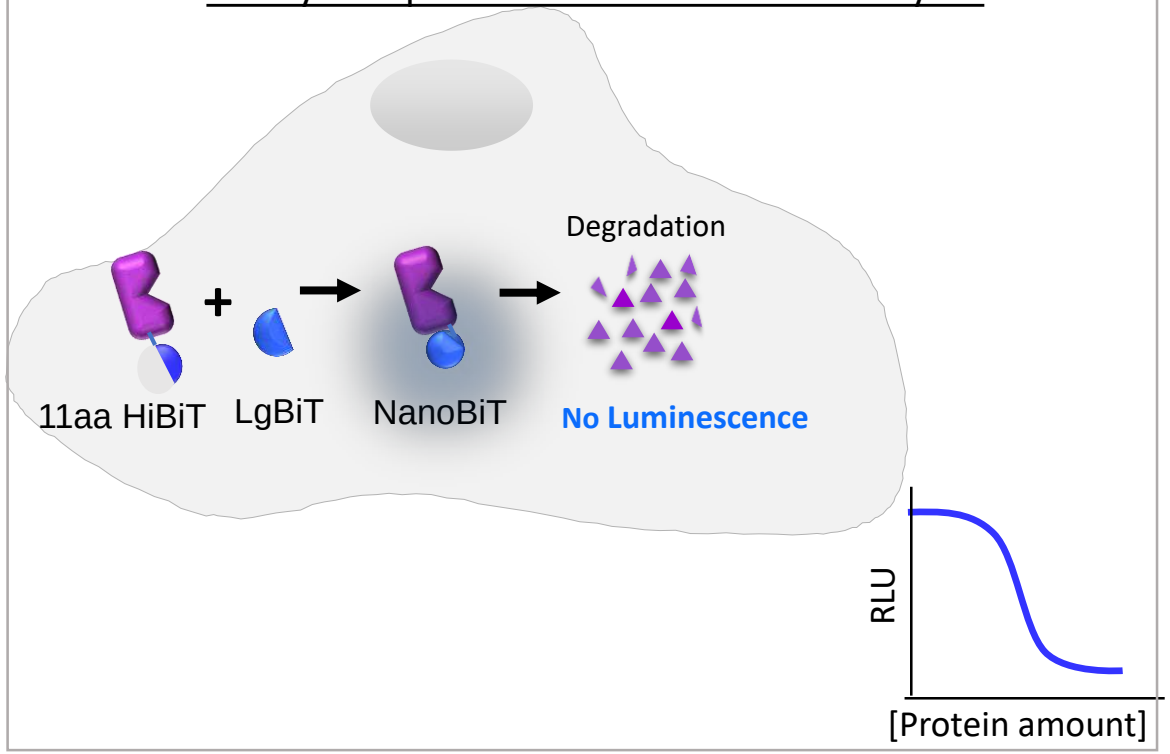


Core technologies for monitoring targeted protein degradation events

Protein Levels:
Degradation and recovery

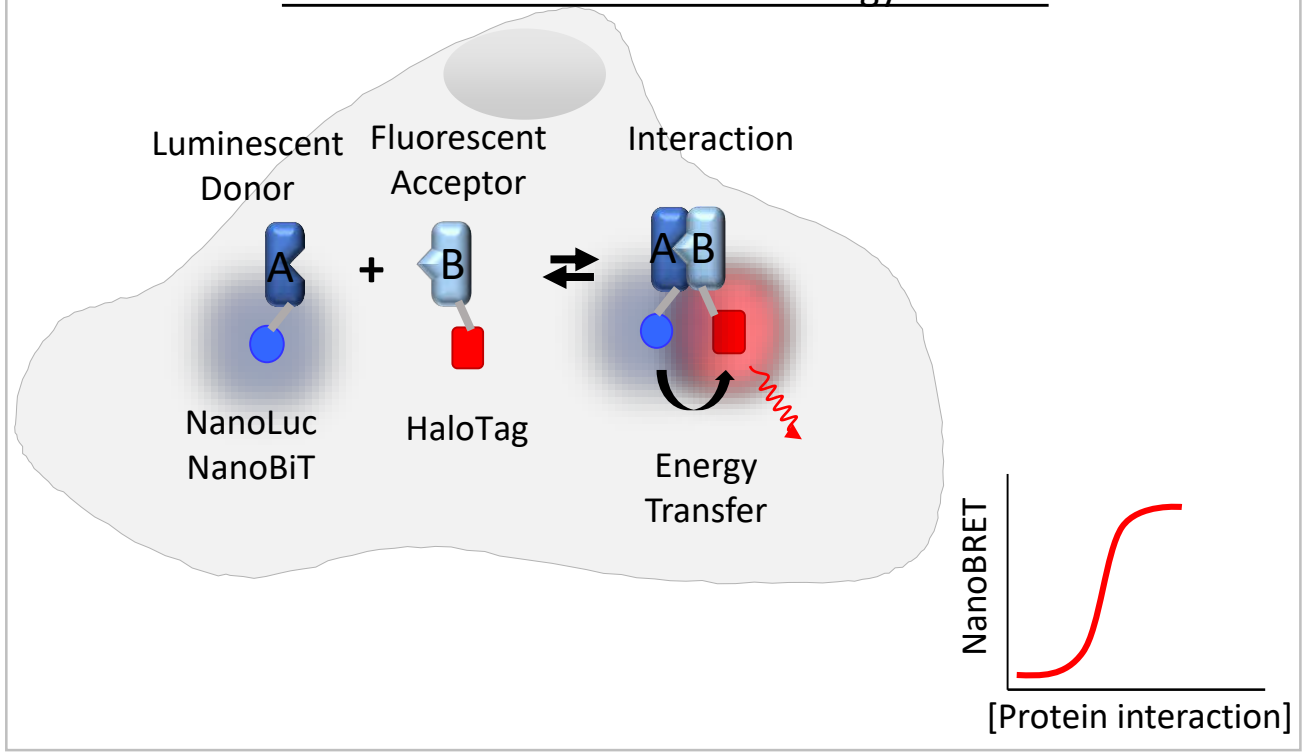
Mechanism:
Protein interactions

HiBiT Technology
Binary Complementation of NanoBiT Enzyme



- Live cell monitoring of protein level
- CRISPR insertion; endogenous expression and regulation
- HTS compatible

NanoBRET
Bioluminescence Resonance Energy Transfer



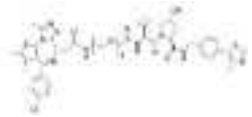
- Study PROTAC ternary complex and Ubiquitination
- Monitor protein:small molecule interactions
- HTS compatible

Case study: Degradation of BET family bromodomain proteins using PROTACs

Significant therapeutic promise for variety of leukemia, lymphomas, and other hematologic cancers

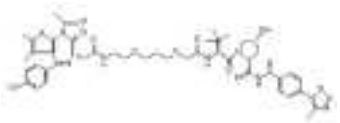
Heterobifunctional degraders/PROTACs

MZ1



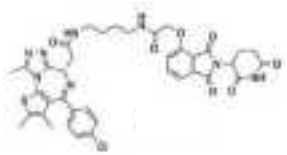
ACS Chem Biol, **2015**, *10*, 1770

ARV-771



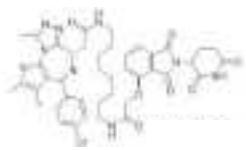
PNAS **2016**, *113*, 7124

dBET1



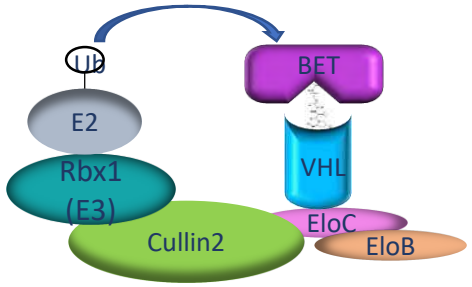
Science, **2015**, *348*, 1376

dBET6

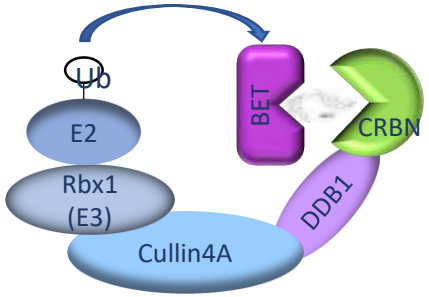


Mol Cell, **2017**, *67*, 5

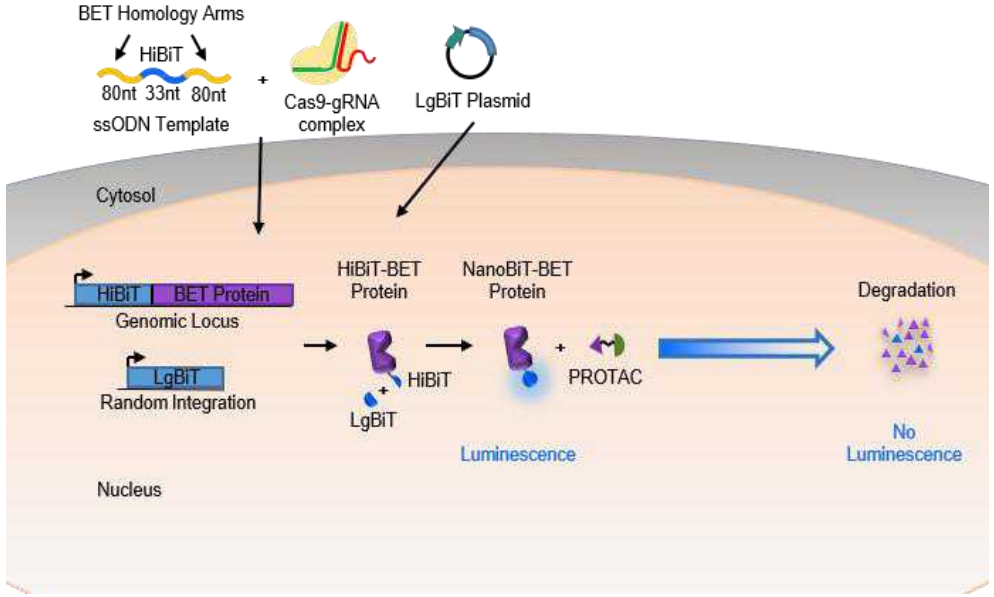
VHL System (MZ1, ARV-771)



CRBN System (dBET1, dBET6)



HiBiT-BET family CRISPR tagging strategy and experimental plan; N-terminal HiBiT-BRD2, BRD3, and BRD4 in HEK293

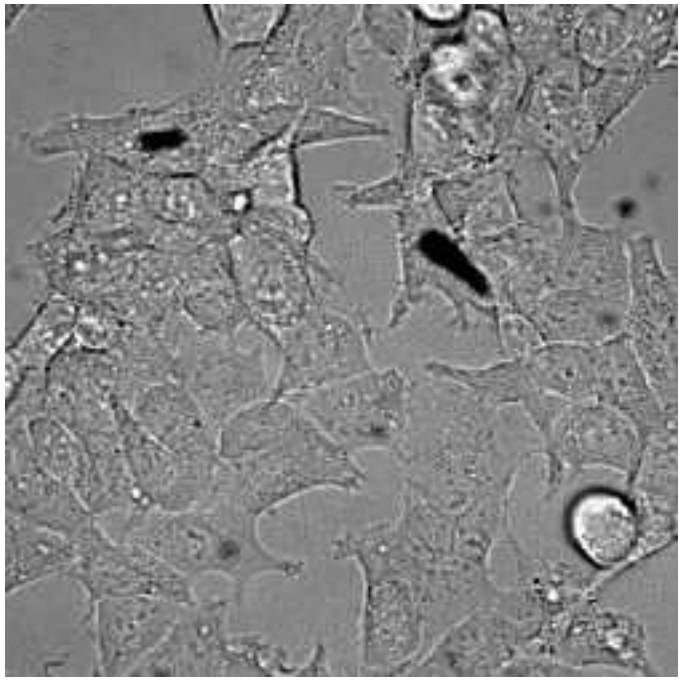


ACS Chem Biology, **2018**, *13*, 2758

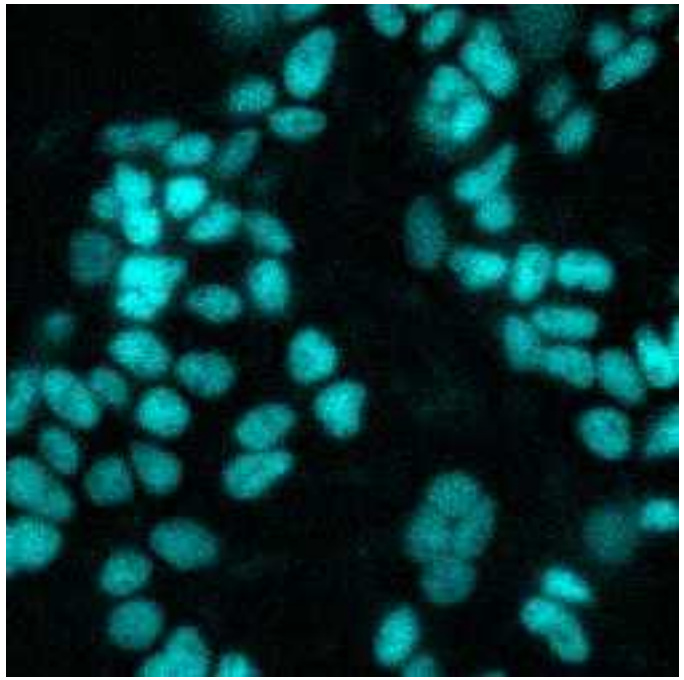
- Can we understand the key steps for efficient and targeted degradation?
- Does this insight help development of therapeutic degradation compounds?

Luminescent imaging capturing endogenously tagged BRD4 degradation

Brightfield



CRISPR HiBiT-BRD4



- Treatment with MZ1 shows uniform loss of BRD4 over 2 hours
- Imaging performed on Olympus LV200

How do we look at degradation quantitatively?

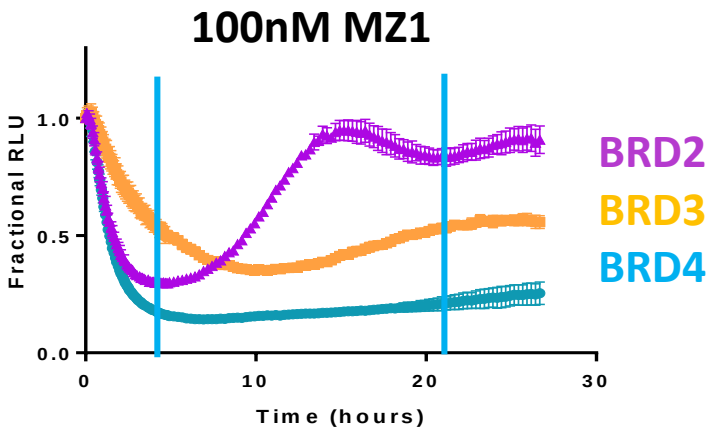
- Degradation rate
- Degradation IC50

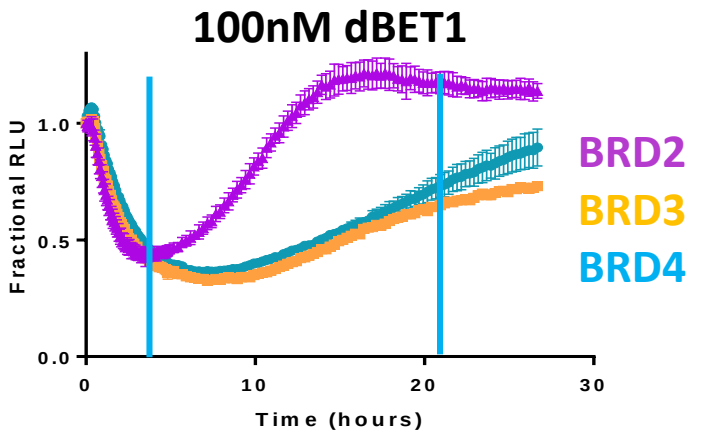
BET family cellular degradation profiles and recovery

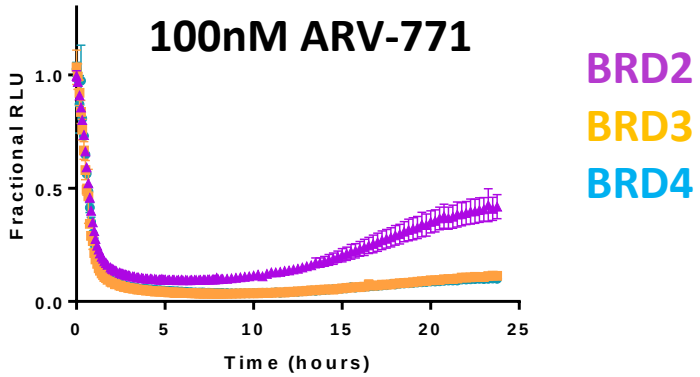
HiBiT (CRISPR)
BRD2, BRD3, or BRD4

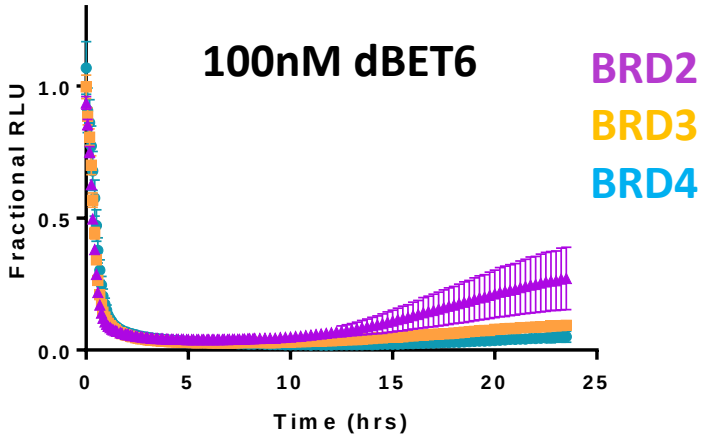
Endogenously tagged protein

\+ PROTAC



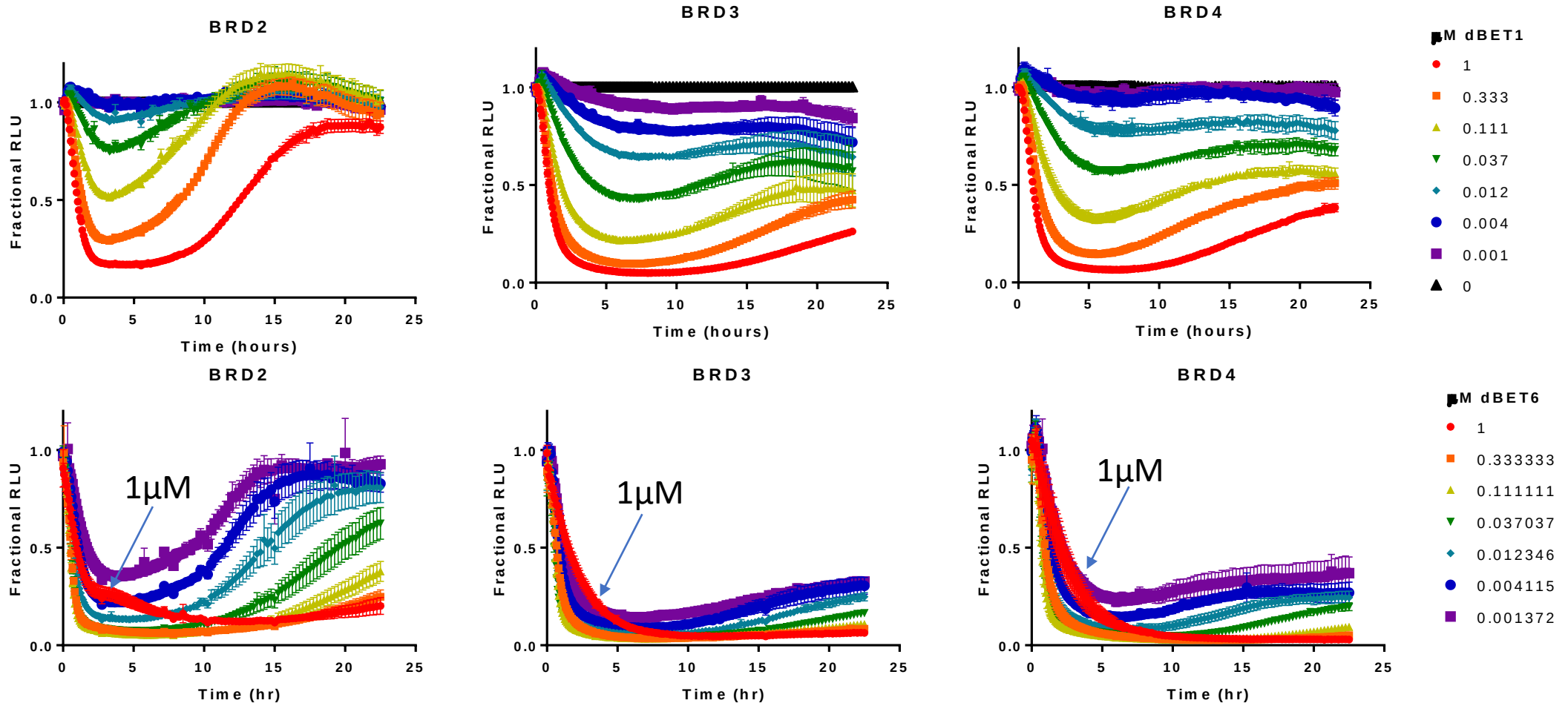






- Significantly different responses to all of the PROTACs
- Each family member reveals unique degradation and recovery response
- Time component is critical
- Where should dose response curves be performed to rank order compounds

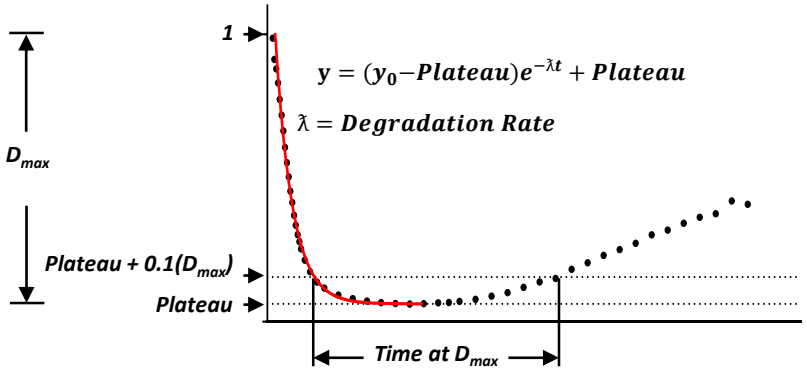
Live cell degradation profiles across concentration series (CRBN degraders)

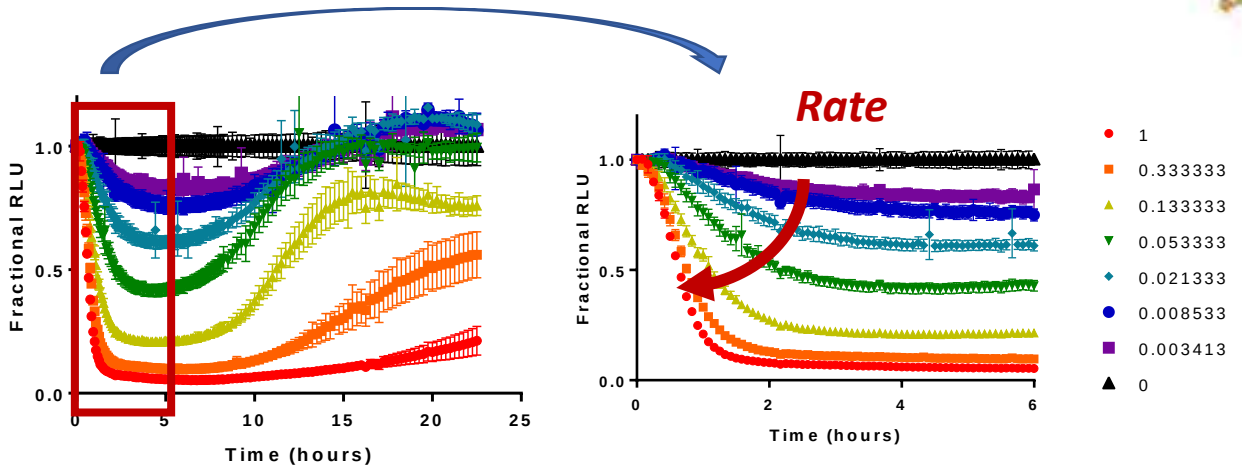


- dBET6 much more potent for degradation of all BET family members as compared to dBET1
- Degradation slows for dBET6 at concentrations greater than 100nM

A new way to look at degradation

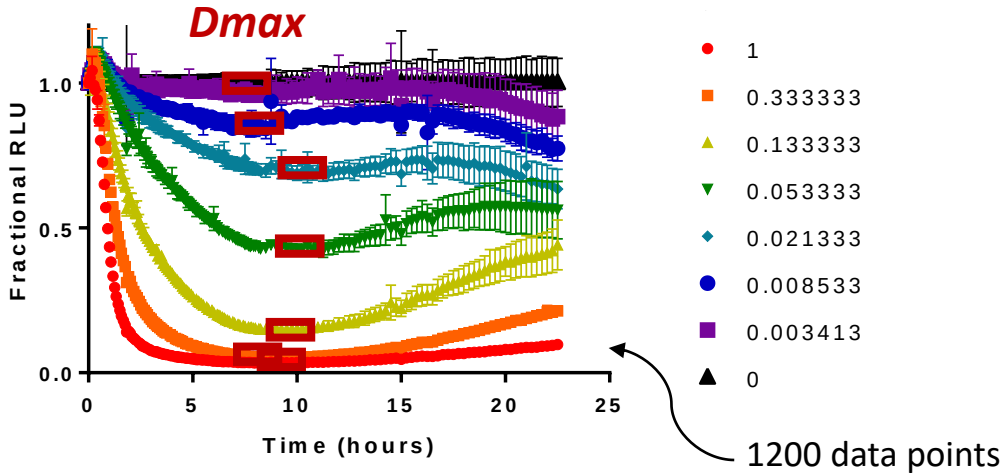
Degradation Profile





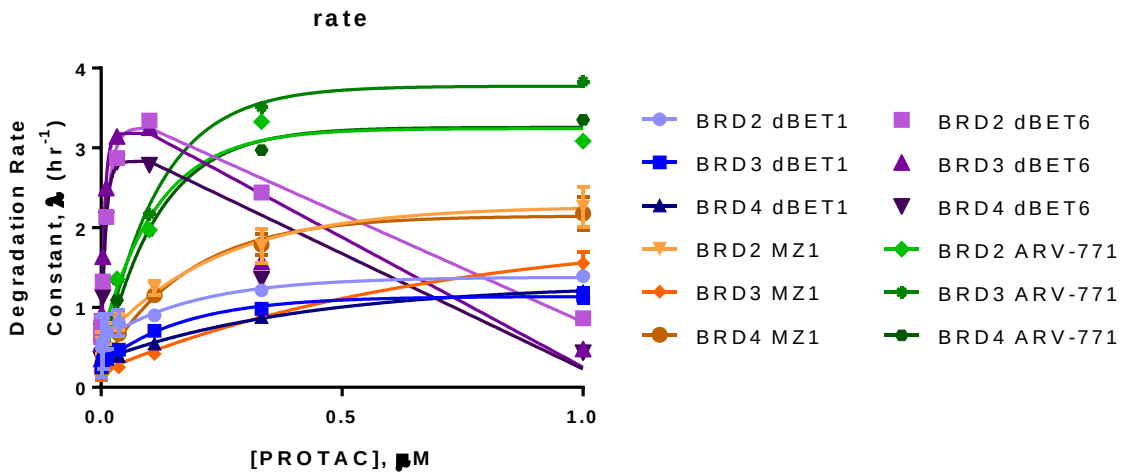
Quantitative Parameters for rank ordering:

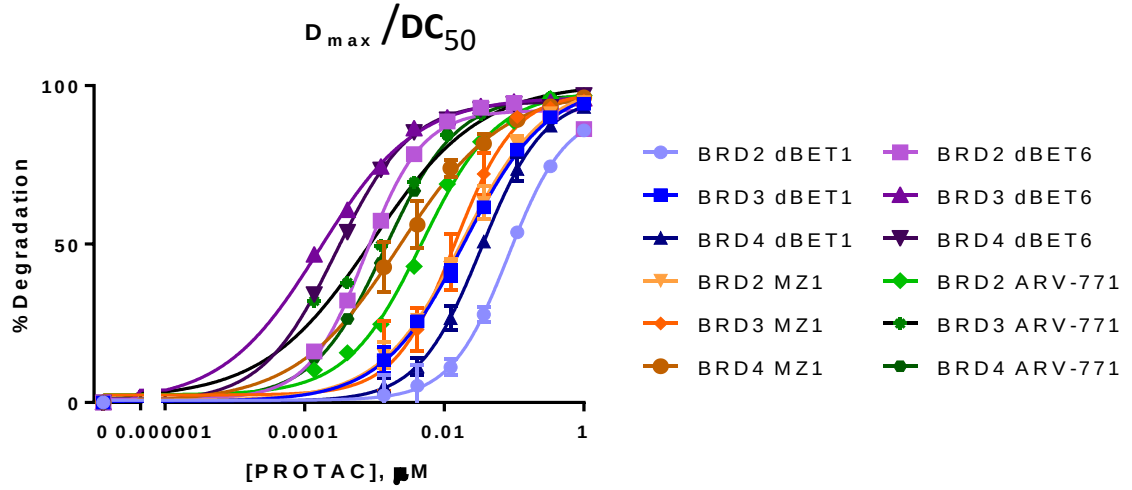
- *Rate of degradation*
- *Dmax*
- *Time at Dmax*



Can calculate multiple parameters in a single degradation profile.

Calculated cellular degradation rates and D_{max} of each BET family member





- Observe target and PROTAC dependent maximal rate of degradation
- The hook effect observed for rate, not observed for Dmax
- DC_{50} trends do not always correlated with rate
- dBET6 shows sharp decline in rate beyond 100nM concentration
- Allows for rank ordering of PROTACs and for each family member

DC_{50} Values (nM)

	MZ1	ARV-771	dBET1	dBET6
BRD2	18	4.8	83	0.74
BRD3	14	1	19	0.16
BRD4	2.3	1.4	34	0.28

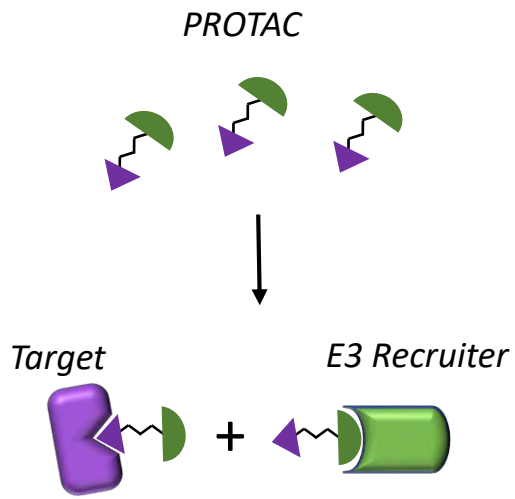
Seeking to understand improvement in dBET1 versus dBET6

DC_{50} *Values (nM)*

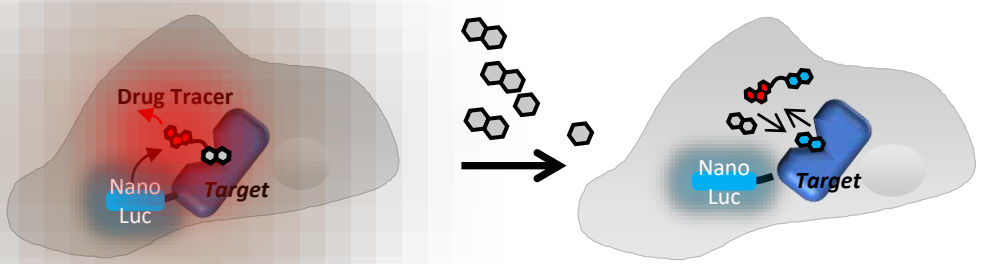
	dBET1	dBET6	Shift
BRD2	83	0.74	112X
BRD3	19	0.16	118X
BRD4	34	0.28	121X

Is this due to improved permeability and/or binding?

Monitor binding of PROTAC to E3 recruiter or target protein in live cells

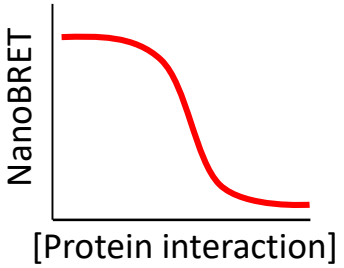


NanoBRET Target engagement



Intracellular binding

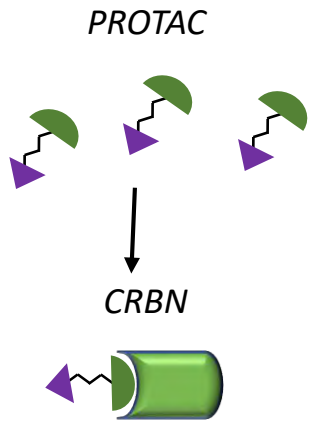
Intracellular affinity



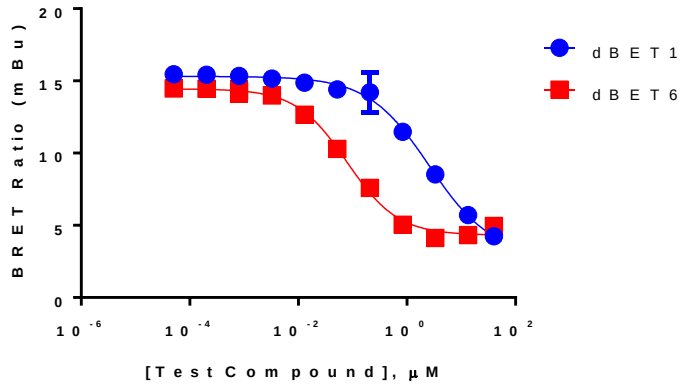
- Bioluminescence Resonance Energy Transfer to monitor protein:small molecule interactions
- Highly specific, biophysical measurement
- Monitor binding affinity, residence time and rank order compounds

Assessing improvement in binding and permeability on CRBN

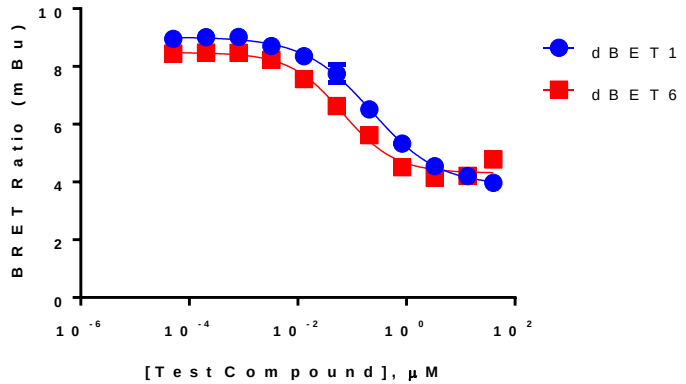
Cereblon NanoBRET Target Engagement



Live Cell



Lysate



IC_{50} Values (nM)

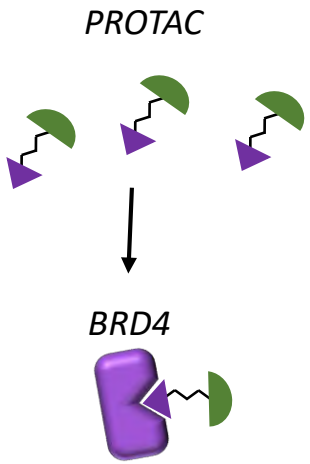
	dBET1	dBET6	Shift
CRBN	2600	79	33X

IC_{50} Values (nM)

	dBET1	dBET6	Shift
CRBN	230	66	3.5X

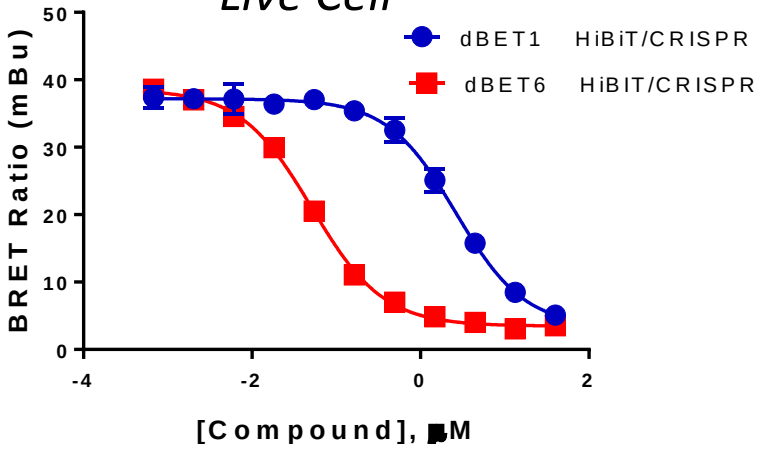
- Binding shift observed in live cells is partially due to improved cellular permeability of dBET6 as compared to dBET1

Assessing improvement in binding and permeability on BRD4



BRD4 NanoBRET Target Engagement

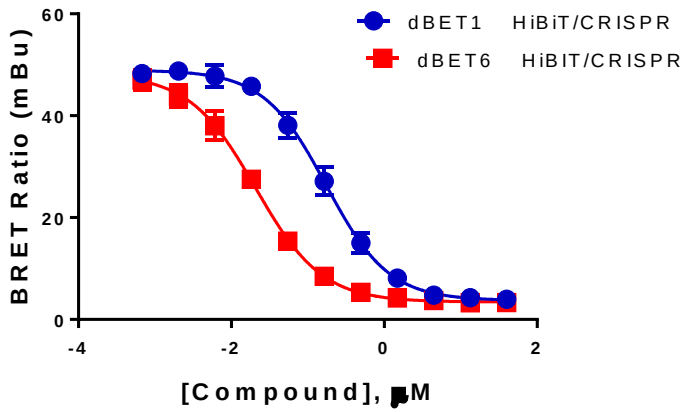
Live Cell



IC_{50} Values (nM)

	dBET1	dBET6	Shift
BRD4	2630	51	51X

Lysate



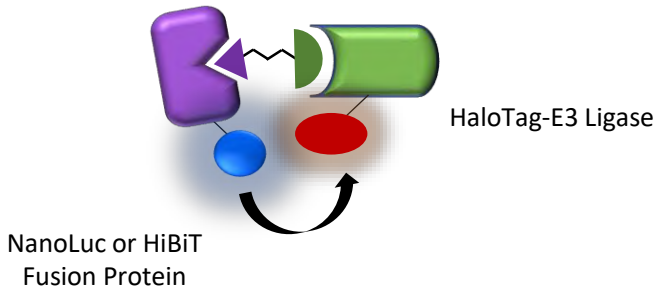
IC_{50} Values (nM)

	dBET1	dBET6	Shift
BRD4	174	21	8.3X

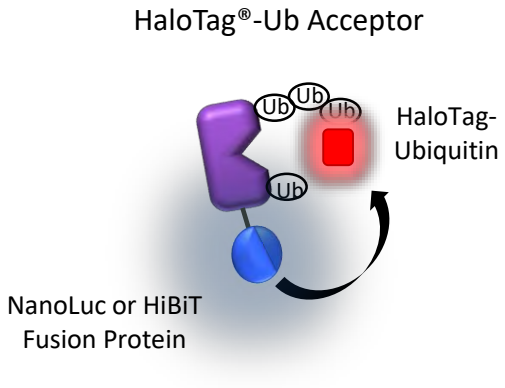
- Target engagement with BRD4 shows dBET6 improvement in BRD4 binding and cellular permeability
- These shifts do not account for the ~120X DC_{50} improvements and vastly different degradation rate plateaus

Monitoring interactions within the degradation pathway by NanoBRET

NanoBRET® Ternary Complex Assay



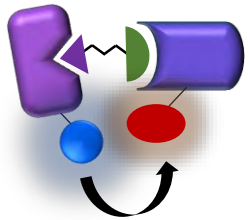
NanoBRET® Ubiquitination Assay

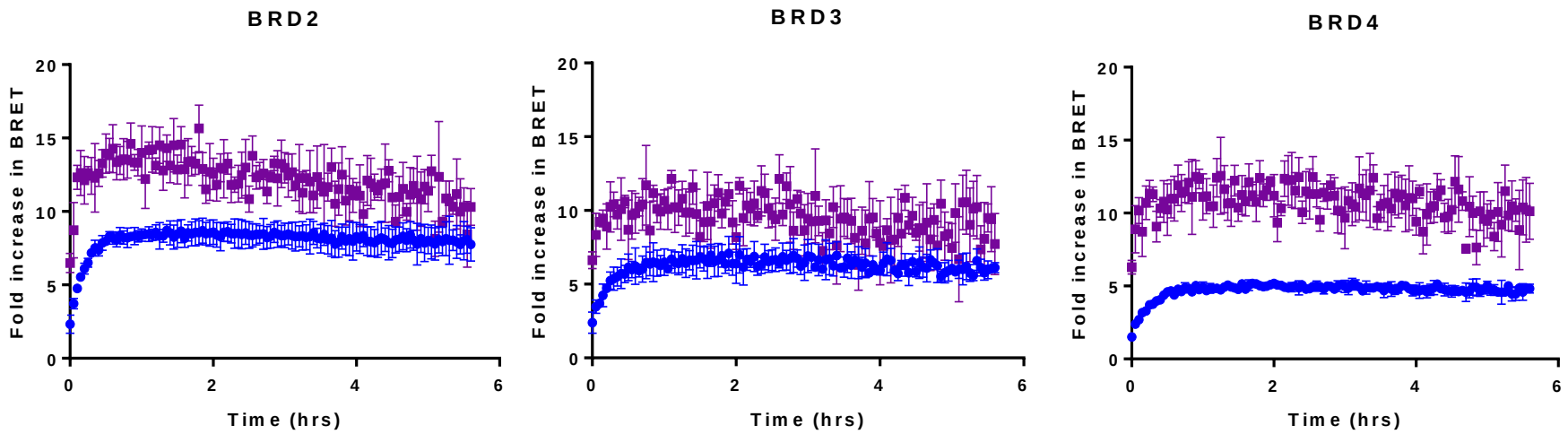


- NanoBRET® assays can used to study dynamics of interactions of protein degradation pathway
- NanoBRET is ratiometric; not impacted by the loss of target

Kinetic monitoring of ternary complexes with CRBN

NanoBRET
HiBiT BET-PROTAC-CRBN

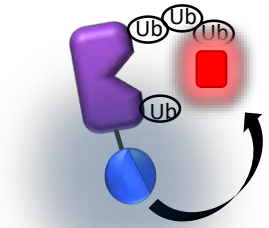


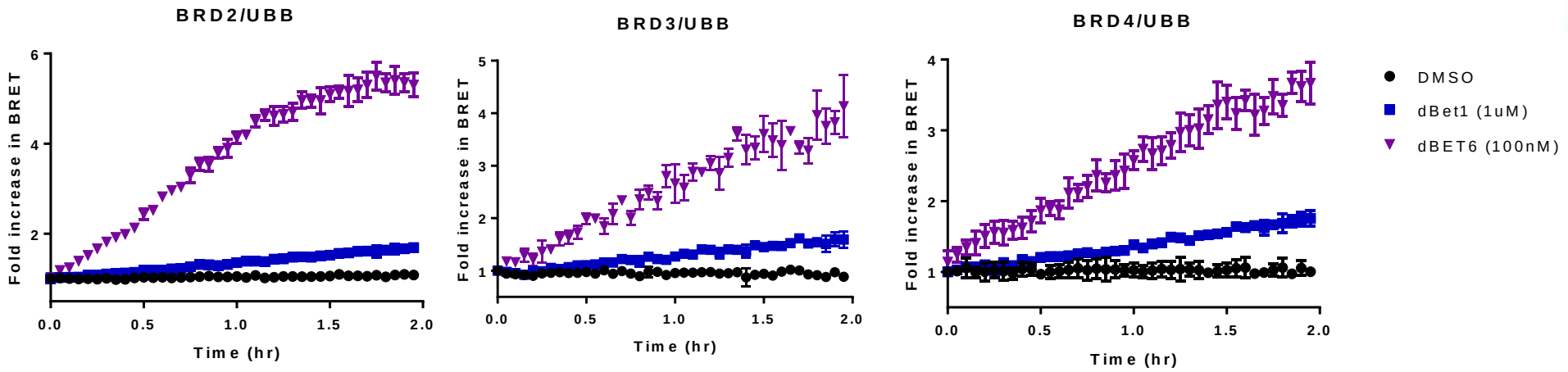


- Both dBET1 and dBET6 show rapid formation of ternary complexes inside cells
- dBET6 show much stronger signal for ternary complex formation, even at 10X lower concentration
- More robust ternary complex leading to more robust ubiquitination?

Kinetic monitoring of BET family ubiquitination

NanoBRET
HiBiT BET-HaloTag-Ub





- Ubiquitination signal increases faster and to greater extent with dBET6 compared to dBET1 across all family members

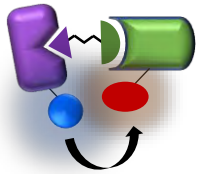
Summary:

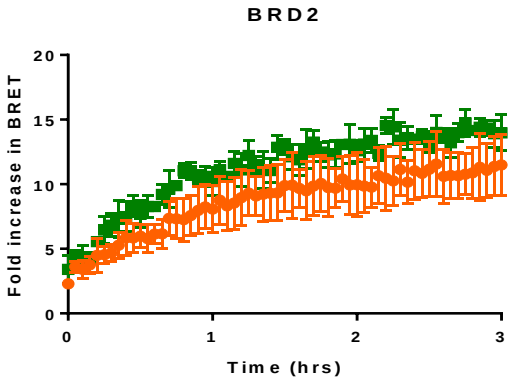
dBET6 has improved potency over dBET1 due to the following:

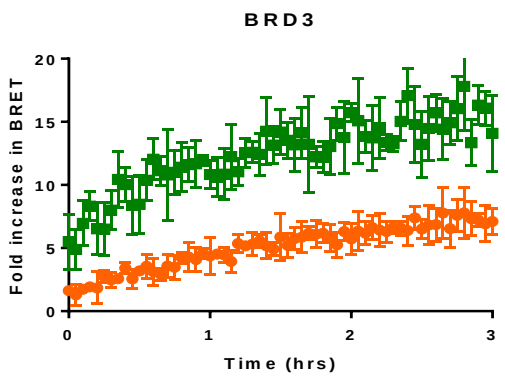
- Improved cellular permeability and binding target protein
- Increased rate of degradation
- Increased rate of ternary complex formation
- More efficient ubiquitination

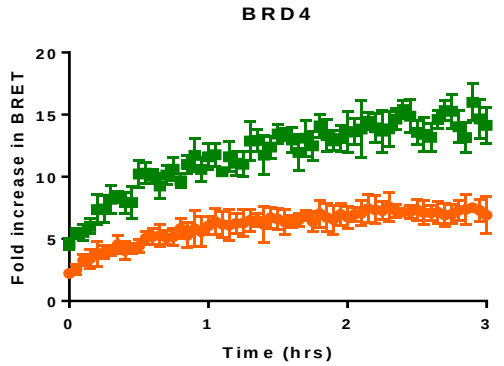
Kinetic monitoring of ternary complexes and ubiquitination with VHL

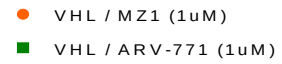
NanoBRET
HiBiT BET-PROTAC-VHL







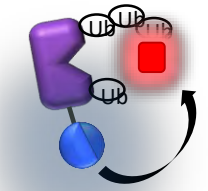


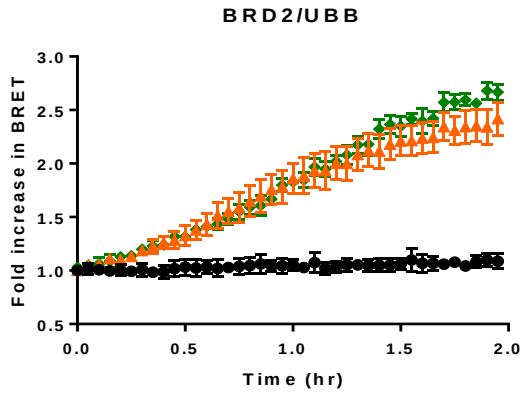


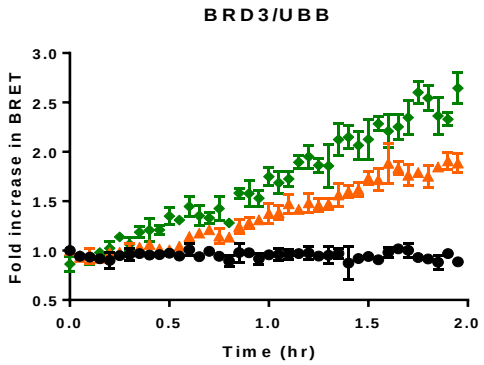
DC_{50} Values (nM)

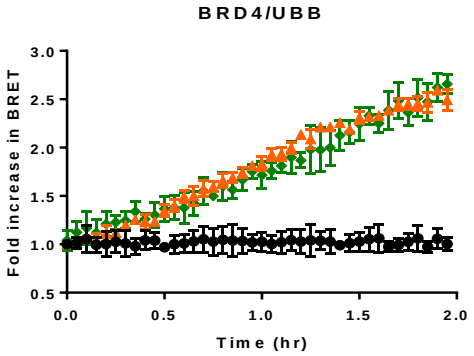
	MZ1	ARV-771
BRD2	18	4.8
BRD3	14	1
BRD4	2.3	1.4

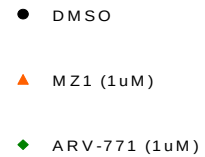
NanoBRET
HiBiT BET-HaloTag-Ub





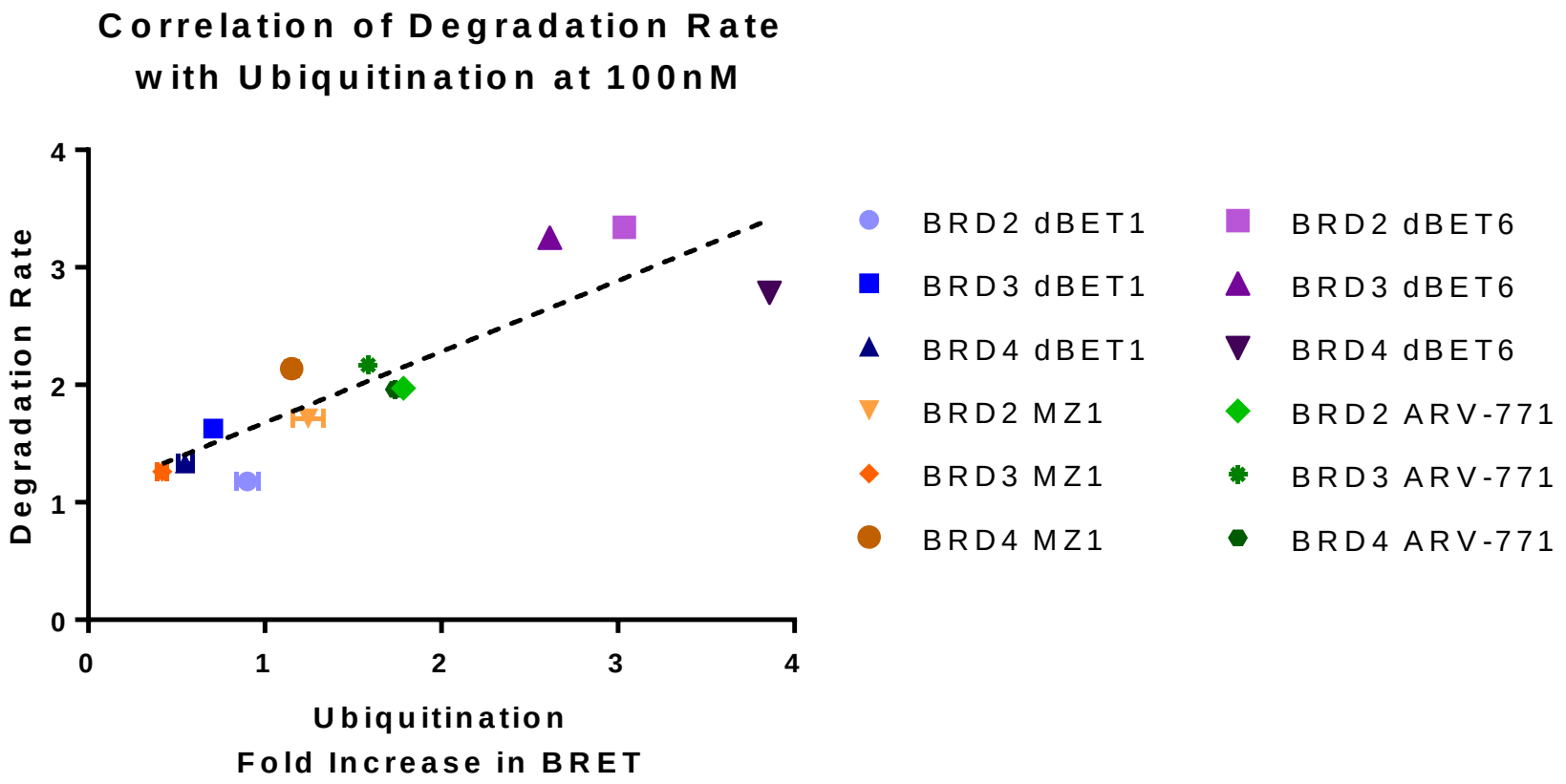






- ARV-771 shows increased levels of ternary complexes, particularly for BRD3
- Both MZ1 and ARV-711 have extended stable complex formation inside cell
- Differential ubiquitination observed on each target by each PROTAC

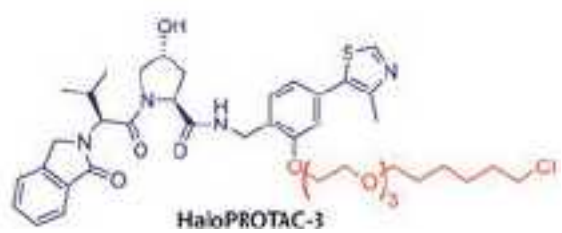
Ubiquitination extent directly correlated with degradation rate



- Observe a linear relationship between degradation rate and level of ubiquitination
- Ubiquitination is key step for determination of degradation rate

Targeted degradation phenotype using HaloTag PROTACs

HaloPROTAC



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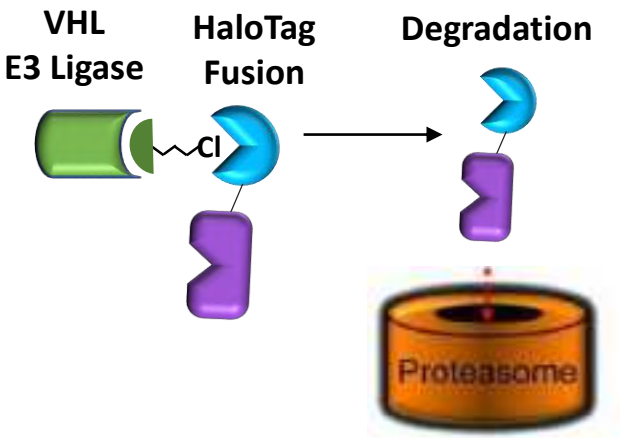
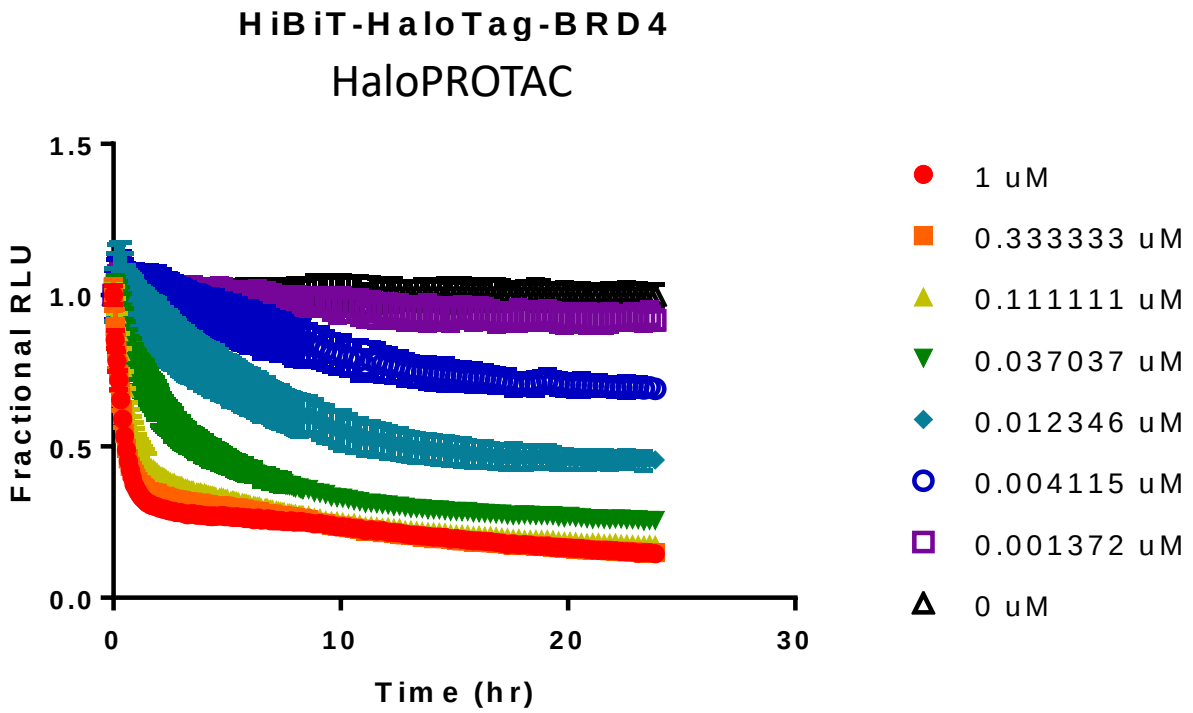


- Study phenotype of degraded targets
- HaloPROTAC targets HaloTag fusion proteins for degradation through VHL recruitment
- Help define targets important for PROTAC development

- Generate endogenously tagged HaloTag fusions
- Pair with HiBiT (HiBiT-HaloTag) for easy detection
- Characterize kinetics of degradation for variety of proteins

Kinetic degradation profiles of HiBiT-HaloTag-BRD4 with HaloPROTAC



- Efficient loss of endogenous HiBiT-HaloTag-BRD4 using HaloPROTAC, even at 100nM concentration
- HaloPROTAC also demonstrates burst/rapid degradation loss
- HiBiT could be appended to any fusion tag PROTAC to enable live-cell kinetic detection

Conclusions

- Modular program to enable live cell kinetic studies of targeted protein degradation
- Can be used with CRISPR to study endogenous proteins
- Enables quantitation of key degradation parameters
- Monitor dynamic formation of ternary complex and protein ubiquitination
- Can assess PROTAC cellular permeability
- HaloPROTACs show rapid and efficient loss of HaloTag fusion, excellent for phenotypic analysis

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