NanoBRET™ in Live Cells as a Method to Assess E3 Ligase and Target Protein Occupancy for PROTACs

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1. Introduction

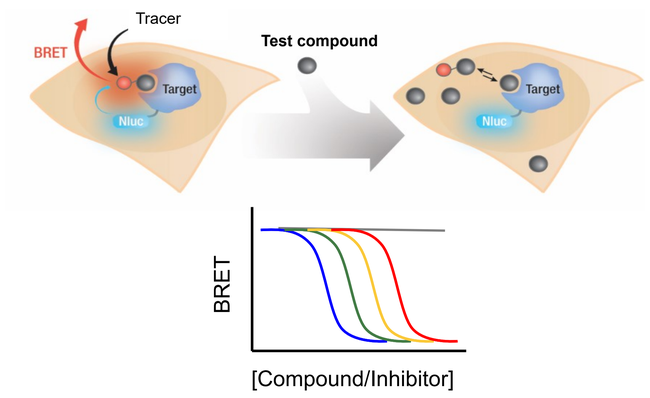
Proteolysis targeting chimeras (PROTACs) are bifunctional molecules that hijack ubiquitin E3 ligases and induce degradation of intracellular proteins through a tightly regulated proteosomal mechanism. Although several successful PROTACs have been developed against key intracellular target classes including bromodomains, kinases, and nuclear hormone receptors, these bivalent molecules often suffer from poor cell permeability due to high molecular weight.

To enable a high-throughput, quantitative readout for PROTAC cell permeability and E3 ligase occupancy in living cells, we have developed a panel of NanoBRET™ target engagement (TE) assays for key E3 ligase including CRBN, VHL, XIAP, cIAP, and MDM2. NanoBRET target engagement (TE) intracellular assays are the first biophysical method to enable the quantitative determination of compound occupancy, potency, and residence time for specific target proteins inside living cells using bioluminescent resonance energy transfer (BRET). This method has previously been applied to several other protein classes including kinases, bromodomains, and HDACs.

Here, we demonstrate that the NanoBRET platform allowed assessment of PROTAC cell permeability & E3 ligase occupancy. Using NanoBRET TE assays for proteins targeted by the E3 ligases, we obtained intracellular PROTAC binding kinetics. We further extend the analysis of PROTAC permeability as a dynamic process in real-time, using BRD-targeting MZ1 and dBET1 as a model system. Together these approaches allow a mechanistic interrogation of intracellular PROTAC permeability, E3 ligase occupancy, target occupancy, and target residence time.

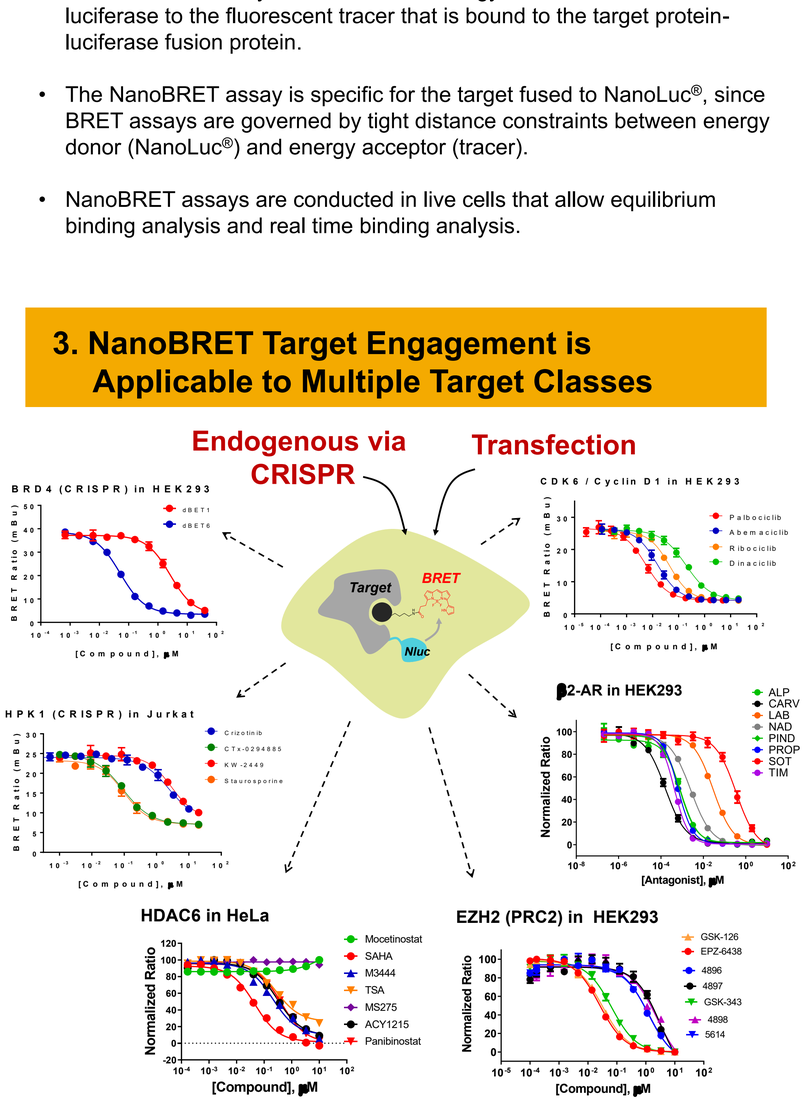
2. Target Engagement (TE) using BRET

Affinity / Potency Determinations



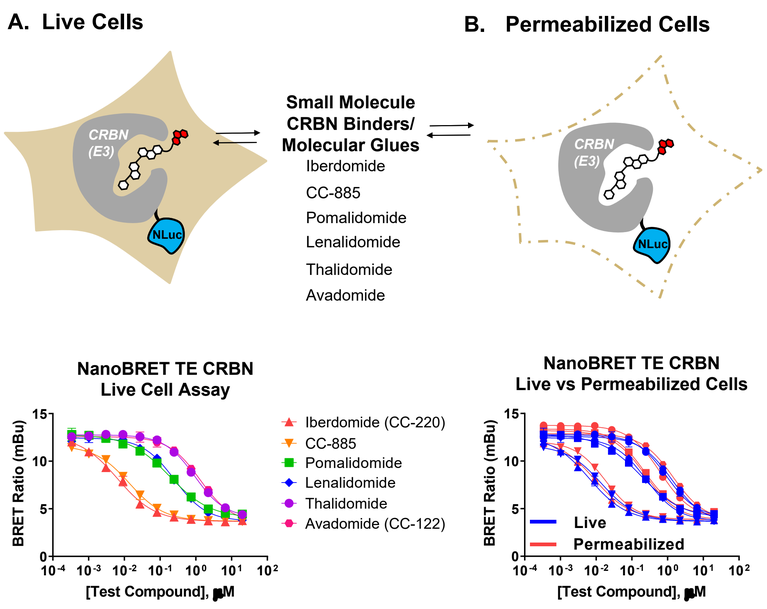
- BRET is achieved by the luminescent energy transfer from NanoLuc® luciferase to the fluorescent tracer that is bound to the target protein-luciferase fusion protein.
- The NanoBRET assay is specific for the target fused to NanoLuc®, since BRET assays are governed by tight distance constraints between energy donor (NanoLuc®) and energy acceptor (tracer).
- NanoBRET assays are conducted in live cells that allow equilibrium binding analysis and real time binding analysis.

3. NanoBRET Target Engagement is Applicable to Multiple Target Classes



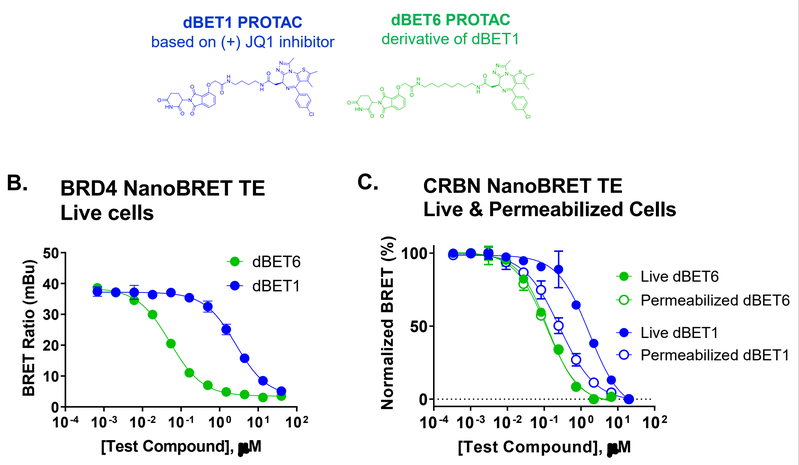
- NanoLuc-target fusion gene delivery is most often done via transfection for transient or stable expression. Additionally, fusion genes can be introduced to cells by CRISPR editing or viral particles.

4. Assess Cellular Binding & Permeability of Small Molecules to CRBN



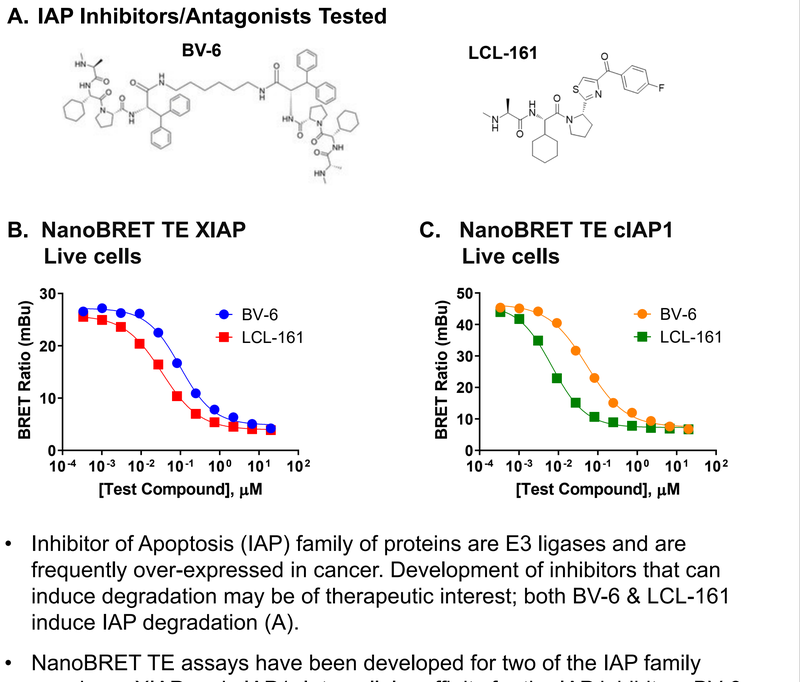
- NanoBRET TE CRBN assay can be run in live or permeabilized cells (A & B), enabling assessment of compound intracellular affinity and permeability.
- The live cell assay was used to quantify the apparent intracellular affinity of a series of CRBN binders that are also molecular glues (A).
- Using permeabilized cells (B), there was little change in IC_{50} observed compared to when the assay was run in live cells, indicating these compounds were readily cell permeable.

5. Quantitate PROTAC Occupancy at CRBN and Protein Targeted for Degradation



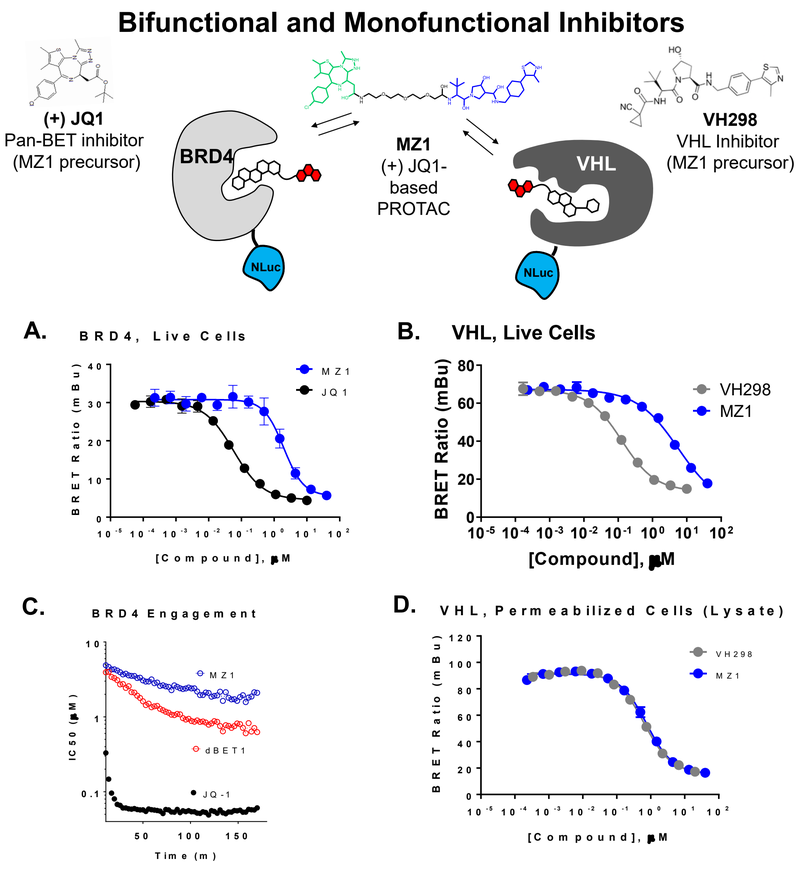
- PROTACs tested vary in the linkers connecting CRBN & BRD ligands (A).
- NanoBRET TE was used to quantitate PROTAC affinity for the BRD4 target in live HEK293 cells (B), showing dBET6 is 100-fold more potent. This can be a result of increased affinity for BRD4 target &/or cellular permeability.
- To understand PROTAC permeability, NanoBRET TE assay can be run in live cells or permeabilized cells (C). Comparing results from NanoBRET TE CRBN assay in live cells to permeabilized cells revealed that (1) dBET6 is more permeable than dBET1; (2) dBET6 is slightly more potent then dBET1.

6. Intracellular Affinity of IAP Inhibitors for XIAP and cIAP in Live Cells



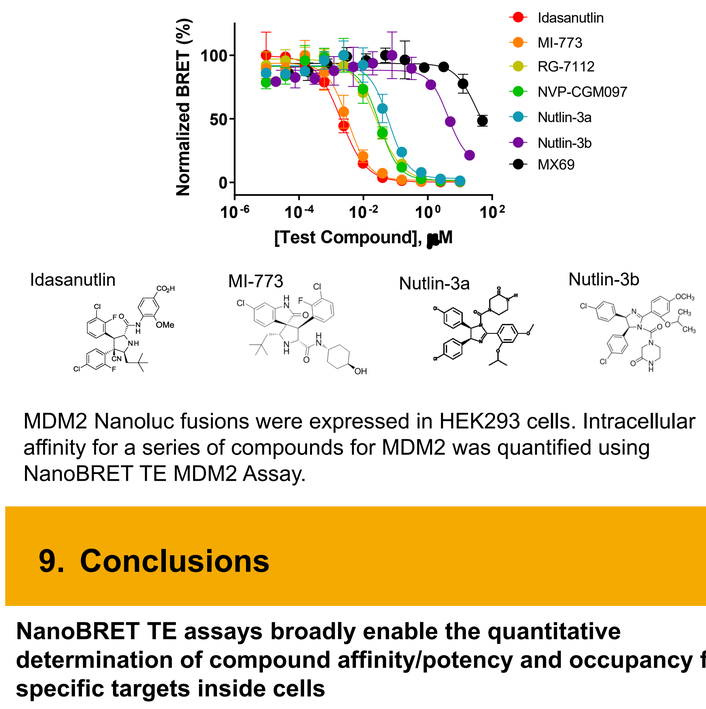
- Inhibitor of Apoptosis (IAP) family of proteins are E3 ligases and are frequently over-expressed in cancer. Development of inhibitors that can induce degradation may be of therapeutic interest; both BV-6 & LCL-161 induce IAP degradation (A).
- NanoBRET TE assays have been developed for two of the IAP family members, XIAP and cIAP1. Intracellular affinity for the IAP inhibitors BV-6 and LCL-161 were determined for both XIAP and cIAP1 in HEK293 cells (B & C).

7. PROTAC & Precursor Permeability Measured with BRD4 & VHL NanoBRET TE Assays



- Using target BRD4 NanoBRET TE assay in live cells, The PROTAC MZ1 is less potent compared to precursor JQ1 (A).
- NanoBRET TE VHL assays were used to assess permeability of PROTAC MZ1 and precursor VH298. In live cells, the MZ1 was ~40-fold less potent than VH298 (B). Since potency values are identical in permeabilized cells (D), the shift in live cell potency is due to decreased permeability of PROTAC MZ1.
- PROTAC & precursor potencies for BRD4 were measured in real time. Plotting compound IC_{50} vs time reveals slow equilibration of PROTACs MZ1 & dBET1 compared to JQ1 precursor (C).

8. Quantitative Measurement of MDM2 TE in Live Cells with Nutlin Derivatives



MDM2 Nanoluc fusions were expressed in HEK293 cells. Intracellular affinity for a series of compounds for MDM2 was quantified using NanoBRET TE MDM2 Assay.

9. Conclusions

NanoBRET TE assays broadly enable the quantitative determination of compound affinity/potency and occupancy for specific targets inside cells

- NanoBRET TE has been successfully used to interrogate live cell compound engagement for hundreds of intracellular targets, spanning a variety of protein classes in the human proteome.
- Cell permeable NanoBRET Tracers have been developed that allow TE assays for >200 full length kinases and five E3 ligases.

PROTACs, molecular glues, and small molecule inhibitors for E3 ubiquitin ligases can be assessed by NanoBRET TE assays

- Assays have been developed for the E3 ligases or adapter proteins including CRBN, VHL, XIAP, cIAP1, and MDM2
- NanoBRET TE assays can be run in live and lytic mode that aids in understanding compound permeability.
- NanoBRET TE live cell assays can be run in real-time to allow examination of kinetics of intracellular binding.

The NanoBRET TE method should facilitate the development of PROTAC and E3 ligase inhibitors with optimal cell permeability and target occupancy

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