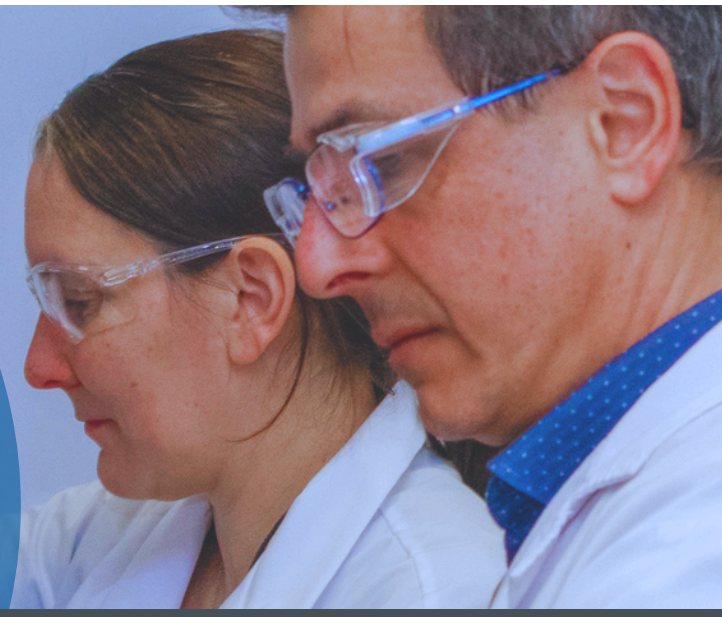
Paraza

Targeted Protein Degradation



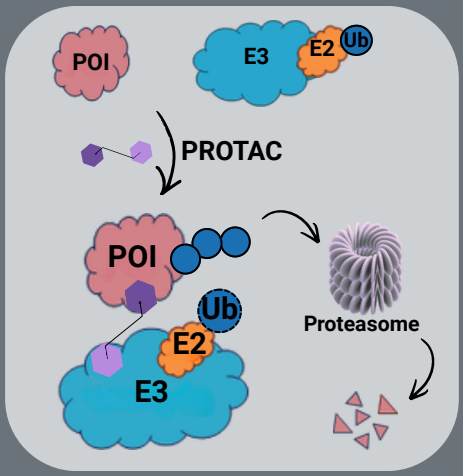
PROTAC® or molecular glues rely on the ubiquitin proteasome system (UPS) to degrade their target protein. Our biology department offers a suite of assays to develop and characterize this new class of therapeutic agents and has already supported more than 16 projects for targeted protein degradation drug development, providing flexible and know-how expertise. Paraza Pharma chemistry team can support PROTAC® and glue design and synthesis, while our DMPK team is ready to profile physicochemical properties/ADME/PK.

Binary Binding to POI or E3 Ligase

- SPR
- Fluorescence Polarization
- TR-FRET
- NanoBRET
- MST
- DSF

Ternary Complex Formation

- Fluorescence Polarization
- TR-FRET
- NanoBRET®/NanoBiT
- Co-operativity Studies
- Pull-down



Protein Degradation

- Western Blot
- Protein Simple JESS™
- HiBiT System
- AlphaLISA™
- MSD

Understanding MOA

- Degradation Kinetics
- Proteasome Dependency
- Competition Assays
- Ubiquitination Assays

Cellular Functional Assay

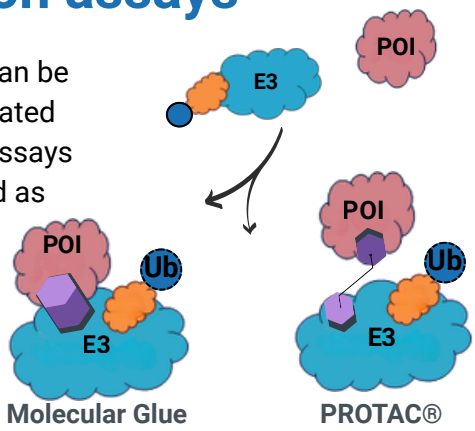
- Downstream Markers
- Cytotoxicity/Apoptosis
- Signaling Pathways
- Cell Cycle
- Gene Expression
- Migration/Invasion

Download all our white papers here:

Ternary complex formation assays

PROTAC® or molecular glue-mediated ternary complex formation can be studied by proximity-based assays in which small molecules are titrated against labeled ubiquitin E3 ligase and protein of interest (POI). In assays such as TR-FRET and NanoBRET®, a fluorescent signal is generated as the target and E3 ligase interact.

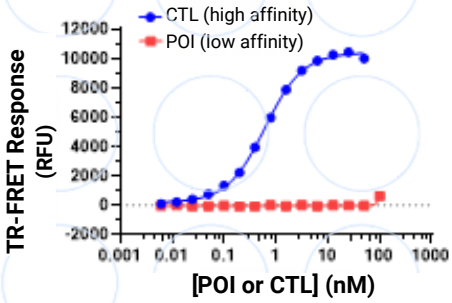
Notably, in the absence of cooperative binding, PROTACs may independently saturate the POI and E3 ligase at high concentrations, leading to a characteristic “hook effect” that is not observed with molecular glues.



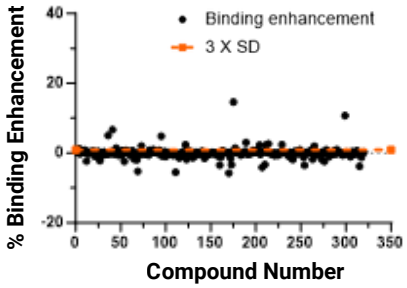
TR-FRET biochemical assay for compound screening

We used TR-FRET to discover molecular glues capable of enhancing interaction between a disease-relevant mutant protein and its natural E3 ligase, to trigger target degradation. Approximately 10 000 compounds were screened and compounds showing binding enhancement were selected for further validation.

A)



B)



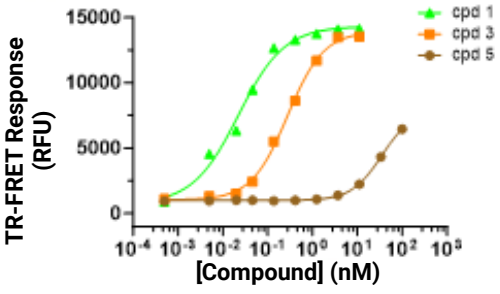
	No POI	High affinity CTL
Average	0.23	1.06
STDEV	0.004	0.048
CV (%)	1.6	4.5
S/B	4.7	
Z'	0.81	

Fig 1. TR-FRET assay to screen molecular glues.
A) TR-FRET assay to measure complex formation of high affinity CTL protein (blue) and low affinity target protein with E3 ligase (red). B) Compound screen in 384-well plates, 10uL volume, 10nM E3 complex and 20 nM POI, compounds tested at 10 µM. Statistics of plate indicate excellent assay performance.

Dose response formats for structure-relationship activity (SAR)

Hits identified during the screen were optimized to improve potency. Two assay formats were used to study SAR: first, compounds were titrated against fixed concentrations of target and E3 ligase to determine EC50s; next, the POI or the positive control protein were titrated against a fixed concentration of compounds to estimate affinity.

A)



B)

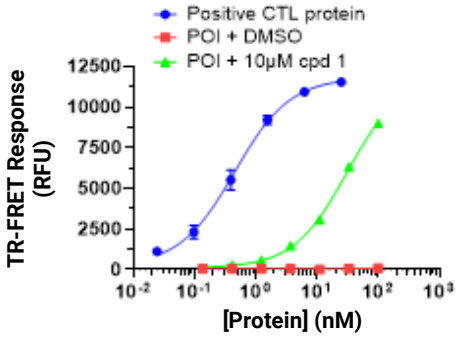


Fig 2. Assay format to profile molecular glues.
A) Active compounds were tested in TR-FRET with 0.3 nM E3 protein and 20 nM POI. B) Binding enhancement with Cpd1 was confirmed when titrating the low affinity POI in TR-FRET assay.

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Quantification of Target Protein Degradation

Protein degradation induced by a PROTAC® or molecular glue can be evaluated by various methods including traditional western blot, JESS™ (Protein Simple), and MSD. Plate-based assays such as AlphaLISA™ and HiBiT offer higher throughput methods suitable for SAR-supporting assays and more complex mechanistic studies. At Paraza, we have experience in optimizing all these assays to follow protein degradation in various cell types.

HiBiT luminescence assay to measure POI levels

HiBiT is a small peptide tag that produce a bioluminescent signal when bound to its complementation partner, LgBiT, to reconstitute NanoLuc®. The HiBit system can be used to follow endogenous protein level and measure the effect of PROTAC®/Molecular glues on POI degradation in cellular models. Through careful optimization of assay conditions, dose-response curves can avoid the "hook-effect" region.

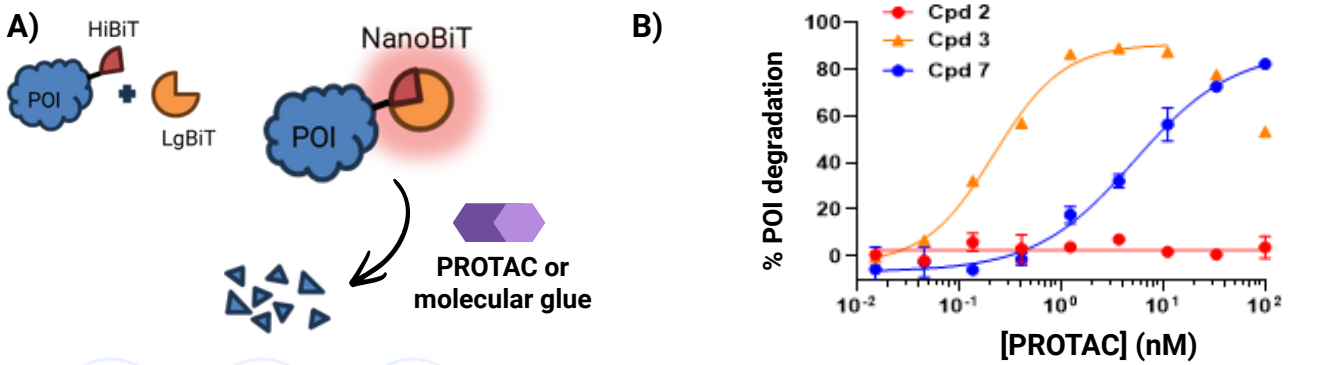


Fig 3. HiBiT assay for PROTAC testing.
A) Principle of the HiBiTsystem. B) A 384-well plate–based assay was implemented with a cell line expressing HiBiT-tagged POI. Treatments with compounds 3 and 7 resulted in POI degradation. For the most potent PROTAC® (Cpd 3), a hook effect is observed at high concentration.

HiBiT cell lines targeting downstream effectors

We used two HiBiT systems to investigate the potency of a lead PROTAC® on the inactive *vs.* active form of a transcription factor by first following its degradation and next, the expression of one of its target genes. PROTAC® 1 degraded the activated transcription factor and led to a dose-dependent downregulation of a downstream target gene.

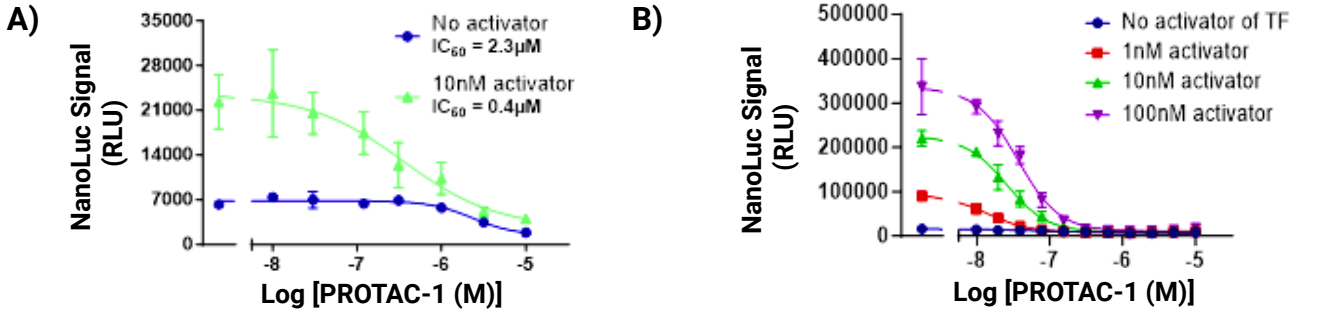


Fig 4. HiBiT assay to monitor PROTAC®-induced degradation of a transcription factor and modulation of its downstream target gene.
A) HitBiT assay to follow transcription factor protein degradation under basal or activated conditions. B) HiBiT assay following a downstream target gene, whose inducible expression depends on the POI.

Confirming Proteasomal Degradation

The goal of induced proximity between the POI and E3 ligase is to promote ubiquitination of the target to tag it for degradation by the 26S proteasome. However, formation of a ternary complex does not always lead to a productive conformation, and direct proof of PROTAC®-induced ubiquitination is important to confirm on-target mechanism of protein degradation. Through the use of proteasome inhibitors (MG132, bortezomib), NEDDylation inhibitors (MLN4924) and E3 ligase-specific inhibitors, involvement of the UPS can be demonstrated.

Ubiquitination assays

Traditionally, protein K48-linked ubiquitination is monitored by immunoprecipitation of the protein of interest under stringent conditions followed by immunoblot against ubiquitin, to generate the characteristic smear of polyubiquitination. More recently, methods such as the NanoBRET® Ubiquitination Live-Cell System (Promega), which combines a HaloTag-Ub fusion protein and a HiBit system for the POI, allow high-throughput monitoring of protein ubiquitination in 96- or 384-well formats.

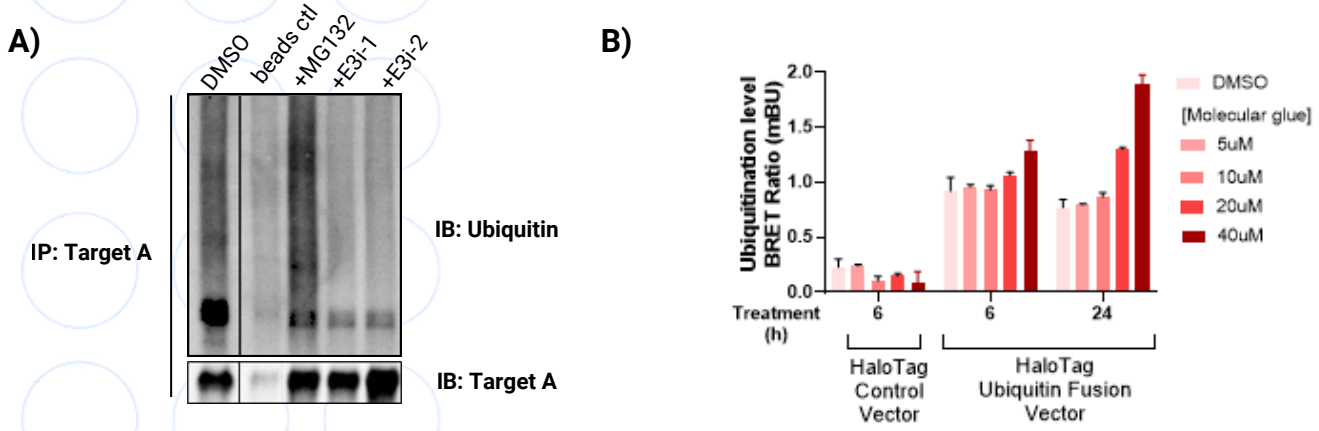


Fig 5. Assays to detect ubiquitination.
A) Detection of ubiquitination by immunoprecipitation. Cell treatments: vehicle (DMSO, lane 1), proteasome inhibitor MG132 (lane 3), specific inhibitors of E3 ligase (lanes 4,5).Control with beads alone (no antibody) is shown in lane 2. B) 6h and 24h PROTAC® treatment showed time and dose-dependent increase of the in POI ubiquitination in HaloTag® Ubiquitin Fusion vector transfected cells.

UPS inhibition assay

UPS inhibitors or E3 ligase competitor ligands can be used to confirm the mechanism of degradation induced by PROTAC®/molecular glues. In the following example, POI degradation induced by PROTAC® was reduced following co-treatment with neddylation inhibitor or a VHL ligand, indicating that the PROTAC® induce degradation of the POI through the UPS.

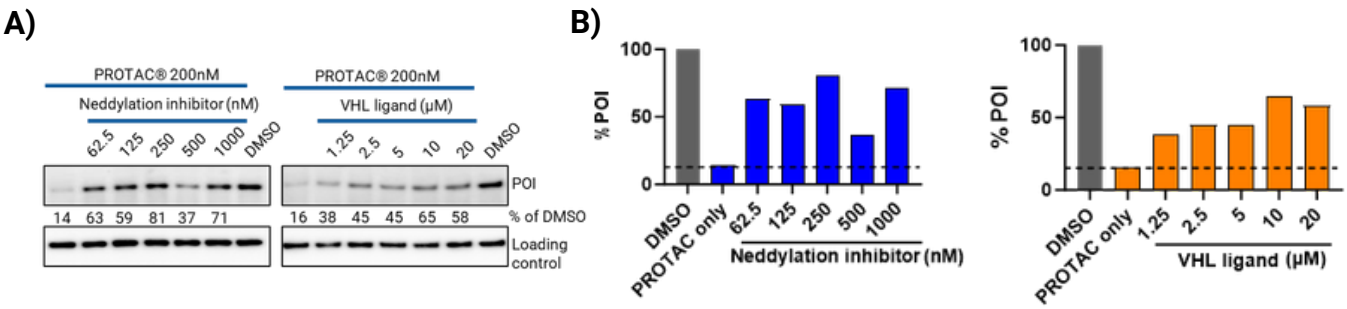


Fig 6. POI degradation induced by PROTAC® decreases with UPS inhibitor or E3 ligase ligand.
A) Cells were treated with the PROTAC® at DC90 and increasing doses of Neddylation inhibitor or VHL ligand. A) Western blot detecting level of POI B) Relative quantification of bands by densitometry, normalized to loading control and expressed as percentage relative to DMSO control.

Downstream Fuctional Assays

Efficient targeted protein degradation will cause downstream phenotypic changes that can be measured in cellular models, including but not limited to apoptosis, cell cycle arrest, impairment of migration and invasion and modulation of downstream signaling pathways. These measurements provide important indications on drug function and help in selection of lead compounds before *in-vivo* testing.

Cell death and cell growth arrest

2D cell culture is a standard *in-vitro* model for evaluating TDP phenotypic responses. In the example below, a PROTAC® was assessed for its impact on apoptosis and cell growth inhibition. When target degradation reached 90% (DC90), a significant increase in apoptotic cell death was observed. 3D cell culture models, like spheroids derived from various cell lines can be used to better mimic tumor conditions. Their growth can be monitored using the BioTek Cytation 5 and the Incucyte® live-cell imaging system.

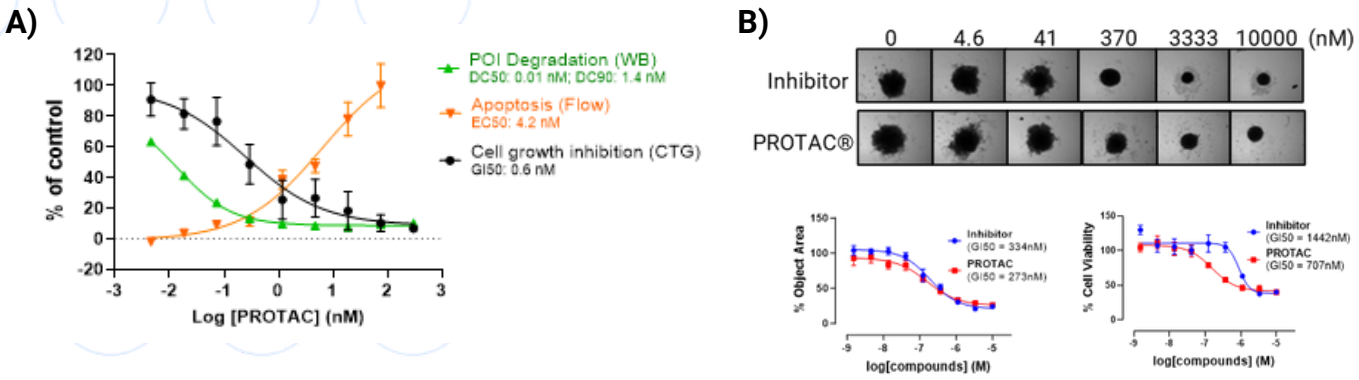
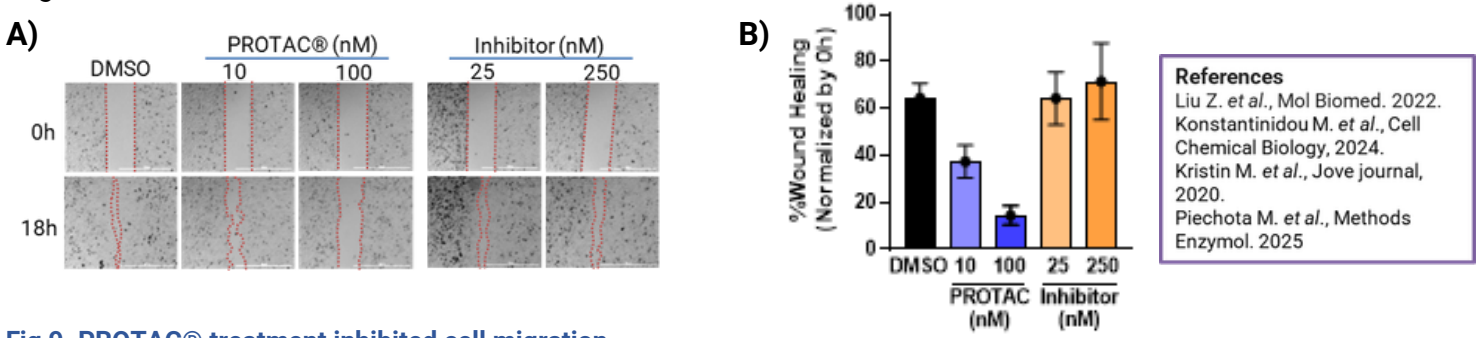


Fig 7. Target degradation and effect on downstream cellular responses.
A) Protein degradation by PROTAC® (by Western blot) leads to increase of apoptosis (quantified by Flow cytometry) and decrease of cell growth (measured by CellTiterGlo®). B) Spheroids were treated with PROTAC® or inhibitor for 7 days and GI50 was determined with 3D-CellTiter Glo or by size measurement using Cytation 5 imaging system.

Cell migration by scratch assay

The wound healing assay (or scratch assay) is used to evaluate the effect of compounds on cell migration. This assay has a role in many disease contexts; in particular results can be used to measure effects of test compounds which may reduce invasion of cancer cells. This technique involves creating a cell-free gap in a confluent cell monolayer to monitor the rate at which cells migrate to close the gap. We show here that the PROTAC® is more potent than its corresponding inhibitor in suppressing cell migration.



References
Liu Z. *et al.*, Mol Biomed. 2022.
Konstantinidou M. *et al.*, Cell Chemical Biology, 2024.
Kristin M. *et al.*, Jove journal, 2020.
Piechota M. *et al.*, Methods Enzymol. 2025

Fig 9. PROTAC® treatment inhibited cell migration.
Cells were treated with a PROTAC® or an inhibitor targeting the same POI. A) Images taken at the same location at time 0h and 18h post treatment using a Cytation 5 imaging system. B) Quantification of gap distance.

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