Recent advances in targeted protein degradation research in cancer.

Here, we review recent literature reports that explore targeted protein degradation with no-wash immunoassays.

The cell relies on two main systems to breakdown unwanted, faulty, or damaged proteins: the ubiquitin–proteasome system (UPS) and lysosomal proteolysis. The UPS typically degrades short-lived, soluble proteins; whereas lysosomal proteolysis handles insoluble, long-lived proteins, as well as larger particles, such as aggregated proteins, organelles, and bacteria (Figure 1).[1] These systems can be "hijacked" by small molecules to degrade pathological proteins—a therapeutic strategy called targeted protein degradation (TPD).[2-4] TPD has gained traction in recent decades, particularly as an anti-cancer therapy, where five anti-cancer TPD therapies have been approved and over 1000 clinical trials involving cancer and TPD were reported between 2012 and 2022.[5,6] TPD offers a number of advantages as a therapeutic strategy for cancer, such as:

- TPD can tackle "undruggable" proteins, which are proteins that traditional therapeutic approaches struggle to act on. Undruggable proteins can lack sites or pockets for ligands to interact with, which can make drugs such as inhibitors ineffective.[6-9]
- TPD agents (or degraders) are efficient as they can trigger degradation of one copy of a target protein, then reattach and act on a different copy of a target protein—acting in a catalytic manner.[6]

Here, we discuss recent literature reports of TPD with a focus on cancer, including designing and testing new degraders, as well as developing TPD for cancer targets.

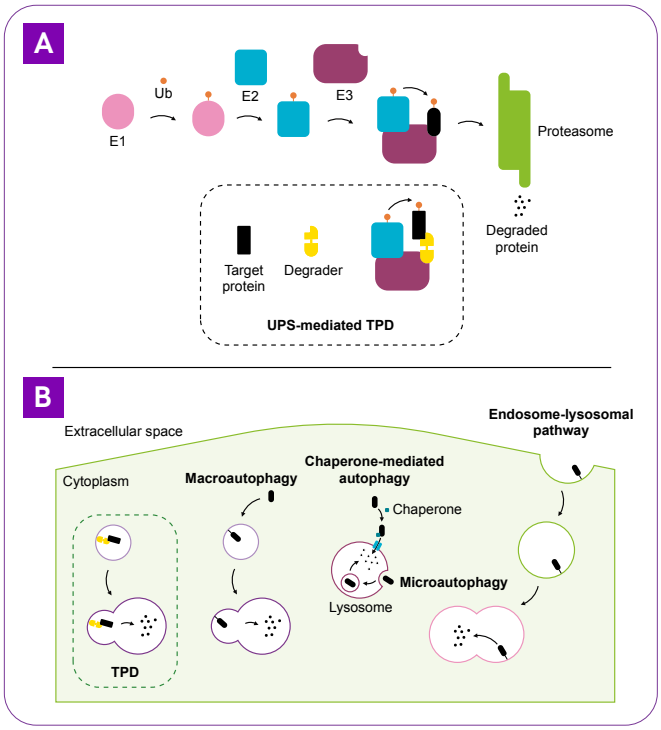

Figure 1. Overview of degradation pathways: UPS (A) and lysosomal proteolysis (B) with example TPD strategies.

Types of degraders

A wide variety of degraders have been developed so far, which can target either the UPS or the lysosomal degradation pathways. Among the many types of degraders that can act through the UPS, two of the most clinically advanced examples are:[10]

- Proteolysis-targeting chimeras (PROTACs) — small molecules that recruit an E3 ligase to a protein of interest, allowing ubiquitination and protein degradation via the UPS. A PROTAC is a small molecule containing a structure that binds to a target protein tethered to a structure that binds to an E3 ligase, making these small molecules heterobifunctional.[6]
- Molecular glue degraders — molecular glues interact with or alter protein surfaces, creating new protein–protein interactions and increasing binding affinity between proteins.[6] Molecular glue degraders can improve the binding affinity between a protein of interest and an E3 ligase, allowing the protein to be ubiquitinated and degraded via the UPS. For example, immunomodulatory imide drugs (IMiDs), such as thalidomide, are a type of molecular glue that bind to the E3 ligase cereblon, altering the surface of cereblon and allowing it to bind to and act on a target protein.[11]

In addition, a variety of degraders that breakdown proteins via lysosome proteolysis have been developed, such as:

- Lysosome-targeting chimera (LYTAC) — a molecule that has a structure that binds to the target protein (such as an antibody) and a structure that attaches to a lysosome-targeting receptor (such as a glycan), triggering lysosomal degradation of the target protein.[1,12]
- Autophagy-tethering compound (ATTEC) — a compound that involves a structure that binds to the phagophore/autophagosome protein, LC3, and a structure that binds to a target protein, leading to degradation of the target protein via the autophagosome–lysosomal pathway.[1,13]
- Antibody-based PROTAC (AbTAC) — a bispecific antibody (an antibody that can bind two different antigens) that binds to a cell surface E3 ligase (such as RNF43) and a cell surface target protein, leading to degradation of the target protein via the endosome–lysosomal pathway.[14,15]

Designing new PROTACs: Application of the ELIOT (E3 LIgase pocketOme navigaTor) platform (Palomba et al.[16])

Despite humans having more than 600 E3 ligases, only a small proportion of these are targeted by PROTACs.[17] This can limit the use of current PROTACs as E3 ligase expression can vary between tissues, restricting where a PROTAC can act and the conditions it can target. Cancer cells have also been shown to develop resistance to two commonly-used E3 ligases in TPD: cereblon and Von Hippel–Lindau (VHL). Consequently, tapping into the wider E3 ligase pool may widen the therapeutic applicability of PROTACs – and TPD generally – as a cancer treatment.[16] To do this, Palomba et al. recently developed the ELIOT (E3 LIgase pocketOme navigaTor, https://eliot.moldiscovery.com) platform—which can help find and design ligands to recruit new E3 ligases for PROTACs (Figure 2).[18]

The ELIOT platform includes human crystallographic structures reported in the Protein Data Bank (PDB) and consists of an E3 ligase pocketome, involving "all possible pockets on the surface of the E3 enzymes".[16] By analyzing the similarity between these pockets through GRID molecular interaction fields (MIFs) – termed cross-relationship analysis – the platform identifies similarities between E3 ligase binding sites to help design ligands that can engage new E3 ligases. As many of the E3 ligase structures in the ELIOT pocketome are ligand free, cross-relationship analysis can be used to repurpose scaffolds or ligands from other similar E3 ligase pockets that do contain ligands in the ELIOT platform. To demonstrate the ability of ELIOT to help design new PROTACs, Palomba et al. provide a case study of the E3 ligase: tripartite motif containing 33 (TRIM33)—which has two isoforms, TRIM33α and TRIM33β, where TRIM33α has a 17-residue amino acid insert in its bromodomain (BRD).[16]

Cross-relationship analysis revealed that TRIM33 has a highly similar binding site to TRIM24—a well-characterized E3 ligase that has 12 ligand-bound crystalized structures already reported. Assuming similar pockets may bind similar ligands, Palomba et al. began the design of TRIM33 ligands by using the common scaffold of the reported TRIM24 ligands. Cross-relationship analysis also revealed key differences between TRIM24 and TRIM33 binding sites. For example, the entrance of the binding pocket is more polar in TRIM33 (both isoforms) due to its glutamate residue (GLU981)—compared to TRIM24, which has an

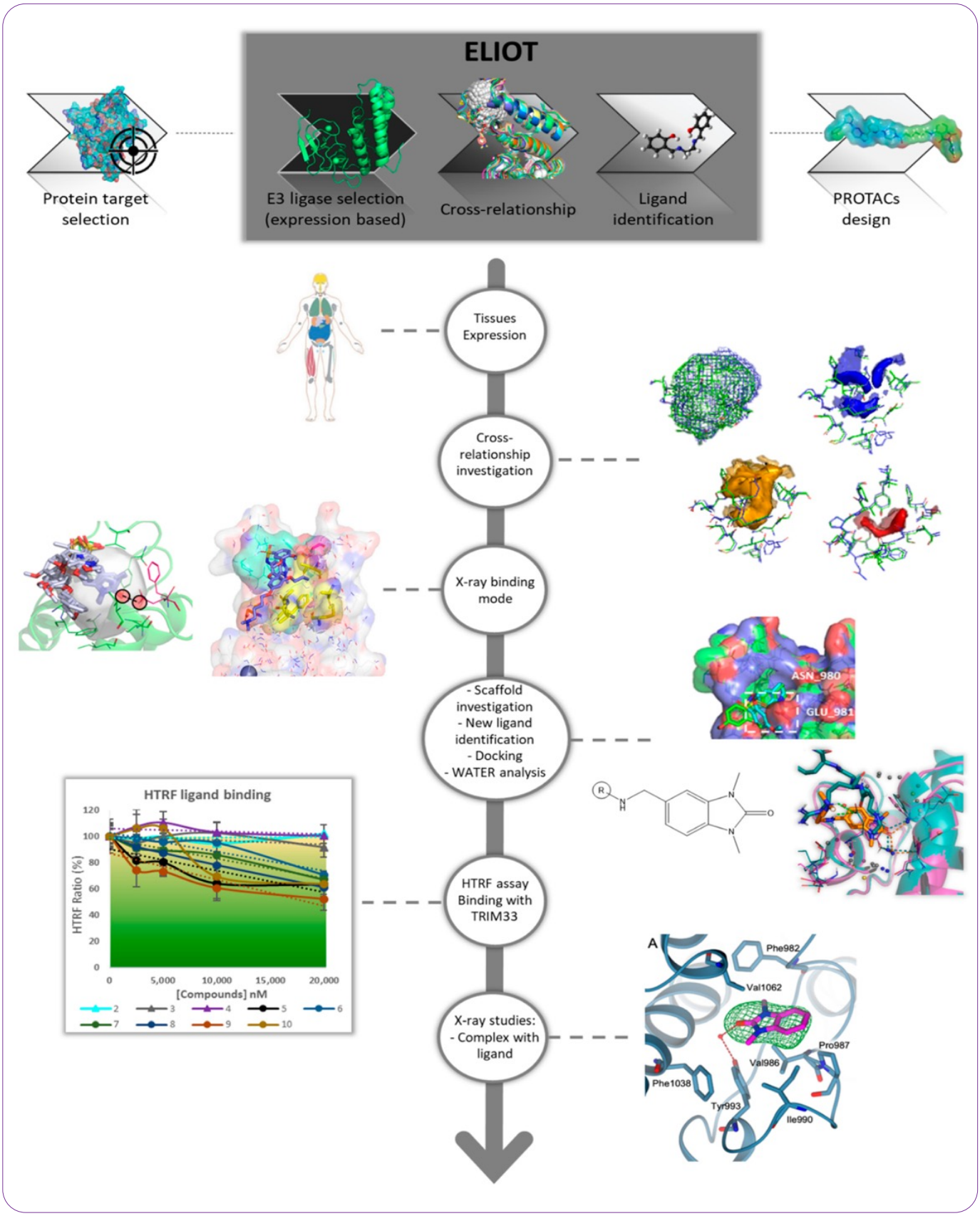

Figure 2. Overview of ELIOT workflow. Figure reproduced from Palomba T, Tassone G, Vacca C, et al. Exploiting ELIOT for Scaffold-Repurposing Opportunities: TRIM33 a Possible Novel E3 Ligase to Expand the Toolbox for PROTAC Design. Int J Mol Sci. 2022;23(22):23 doi:10.3390/IJMS232214218/S1.[16]

alanine residue (ALA923). GLU981 may regulate access to the binding site as it takes up more volume at the entrance than ALA923. Consequently, ligands were selected that used the scaffold of TRIM24 ligands but included modifications to improve interaction with the TRIM33 binding site, such as more spacing between the aromatic ring of the core and the amino group to push the amino group closer to GLU981 (see Figure 3 for the selected TRIM33 ligand structures).

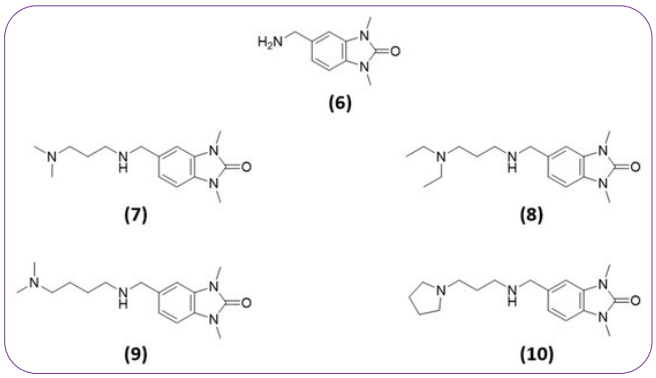

Figure 3. Structure of the ligands selected for TRIM33 from cross-relationship analysis. Figure reproduced from Palomba T, Tassone G, Vacca C, et al. Exploiting ELIOT for Scaffold-Repurposing Opportunities: TRIM33 a Possible Novel E3 Ligase to Expand the Toolbox for PROTAC Design. Int J Mol Sci. 2022;23(22):23 doi:10.3390/IJMS232214218/S1.[16]

The binding pocket similarities were further investigated using computational, docking, and water analyses, revealing that the selected TRIM33 ligands (Figure 3) fitted well in the TRIM33 pocket and water molecules also had a role in binding. The TRIM33 ligands were synthesized and binding to both TRIM33 isoforms was assessed using a series of HTRF assays using Revvity's HTRF EPIgeneous Binding Domain Discovery Kit (part number 62BDDPEG; Figure 4). After selecting a biotinylated histone peptide (biotinylated histone $H_3K_{14}Ac$) that bound TRIM24, TRIM33α, and TRIM33β, a competitive HTRF assay format was performed to assess the binding of TRIM33 ligands, TRIM24 ligands, and the scaffold ligand to TRIM33α and TRIM33β (Figure 4). TRIM33 ligands were found to bind to both TRIM33 isoforms, but TRIM24 ligands did not bind to either isoform.

Finally, three of the TRIM33 ligands (compounds 8–10, see Figure 3) were taken forward for X-ray crystallography, along with two TRIM24-binding ligands. All TRIM33-binding ligands were successfully co-crystalized with TRIM33α, while none of the TRIM24 ligands co-crystalized with TRIM33α—validating the HTRF binding and docking results. Collectively, these findings demonstrated how the ELIOT platform can be successfully used as a scaffold-repurposing strategy to identify new E3 ligases and their ligands for PROTAC design.

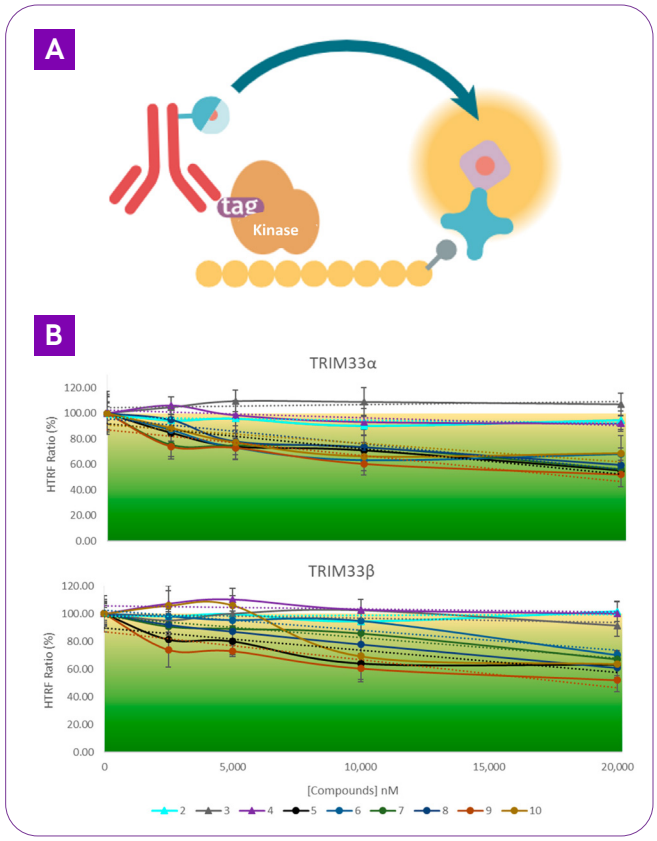

Figure 4. A) Overview of signal generation in HTRF-based assay. B) Results from HTRF-based competition binding assays assessing binding to TRIM33α and TRIM33β, with increasing concentrations of TRIM24-binding ligands (compounds 2–4, blue, grey, and purple triangles, respectively), scaffold ligand (compound 5, black circles), selected TRIM33-binding ligands (compounds 6–10, blue, green, dark blue, orange, and dark yellow circles, respectively). Figure reproduced from Palomba T, Tassone G, Vacca C, et al. Exploiting ELIOT for Scaffold-Repurposing Opportunities: TRIM33 a Possible Novel E3 Ligase to Expand the Toolbox for PROTAC Design. Int J Mol Sci. 2022;23(22):23 doi:10.3390/IJMS232214218/S1.[16]

Developing a new type of degrader: Intramolecular bivalent glues (Hsia et al.[19])

Bromodomain and extraterminal (BET) proteins (such as BRD4) recognize acetylated histones, playing a role in regulating gene expression. BRD4 expression is often elevated or altered in cancer and is involved in the growth and survival of cancer cells—where BRD4 inhibitors can limit growth and trigger cell death via apoptosis in cancer cells[20]. Hsia et al. studied the mechanism of action of a new type of degrader – intramolecular bivalent glues (IBGs) – that act as targeted protein degraders of the BET proteins: BRD2 and/or BRD4.[19]

Hsia et al. first synthesized IBG1 (Figure 5), which is made up of a BET inhibitor (JQ1) linked to E7820—an aryl sulfonamide molecular glue that degrades the mRNA splicing factor: RBM39.19 E7820 can target RBM39 in cancers such as acute myeloid leukemia (AML), which can have splicing factor mutations[21]. E7820 acts by modifying the surface of DCAF15 ¬– a cullin RING E3 ubiquitin ligase (CRL) substrate receptor – and increase its affinity for RBM39.[22]

Hsia et al. showed that IBG1 efficiently killed AML (MV4;11) and colorectal cancer (HCT-116) cell lines.19 Initial experiments into the mechanism of action of IBG1 – using a proteasome inhibitor and a neddylation inhibitor – showed that IBG1 degraded BRD2 and BRD4 through the UPS. However, BRD2 and BRD4 protein degradation was independent of DCAF15 as both proteins were broken down in the absence of DCAF15 (using DCAF15-knockout HCT-116 cells, see Figure 5). By performing CRISPR screens, Hsia et al. showed that subcomponents of the CRL4-DCAF16 ligase complex are needed for IBG1-mediated degradation of BRD4.19 These results were validated in experiments using cells that lacked CRL4-DCAF16 subunits, with BRD2 and BRD4 degradation blocked in these cell types.

To gain further insight into the interactions between IBG1, DCAF16, and BRD4, Hsia et al. performed a series of experiments involving ITC, TR-FRET, SEC, and cell-based assays.[19] These experiments used a BRD4 derivative ($BRD4^{Tandem}$) that contained both bromodomains (BD1 and BD2) of the BRD4 protein, as well as derivates that only contained either BD1 ($BRD4^{BD1}$) or BD2 ($BRD4^{BD2}$). These experiments showed that DCAF16, IBG1, and $BRD4^{Tandem}$ form a ternary complex, requiring both bromodomains for the complex to form. While DCAF16 was found to bind $BRD4^{Tandem}$ – but not to $BRD4^{BD1}$ or $BRD4^{BD2}$ – IBG1 stabilizes and strengthens the BRD4–DCAF16 interaction. And, Hsai et al. performed AlphaLISA displacement assays. AlphaLISA is a luminescent-based immunoassay that relies on coated donor and acceptor beads to detect a molecule of interest (Figure 6A). When donor and acceptor beads are in close enough contact (such as when bound to the molecule of interest through antibodies), irradiation of the sample causes energy transfer from the donor bead to the acceptor bead—generating a luminescent signal. In the AlphaLISA displacement assay (AlphaLISA Nickel Chelate Acceptor Beads part number AL108C, AlphaScreen Anti-6xHis Donor Beads part number AS116D; Figure 6B), Hsai et al. incubated $BRD4^{Tandem}$ or BD1 with variable concentrations of IBG1, in the presence and absence of DCAF16 (Figure 6), showing that DCAF16 increases IBG1's affinity for $BRD4^{Tandem}$ (IC50 with DCAF16 present = 12.8 nM vs IC50 with DCAF16 absent = 462 nM).

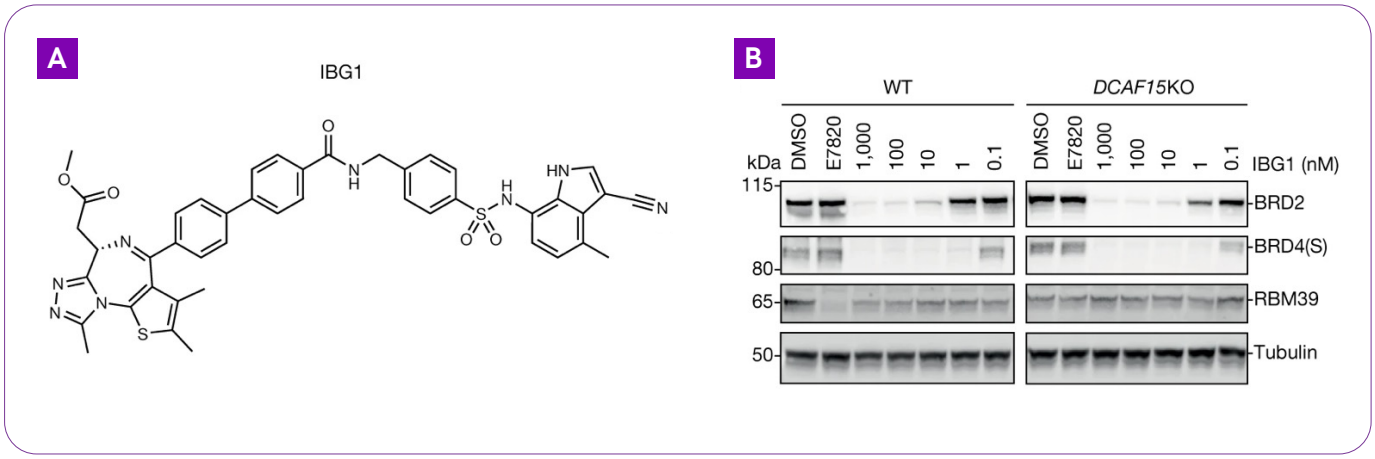

Figure 5. A) Structure of IBG1. B) Western blot analysis analysing BRD2 and BRD4 degradation of BRD2 and BRD4 in the presence (left, wildtype or WT cells) and absence of DCAF15 (right, DCAF15-knockout cells or DCAF15-KO). Figure reproduced from Hsia O, Hinterndorfer M, Cowan AD, et al. Targeted protein degradation via intramolecular bivalent glues. Nature 2024 627:8002. 2024;627(8002):204-211. doi:10.1038/s41586-024-07089-6.[19]

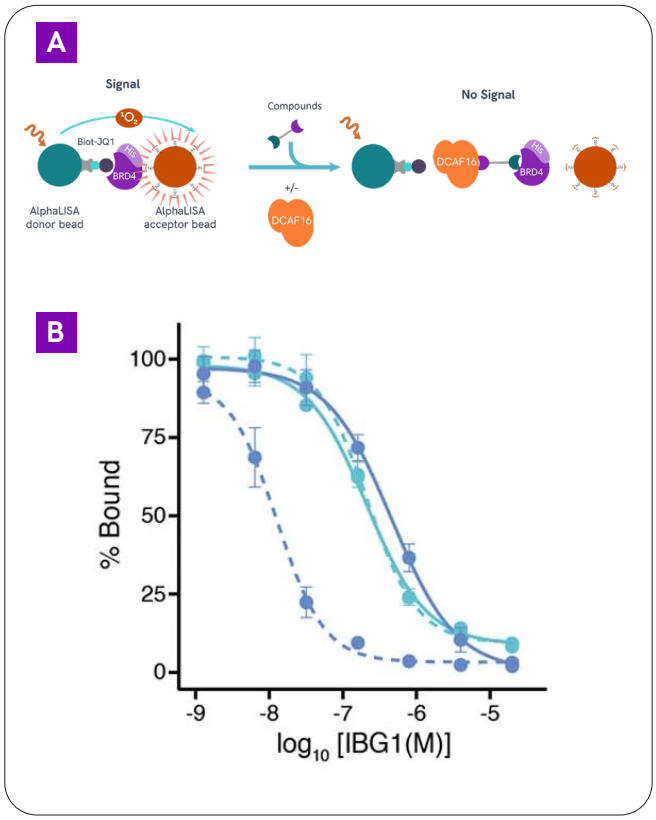

Figure 6. A) Overview of AlphaLISA signal generation. B) Results from AlphaLISA displacement assay assessing binding of BRD4tandem (dark blue circles) and BD1 (light blue circles) binding with IBG1 in the presence (dotted line) and absence (solid line) of DCAF16. Figure reproduced from Hsia O, Hinterndorfer M, Cowan AD, et al. Targeted protein degradation via intramolecular bivalent glues. Nature 2024 627:8002. 2024;627(8002):204-211. doi:10.1038/s41586-024-07089-6.[19]

Structural insights were then gathered using cryoEM, where Hsia et al. solved the structure and showed that both bromodomains (BD1 and BD2) are bound to DCAF16 (Figure 7).[19] JQ1 and E7820 were found to bind the acetyllysine pockets of BD1 and BD2, respectively. Consequently, both bromodomains are bound by IBG1.

Hsia et al. also investigated other IBG molecules: IBG3 and IBG4 (Figure 8).19 IBG3 involves two JQ1 molecules joined together by the IBG1 linker. IBG3 was found to have a much higher efficiency at degrading BRD4 compared to IBG1,

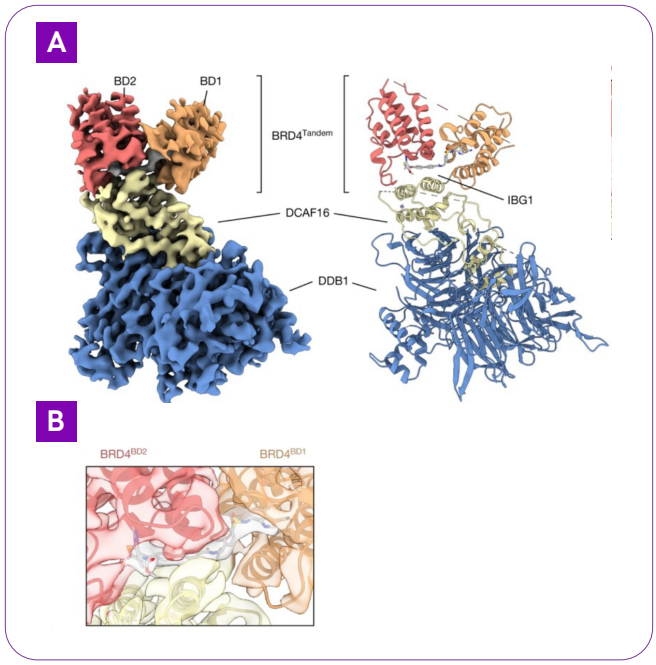

Figure 7. CryoEM experiments to solve the structure of the DCAF16– DDB1(ΔBPB)–IBG1–BRD4Tandem complex with A) showing the electron density of the complex and B) showing the electron density at the interface of the complex, showing IBG1 interacts with both BRDs. Figure reproduced from Hsia O, Hinterndorfer M, Cowan AD, et al. Targeted protein degradation via intramolecular bivalent glues. Nature 2024 627:8002. 2024;627(8002):204-211. doi:10.1038/s41586-024-07089-6.[19]

with higher affinities than the PROTAC dBET6 too (Figure 8). IBG4 involves JQ1 linked to pyrazolopyrimidine (a BRD4 degrader). CRISPR screens revealed that IBG4 recruits a different E3 ligase to IBG1: the CRL4–DCAF11 complex. IBG4 was found to efficiently degrade BRD4 but not BRD2—unlike IBG1, which efficiently degraded both BET proteins.

Consequently, Hsia et al. developed a new type of degrader – IBGs – that bind and bridge two sites on a target protein, stabilizing ternary complexes to allow for efficient protein degradation.[19]

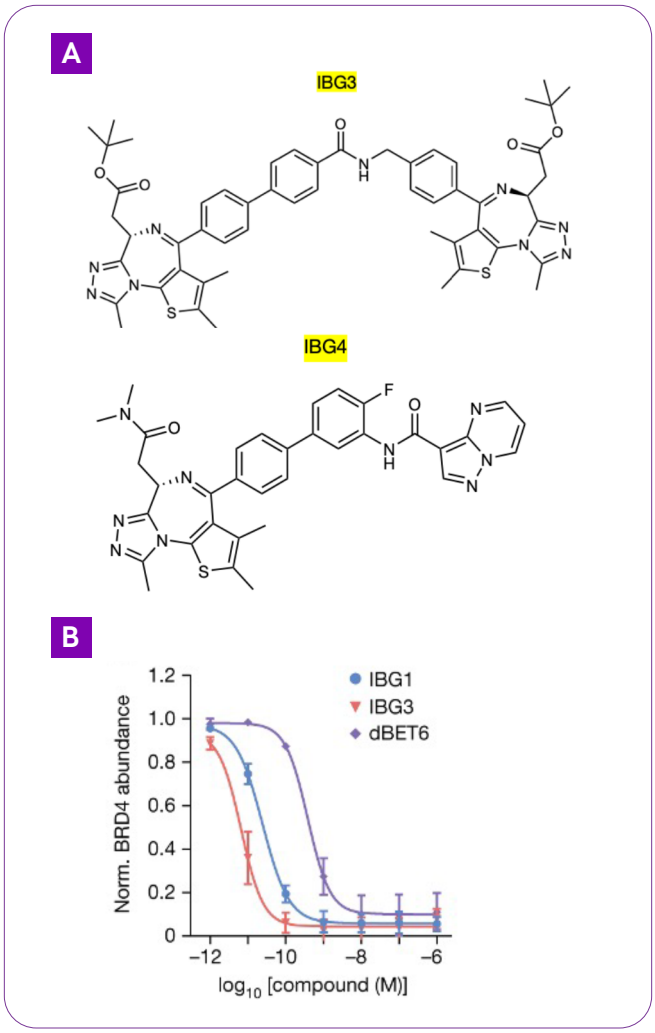

Figure 8. A) Structures of IBG3 and IBG4. B) Results from a FACS-based assay assessing BRD4Tandem levels in KBM7 reporter cells with increasing concentrations of IBGs (IBG1 and IBG3, blue circles and red triangles, respectively) and PROTAC (dBET6, purple diamonds). Figure reproduced from Hsia O, Hinterndorfer M, Cowan AD, et al. Targeted protein degradation via intramolecular bivalent glues. Nature 2024 627:8002. 2024;627(8002):204-211. doi:10.1038/s41586-024-07089-6.[19]

Developing new TPD-based cancer therapeutics: NRX-0492 in chronic lymphocytic leukemia (Zhang et al.[23])

Bruton tyrosine kinase (BTK) is needed for B-cell receptor signaling, which plays a key role in chronic lymphocytic leukemia (CLL).[24] BTK inhibitors are used to treat CLL, but cancer cells can develop resistance through mutations—particularly at the cysteine residue C481, which stops covalent drug binding. Zhang et al. aimed to develop a degrader (NRX-0492) that could breakdown both wildtype and mutated forms of BTK in CLL cells through TPD.[23]

Zhang et al. first synthesized NRX-0492, which involves a "hook" that non-covalently attaches to the target protein (BTK) and a "harness" that recruits the E3 ligase adaptor protein: cereblon (Figure 9A). Through FRET-based competition assays (HTRF Streptavidin-Tb part number 610SATLF), NRX-0492 was found to have high affinity for cereblon and wildtype and mutated forms (C481S or T474I) of BTK, with the following binding affinities (Figure 9B):

- Cereblon IC50 = 9 nM
- Wildtype BTK IC50 = 1.2 nM
- Mutated BTK (C481S) IC50 = 2.7 nM
- Mutated BTK (T474I) IC50 = 1.2 nM.

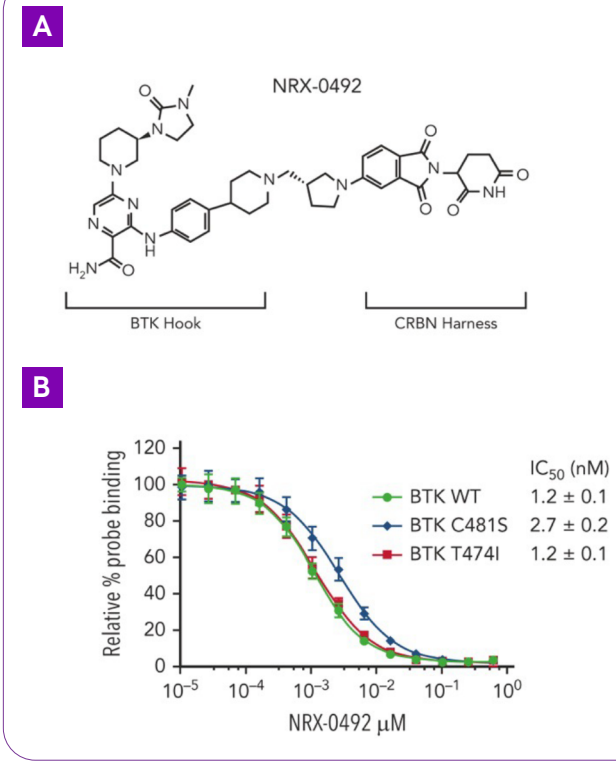

Figure 9. A) Structure of NRX-0492. B) Results from FRET-based competition assay assessing binding between NRX-0492 and wildtype (green circles) and mutant (blue diamonds and red squares) BTK. This research was originally published in Blood. Zhang et al. NRX-0492 degrades wild-type and C481 mutant BTK and demonstrates ***in vivo*** activity in CLL patient-derived xenografts. Blood. 2023;141:1584-1596. © the American Society of Hematology.[23]

After solving the structure for the wildtype kinase domain of BTK with the BTK-binding domain (or "hook") of NRX-0492, Zhang et al. performed HTRF assays using Revvity's HTRF Total BTK Detection Kit (part number ADK064PEG) to further assess the mechanism of action of NRX-0492 (Figure 10).

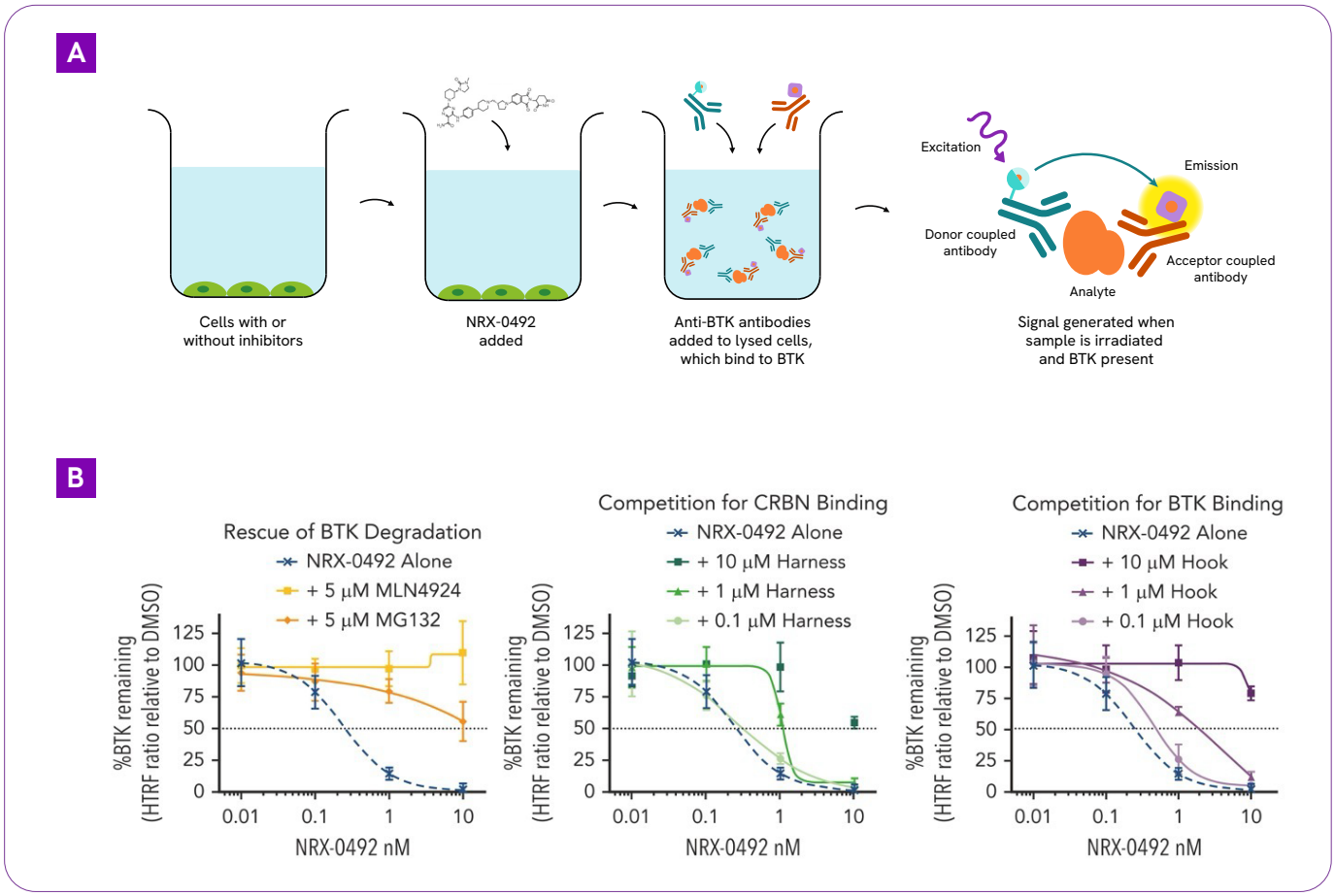

Figure 10. A) Schematic of HTRF-based assay measuring BTK degradation. B) Results from HTRF-based assays measuring BTK degradation (reported as a percentage of BTK remaining) mechanism of action using MD8 cells. Left: Comparison of NRX-0492 in the presence and absence of either a proteasome or neddylation inhibitor (MG132 and MLN4924, respectively), showing the inhibitors blocked or reduced degradation of BTK. Middle: Increasing cereblon-binding ligand (harness) levels in the presence of NRX-0492, showing excess levels of harness reduced BTK degradation. Right: Increasing BTK-binding ligand (hook) levels in the presence of NRX-0492, showing excess levels of hook reduced BTK degradation. This research was originally published in Blood. Zhang et al. NRX-0492 degrades wild-type and C481 mutant BTK and demonstrates in vivo activity in CLL patient-derived xenografts. Blood. 2023;141:1584-1596. © the American Society of Hematology.[23]

BTK degradation was reduced when the degrader was added in the presence of excess CRBN-binding ligand (harness), excess BTK-binding ligand (hook), proteasome inhibitor (MG132), or neddylation inhibitor (MLN4924). Consequently, these findings suggest that NRX-0492 binds BTK and cereblon and efficiently degrades wildtype and mutated forms of BTK through proteasomal degradation.

NRX-0492 degradation of BTK was also assessed through a series of *in vitro* and *in vivo* studies. NRX-0492 successfully degraded wildtype and mutant BTK in TMD8 cells (which expressed wildtype or mutant BTK, respectively), as well as BTK in CLL cancer cells and peripheral blood mononuclear cells (PBMCs) from CLL patients. For *in vivo* studies, Zhang et al. used a patient-derived xenograft mouse model of CLL:

- For the first set of *in vivo* experiments, mice were injected with PBMCs from an untreated patient. NRX-0492 significantly lowered BTK in CLL cells in the blood and spleen of mice and significantly reduced proliferation in the blood and spleen of mice (Figure 11A–B).
- For the next set of *in vivo* experiments, mice were injected with PBMCs from a patient that had received ibrutinib for 7 years. BTK was degraded in CLL cells from the blood after one week and CLL cells that had infiltrated the spleen after three weeks (Figure 11C).

Consequently, Zhang et al. developed and characterized a new type of degrader (NRX-0492) that is capable of degrading both wildtype and mutant forms of BTK in CLL cells (*in vitro* and *in vivo*).

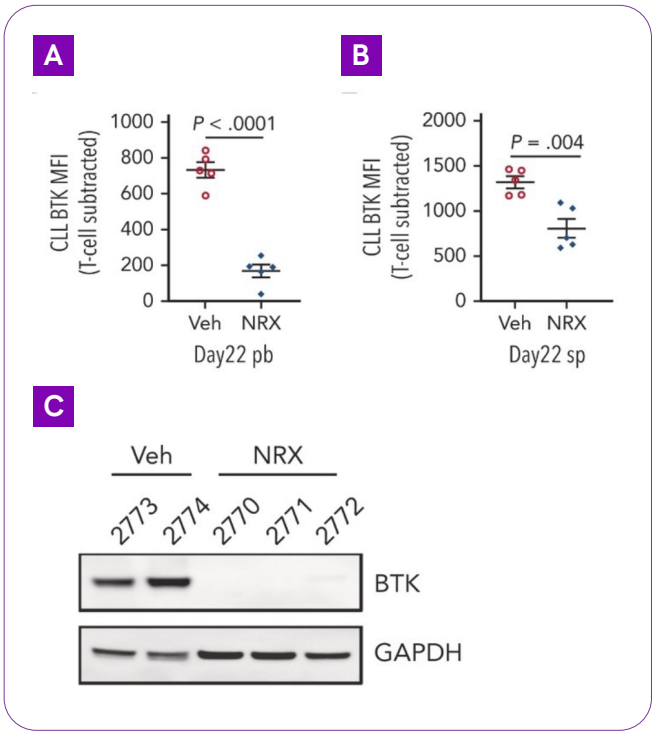

Figure 11. FACS-based assays measuring BTK levels (reported as mean fluorescent intensity or MFI using AF647-BTK) in CLL cells from PDX mice after control (Veh) or NRX-0492 (NRX) treatment. Here, assessed CLL cells were collected from the blood (a) or the spleen (b) after 22 days. c) Western blot analysis of spleen-derived CLL cell samples from PDX mice (using ibrutinib-resistant CLL patient cells) after NRX-0492 (NRX) or control (Veh) treatment. Assessed CLL cells were collected after 20 days. This research was originally published in Blood. Zhang et al. NRX-0492 degrades wild-type and C481 mutant BTK and demonstrates in vivo activity in CLL patient-derived xenografts. Blood. 2023;141:1584-1596. © the American Society of Hematology.[23]

PROTAC degraders targeting cyclin-dependent kinases (CDKs)

Cyclin-dependent kinases (CDKs) regulate transcription and the cell cycle and are activated once bound to cyclins.[25] Disruption of cell cycle regulation, such as through dysregulated CDK/cyclin activity, can lead to uncontrolled cell growth and division and diseases such as cancer.[26,27] Consequently, targeting CDK activity can offer an anti-cancer therapeutic strategy.[26] Revvity has recently assessed PROTAC degraders for multiple CDKs, including CDK4/CDK6 and CDK9, which are discussed below.

CDK4/CDK6

CDK4 and CDK6, along with cyclin D1, play a key role in regulating and initiating the cell cycle. In growth I phase (G1) cyclin D1 forms a complex with CDK4 and CDK6, allowing phosphorylation of retinoblastoma protein (Rb).

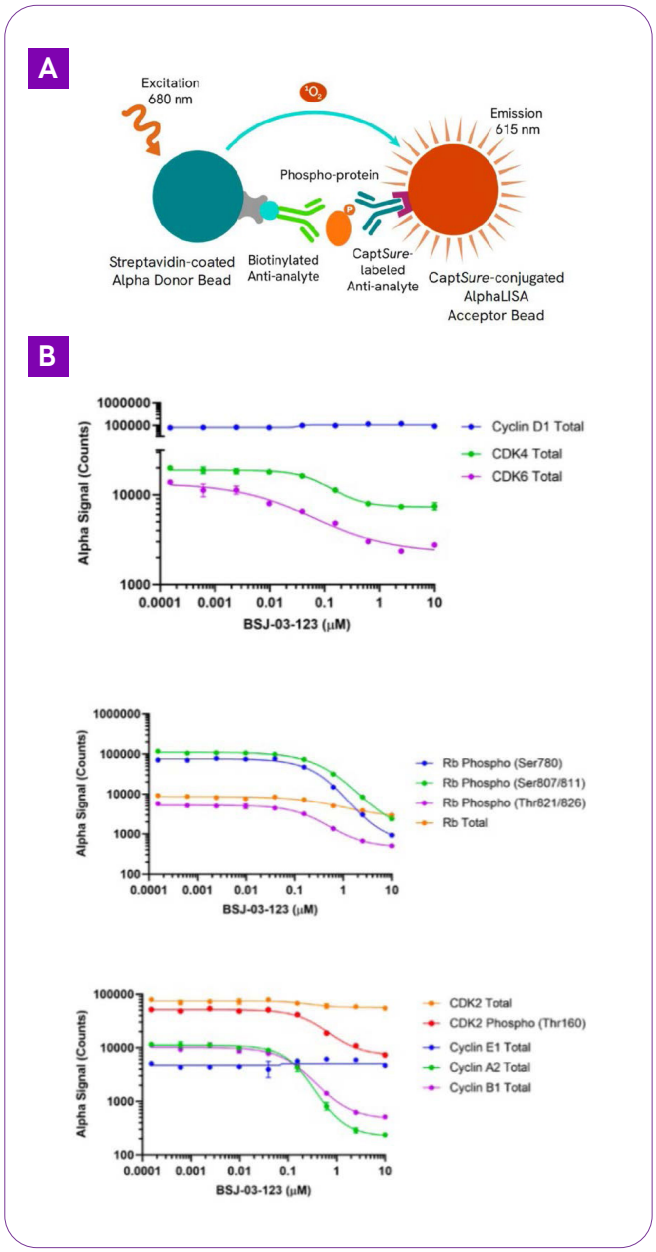

Figure 12. A) Overview of assay format, B) Results from an AlphaLISA immunoassay measuring cell cycle regulatory protein levels with increasing concentrations of BSJ-03-123.

Non-phosphorylated Rb is an inhibitor of E2F-mediated gene transcription, binding to E2F and blocking its action. When Rb is phosphorylated it releases E2F, activating E2F-mediated gene expression—which allows the cell to enter the cell cycle.[27] With CDK4/CDK6 playing a key role in initiating the cell cycle, Ballard et al. tested the effects of a CDK6-targeting PROTAC (BSJ-03-123) on cell cycle-related proteins in a breast cancer cell line (MCF-7).

Ballard et al. treated MCF-7 cells with BSJ-03-123 – a PROTAC that binds to CDK6 and cereblon – and measured the effects of the degrader on the levels of Rb and

phosphorylated Rb, along with levels of the following cell cycle-related proteins:

- Cyclin D1
- CDK4
- CDK6
- CDK2
- Phosphorylated CDK2
- Cyclin E1
- Cyclin A2
- Cyclin B1.[27]

The protein levels were assessed from the cell lysates using AlphaLISA assays (Figure 12). The experiments showed that BSJ-03-123 treatment led to targeted degradation of CDK4 and CDK6, as well as a decrease in levels of phosphorylated Rb. Levels of various cell cycle proteins (cyclin A2, cyclin B1, and phosphorylated CDK2) were also impacted by BSJ-03-123 treatment. Consequently, these experiments demonstrate how BSJ-03-123 enables the targeted degradation of CDK6 (and CDK4), and how this degradation can impact levels of other proteins involved in regulating the cell cycle.[27]

Table 1. Assay kits used in the CDK4/CDK6 experiments.

Target	Part number
Cyclin A2 (Total)	ALSU-TCYCA2-A500
Cyclin B1 (Total)	ALSU-TCYCB1-A500
Cyclin D1 (Total)	ALSU-TCYCD1-A500
Cyclin E1 (Total)	ALSU-TCYCE1-A500
Phospho-CDK2 (Thr160)	ALSU-PCDK2-A500
CDK2 (Total)	ALSU-TCDK2-A500
CDK4 (Total)	ALSU-TCDK4-A500
CDK6 (Total)	ALSU-TCDK6-A500
Phospho-Rb (Ser780)	ALSU-PRB-C500
Phospho-Rb (Ser807/811)	ALSU-PRB-A500
Phospho-RB (Thr821/826)	ALSU-PRB-B500
Rb (Total)	ALSU-TRB-A500

CDK9

CDK9 regulates transcription and dysregulation of CDK9 activity has been linked to numerous cancer types.[28,29] Carlson and Douayry assessed the ability of a CDK9-degrading PROTAC (Thal-SNS-032) to degrade CDK9 through the proteasome in a cervical cancer (HeLa) and breast cancer (MCF-7) cell line.[30] Thal-SNS-032 involves a CDK9-binding ligand (SNS-032) linked to a cereblon-binding ligand (thalidomide).

To test targeted degradation of CDK9 by Thal-SNS-032, HeLa cells were treated with Thal-SNS-032 in the presence and absence of a cereblon inhibitor (thalidomide) and a CDK9 inhibitor (SNS-032) and assessed using HTRF (Figure 13) and AlphaLISA assays. These experiments showed efficient targeted degradation of CDK9 by Thal-SNS-032, with reduction in CDK9 levels by around 80% in the HTRF assay and 95% in the AlphaLISA assay. Inhibitors blocked degradation of CDK9, showing that Thal-SNS-032 binds to CDK9 and acts by recruiting cereblon for proteasomal degradation—as Thal-SNS-032 treatment did not impact GAPDH levels, which is used as a marker for global cellular protein levels.[30]

Further analysis in HeLa and MCF-7 cells, using HTRF and AlphaLISA immunoassays, revealed half-maximal degradation concentrations (DC50) as follows:

- HeLa DC50 = 166 nM HTRF and 74.8 nM AlphaLISA
- MCF-7 DC50 = 60 nM HTRF and 85.9 nM AlphaLISA.

Consequently, Carlson and Douayry showed that Thal-SNS-032 efficiently degrades CDK9 in two cancer cell lines by recruiting cereblon for targeted degradation.[30]

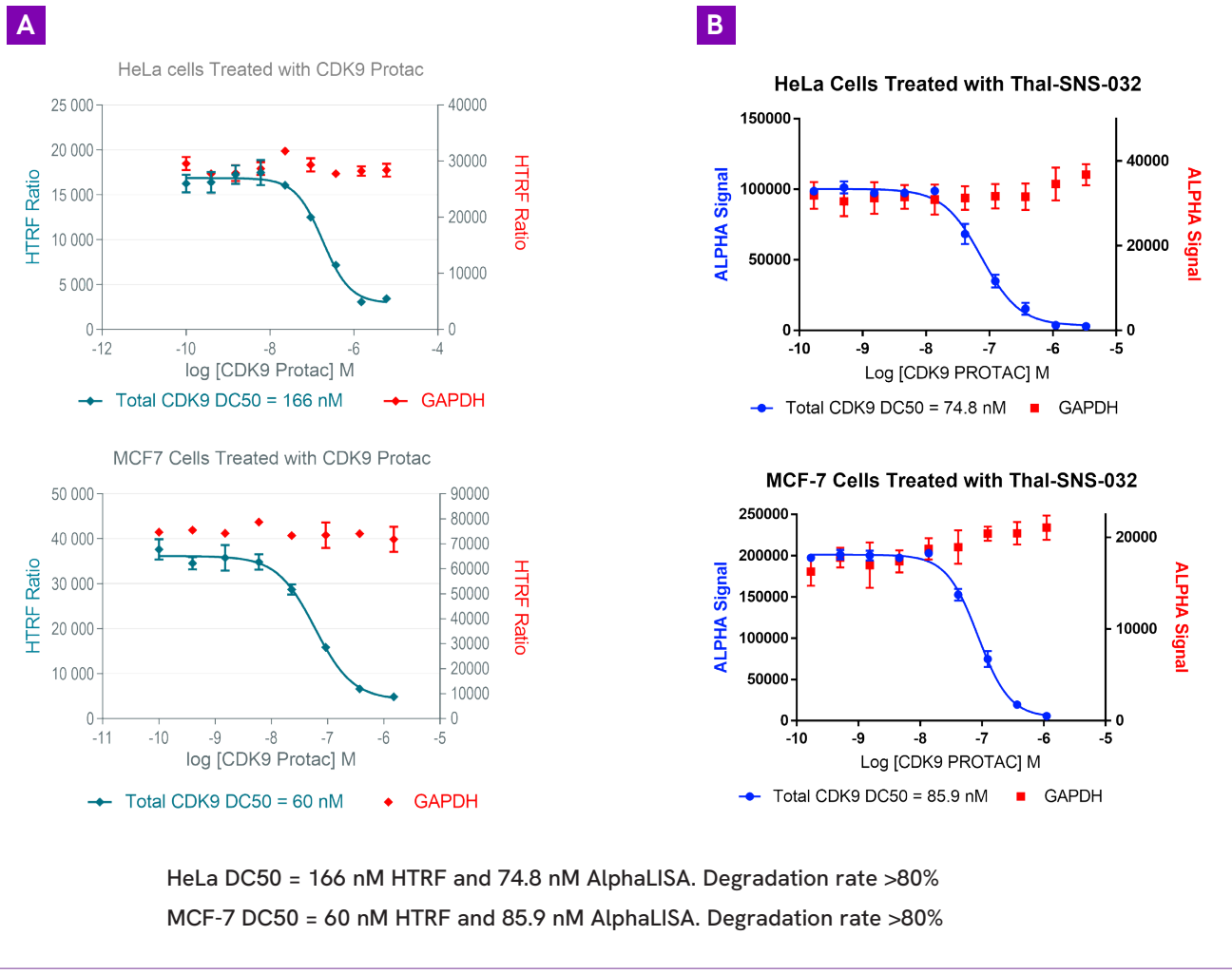

Figure 13. A) Total CDK9 and GAPDH HTRF Assays. Dose-response treatment of CDK9 PROTAC molecule Thal-SNS-032 in HeLa and MCF-7 cells treated for 4 hours. CDK9 half-maximal degradation values (DC50) of 166 nM and 60 nM were achieved in HeLa and MCF-7 cells, respectively. There was no observable change to the GAPDH protein level. B) Total CDK9 and GAPDH AlphaLISA SureFire Ultra Assays. Dose-response treatment of CDK9 PROTAC molecule Thal-SNS-032 in HeLa and MCF-7 cells. CDK9 half-maximal degradation values (DC50) of 74.8 nM and 85.9 nM were achieved in HeLa and MCF-7 cells, respectively, with no decrease to the GAPDH protein level.

Summary

TPD offers a viable therapeutic strategy for cancer treatments – as highlighted by FDA-approved TPD therapies already being available – and offers advantages, such as being able to target "undruggable" proteins or drug-resistant proteins (e.g., mutated BTK in CLL).[6,23] As TPD research has expanded, many different types of degraders have been developed, which can target the UPS or lysosomal proteolysis pathways. This has opened up the type of proteins and cellular material that can be targeted for degradation, such as aggregated or cell surface proteins.[5,6] With many E3 ligases not yet exploited by degraders, designing molecules that can engage new E3 ligases may help widen the therapeutic application of this promising anti-cancer strategy.[16]

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