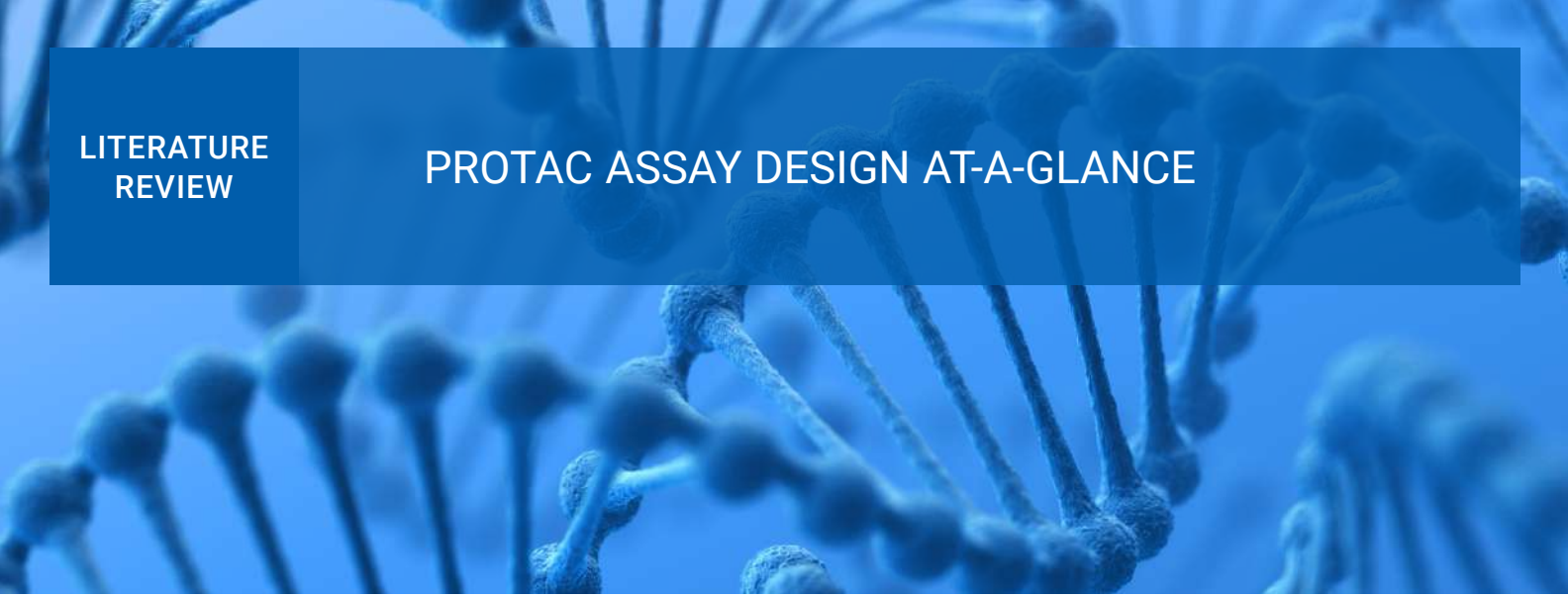

LITERATURE REVIEW

PROTAC ASSAY DESIGN AT-A-GLANCE

Streamlined Set-up and Access to Multiple Read-out Options for Research in Oncology

Abstract

Investigations of small molecules, antibodies, siRNA, or even toxins are among the best known and the most frequently used strategies in drug development, aiming to inhibit the activity of specific proteins involved in diseases. Each of them has pros and cons, such as cross activity issues, drawbacks linked to their size, lack of efficacy and cell permeability, or their undesired off-target effects. The creativity of researchers is unlimited and constantly pushes them to invent new approaches for the development of better therapeutic strategies.

This is how PROTACs (PROteolysis TArgeting Chimeras) emerged, which appear to be a very promising technology for cancer therapy and, more globally, for the treatment of many diseases. PROTACs offers many benefits like target depletion to overcome mutations and resistance, working at low concentrations due to their catalytic turnover and enhanced target selectivity. A smart molecular key to unlock the “undruggable” proteome which includes estimated 85% of human proteins, kicking over a barrier and opening the route towards revolutionary medicine in the 21st century.

This literature review presents recent publications about PROTAC development, and describes the homogeneous assay set-ups used in a variety of different studies.

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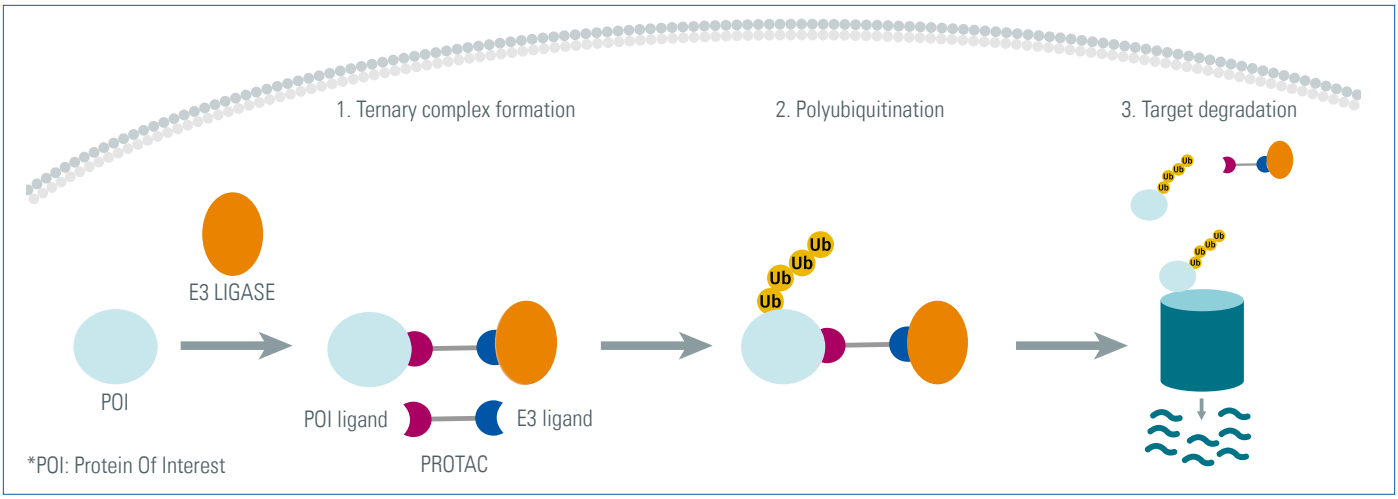


Figure 1: Catalytic mode of action of heterobifunctional degrader molecules. Degraders initiate the formation of a ternary complex between E3 ubiquitin ligase and a target protein which results in polyubiquitination of the target protein, its recognition by the proteasome, and subsequent degradation.

Adapted from Tinworth et al. (2016) Med. Chem. Comm. 7 2206.

About PROTACs

PROteolysis-TArgeting Chimeras (PROTACs) are hetero-bifunctional molecules that recruit an E3 ubiquitin ligase to a given substrate protein, resulting in its targeted degradation. Basically, two different ligands, one that binds to an E3 ligase moiety and another (also called warhead), specific for the target protein, are linked together by a chemical linker. The PROTAC entity will bring the two bound partners into proximity and will form a ternary complex. The E3 ligase will recruit an E2 conjugated enzyme and will poly-ubiquitinate the protein of interest (POI) on specific sites. Poly-ubiquitination will engage the POI in the cell proteasome machinery for its degradation, while the PROTAC entity (not degraded by the proteasome) dissociates from the complex and will initiate a new catalytic cycle with a new target protein. Having two specific ligands bridging two proteins in an artificial protein-protein interaction complex, and thus creating the optimal ternary complex for ubiquitination, are the key features for a potent PROTAC design.

Assessment of Linker Effect on PDL1-PROTAC Activity

Programmed death-ligand 1 (PD-L1) represents one of the most important immune escape mechanisms in tumors. To inhibit PD1/PD-L1 interaction and restore T cell activity, several monoclonal antibodies (MAbs) have been approved by the FDA in recent years (Nivolumab and Keytruda for PD1, both approved in 2014; Avelumab and Atezolizumab for PD-L1, approved in 2014 and 2016 respectively). But MAbs have some drawbacks, like poor bioavailability and high production costs, which have incited searchers to look for alternatives.

B. Cheng *et al.* designed PROTAC molecules based on resorcinol diphenyl ethers, which are small molecules inhibiting PD1-PDL1 interaction. They chose pomalidomide as the cereblon E3 ligase ligand and synthetized a series of novel PROTAC molecules. They worked on warhead optimization on one side, and linkers on the other.

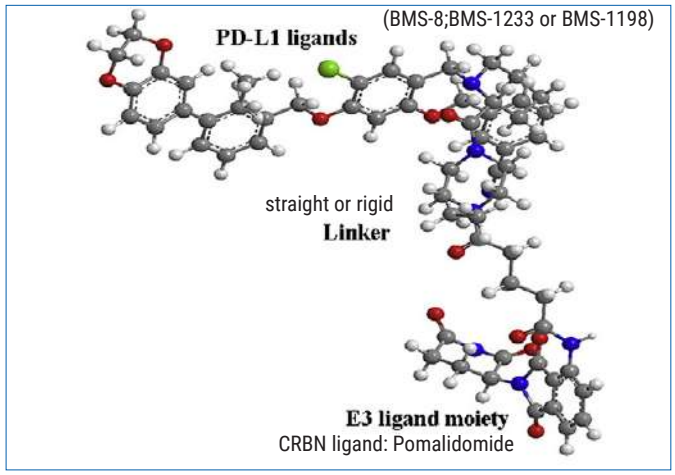


Figure 2: PROTAC compound tridimensional structure.

Copyright: Cheng, B. et al. (2020). Discovery of novel resorcinol diphenyl ether-based PROTAC-like molecules as dual inhibitors and degraders of PD-L1. European Journal of Medicinal Chemistry, 199, 112377

Three different warheads were used:

- BMS-8 on P1-P3
- BMS-1233 on P4-P17
- BMS-1198 on P18-P28

In these three series, the authors used either rigid or flexible linkers, and tested different linker lengths.

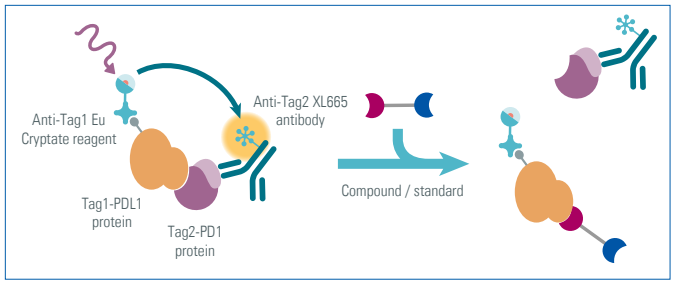


Figure 3: PD1 PD-L1 HTRF assay principle.

To evaluate the binding affinity of the 28 synthetized molecules towards PD-L1 and assess the effect of the different linkers, the authors used the HTRF biochemical PD1-PDL1 kit (Cisbio #64SPD1PEG-#64SPD1PEH).

Warhead Optimization

P1-3 compounds with a BMS-8 warhead displayed a modest inhibitory activity.

The second series (P4-17) with a BMS-1233 warhead showed an activity from 26 to 200nM, which is globally better.

The third series, P18-28 with a BMS-1198 warhead, demonstrated similar activity to the previous serie.

Authors concluded that BMS-8 warhead inhibits of PD-1/PD-L1 interaction.

Linker Optimization

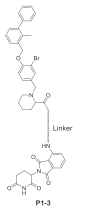
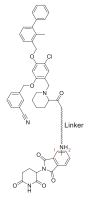
Among the series with the same warhead, either a rigid piperazine linker or a straight/flexible linker were used. The authors observed that the compounds P9 and P10 (BMS-1233 series) and P22 and P23 (BMS-1198 series) had better affinity than other compounds, as P9 and P10 had respectively IC50 values = 26.1 and 44.5nM, and P22 and P23 IC50 values = 39.2nM and 25.2nM respectively. This may be due to their rigid piperazine linker.

Thanks to this approach, the authors demonstrated that compounds with a flexible or a straight linker display higher IC50 values compared to those with a rigid piperazine linker (IC50 values >100nM compared to IC50 between 25 and 39nM for rigid linkers).

These results enabled the authors to conclude that rigid piperazine linkers are more favorable in the inhibition of PD-1/PD-L1 than flexible/straight linkers.

Going further, B. Cheng *et al.* explored linker length role on the PROTAC compound activity. P6, P7, P15, P17-21, and P28 have a chain > 6 atoms and present IC50 values > 200 nM). But compounds with a shorter chain (< 6 atoms) present an IC50 from 52.8 to 193nM (P11-13). So shorter chains are to be preferred over chains > 6 atoms.

Table 1: Activities of P1-28 compounds in inhibition of PD-1/PD-L1 interaction. Blue area: BMS-8 warhead. Gray area: BMS-1233. Red area: BMS-1198.

Structure	ID	Linker	IC_{50} (nM) a
	P1		>200
	P2		>200
	P3		197.4 ± 7.4
	P4		122.5 ± 12.2
	P5		120.8 ± 9.7
	P6		>200
	P7		>200
	P8		101.9 ± 11.3
	P9		26.1 ± 5.1
	P10		44.5 ± 6.2
	P11		117.6 ± 13.5
	P12		97.3 ± 11.5
	P13		52.8 ± 5.5
	P14		170.9 ± 7.9
	P15		>200
	P16		98.8 ± 4.3
	P17		>200
	P18		>200
	P19		>200
	P20		>200
	P21		>200
	P22		39.2 ± 5.8
	P23		25.2 ± 4.3

Copyright: Cheng, B. et al. (2020). Discovery of novel resorcinol diphenyl ether-based PROTAC-like molecules as dual inhibitors and degraders of PD-L1. European Journal of Medicinal Chemistry, 199, 112377

With these first results, the authors were able to focus on compounds P9 and P23, which had the best activity. P9 had a BMS12-33 warhead, a rigid piperazine linker, and an IC50 of 26.1nM. P23 had a 6-atom rigid piperazine linker and an IC50 value of 25.2nM.

To conclude regarding this assay, for their future experiments B. Cheng *et al.* selected one of their best compounds, P22, which has a short rigid piperazine linker and a BMS-1198 warhead.

ALK-PROTAC-VHL Complex Development

The PROTACs in this study were designed to target Anaplastic Lymphoma Kinase (ALK), a transmembrane receptor tyrosine kinase belonging to the insulin receptor superfamily and which is involved in the carcinogenesis process of several human cancers, such as anaplastic large cell lymphoma, lung cancer, inflammatory myofibroblastic tumors or neuroblastoma. Cerinitib, a potent ALK antagonist, was used as the kinase ligand, and a Von Hippel-Lindau (VHL) ligase ligand was attached to the other side of the linker. Ceritinib was attached either with two different linkers using an amide linkage (compounds 6a and 6b), or an amine linkage (8a, b, c, and d).

To discriminate among the different PROTACs synthetized, their potency to form a ternary complex was assessed in-vitro by evaluating binding affinity toward ALK and VHL.

First, anti-ALK activity was assessed using HTRF assays with ALK kinase domain. The protocol is shown in figure 5.

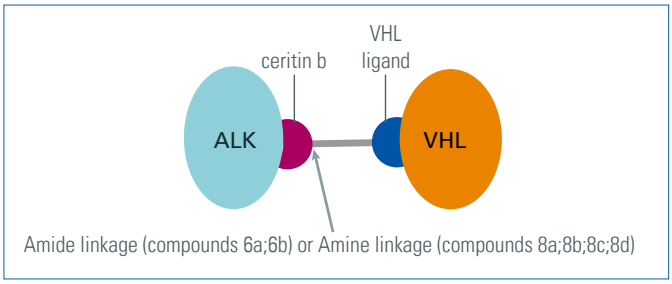


Figure 4: PROTAC specific to ALK compound structure.

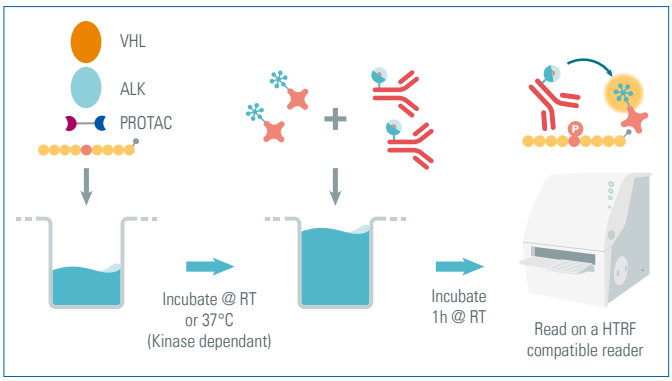
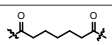
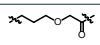
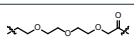


Figure 5: ALK activity inhibition detection assay protocol.

Results of the assay are shown in table 2.

Table 2: In vitro evaluation of ALK degraders.

No.	Name	Linker (=Y)	ALK inh bition (C_{50}, µM)	Alpha screen (IC_{50}, µM)	ALK degradation at 1 uM (%) SU-DHL-1
6a	TD-004		0.11	1 5	93
6b	TD-016		0.14	0 68	97
8a	TD-009		0.032	1 2	51
8b	TD-013		0.089	5 3	30
8c	TD-020		0.02	2 9	51
8d	TD-002		0.016	1.1	54 (H3122)

Compounds linked with a tertiary amine linkage to ceritinib (8a, b, c, and d) showed better inhibitory potency compared to amide linkage (compounds 6a and b).

This experiment was completed by an Alpha assay to assess VHL E3 ligase binding. The protocol is described in figure 5.

To confirm binding affinity to VHL, alpha screen assays were used with a biotinylated residue of HIF-1a peptide, which is known to bind to VHL. All compounds could bind to VHL with micromolar affinity (Table 2).

After confirming the compound activity on cells and that the TD-004 compound showed excellent efficacy in an in-vivo xenograft mouse model, the authors concluded that ALK degrader could be used for developing novel ALK treatments.

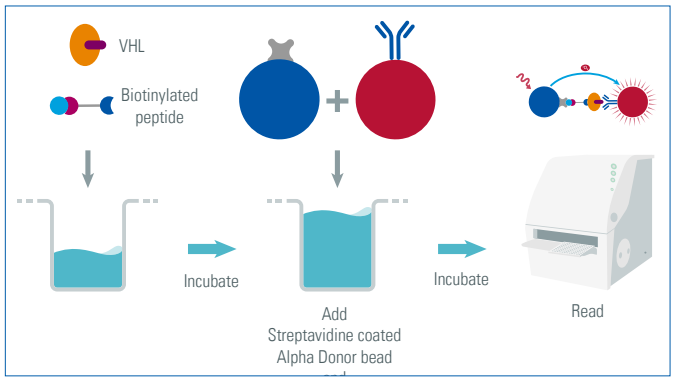


Figure 6: Alpha assay protocol.

BTK-PROTAC-CRBN Ternary Complex Formation

Bruton's Tyrosine Kinase (BTK) is a Tec family kinase which is part of the B Cell Receptor (BCR) pathway. It is an important regulator of cell proliferation and survival in B-cells, and regulates processes like differentiation and signaling. Mutations in BTK can lead to an immunodeficiency state called agammaglobulinemia. BTK is highly expressed in B cell malignancies including Chronic Lymphocytic Leukemia (CLL), mantle cell lymphoma, and multiple myeloma.

The first BTK inhibitor approved by the FDA is a small molecule named ibrutinib that binds covalently to BTK and has been proven to be an effective treatment for various B-cell malignancies. However, it has off-target activity and induces side effects. Moreover it induces drug resistance, which has raised the need for new treatments.

Zorba, A. *et al.* designed a PROTAC approach to engage BTK in proteasome mediated degradation by CRBN, an E3 ligase component.

They synthetized a library of 11 compounds with different PEG-linkers of various lengths.

The library was sorted into two kinds of linkers: Short (compounds 1-4, from 5 to 9 atom linkers) and Longer (compounds 6-11, from 14 to 20 atom linkers)

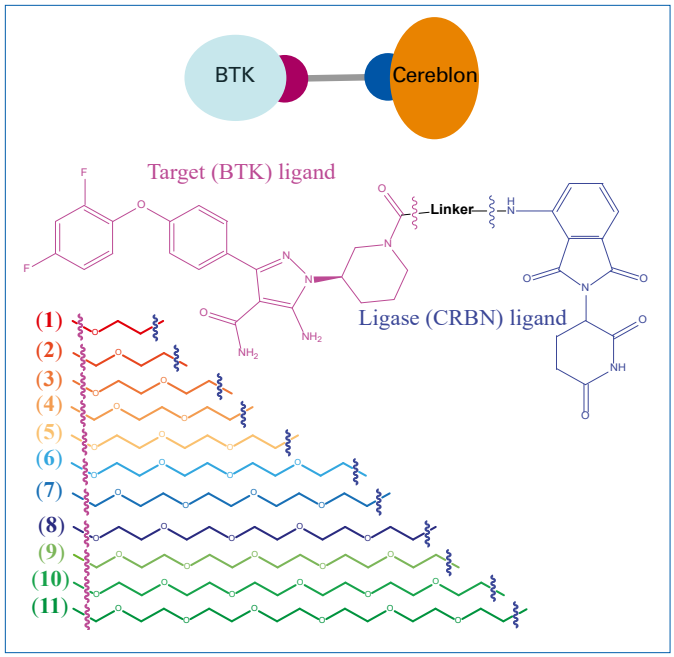


Figure 7: BTK and CRBN parent molecules (Top) from which 11 PROTACs of varying linker lengths were generated (Bottom).

Copyright: Zorba, A. et al. (2018). Delineating the role of cooperativity in the design of potent PROTACs for BTK. Proceedings of the National Academy of Sciences, 115(31), E7285–E7292.

To evaluate the linker length effect on {target–PROTAC–ligase} ternary formation and screen for the most potent PROTAC, an in vitro HTRF assay was set up. Recombinant biotinylated BTK and CRBN proteins were incubated with Streptavidin donor and acceptor conjugates respectively (Figure 8). The results of this assay are presented in figure 9.

The CRBN-PROTAC-BTK showed a bell-shaped dose–response curve (Figure 9), consistent with the three-body binding equilibria in which excess bridging molecules out-compete ternary complex formation.

Similar binding affinities were observed for the longest PROTACs (linkers 6-11). Shorter PROTACs had lower binding affinities.

As well as this HTRF assay, the authors observed that PROTAC compounds with short linkers (compounds 1-4) formed much fewer ternary complexes in vitro than larger compounds (6-11). To confirm these results, Ramos cells were incubated with compounds during 24h, and the lysates were analyzed by Western Blot. PROTACs with shorter linker lengths were largely ineffective in cells, while longer-linker PROTACs degraded BTK. Compound 10 gave the most potent cellular knockdown of BTK. After compiling all these results, Zorba, A. *et al.* predicted that the most potent PROTAC would be N° 10, which has a long linker (20 atoms).

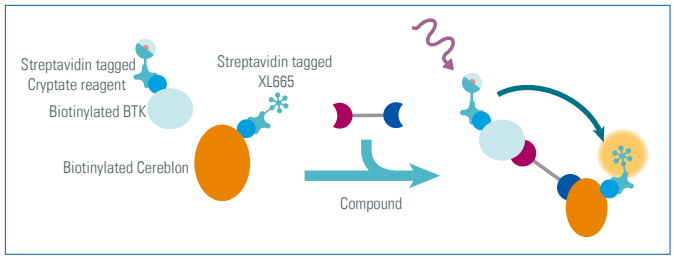


Figure 8: HTRF assay principle.

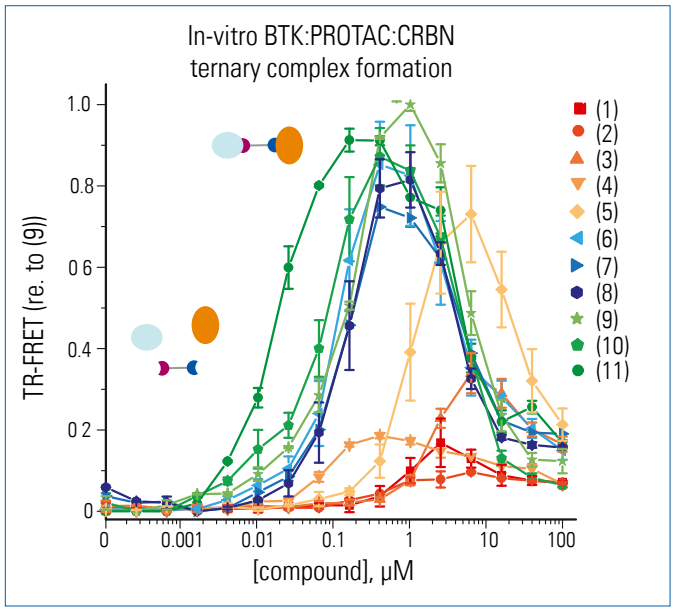


Figure 9: TR-FRET–based assay to evaluate the effect of linker length on {BTK–PROTAC–CRBN} ternary complex formation. 200 nM biotinylated BTK and 500 nM biotinylated CRBN were incubated with varying PROTAC concentrations for 30 min before endpoint data collection at 620 nm (donor) and 665 nm (acceptor). Curves are shown relative to PROTAC (9), whose maximum was normalized to 1.

Copyright: Zorba, A. et al. (2018). Delineating the role of cooperativity in the design of potent PROTACs for BTK. Proceedings of the National Academy of Sciences, 115(31), E7285–E7292.

PROTACs Isoform Specificity (P38α, p38δ)

PROTAC complexes are very specific, and Smith, B.E. *et al.* hypothesized that a single compound could discriminate between closely related proteins. They focused on the p38 MAPK family, which are kinases responding to environment stress and playing a role in many diseases. They have been well studied, and several inhibitors have been developed, but unfortunately none of them has received FDA approval. Isoform p38δ even looks intractable to functional inhibition. Smith. et al developed PROTAC compounds based on a single warhead, foretinib, and a VHL ligand. The structures are presented below.

Two complexes were synthetized with the same warheads and E3 ligase, but different linker lengths (13 atoms for SJFα and 10 atoms for SJFδ) and with different attach points to the VHL recruiting ligand (amide link for SJFα versus phenyl link for SJFδ).

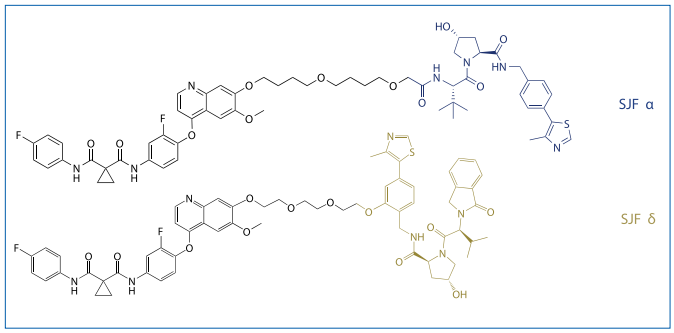


Figure 10: SJFα PROTAC (13-atom linker, amide attachment) and SJFδ PROTAC (10-atom linker, phenyl attachment)

Copyright: Smith, B.E. et al. (2019). Differential PROTAC substrate specificity dictated by orientation of recruited E3 ligase. Nature Communications, 10(1), 131.

They evaluated the ternary complex formation using a proximity-based luminescence assay (AlphaLISA).

These assays demonstrate a significant difference between the behavior of the two compounds. When incubated with p38α, a significant p38α:SJFα:VHL ternary complex could be detected. With SJFδ, no such VHL:PROTAC: p38α ternary complex was detected.

This result was confirmed in a cellular model. When cells co-expressing p38α were incubated with SJFδ or SJFα, p38α from only SJFα-treated cells displayed poly-Ub conjugation. This may suggest that only SJFα would induce p38α ubiquitination at a cellular level.

To conclude, Smith, B.E. *et al.* managed to develop an isoform-selective p38 MAPK targeting using one warhead and one E3 ligase. They demonstrated that it is possible to target specifically a protein isoform over another by varying linker design. They identified two PROTACs which target different isoform of the p38 mAPK family.

BRD4-PROTAC-E3 Ligase Optimization

Despite all the research into linker length and composition, little is known about designing an ideal linker. PROTAC compound creation remains complex. Wurz, R.P. *et al.* developed a click chemistry approach to generate several PROTAC compounds using either CRBN or VHL as an E3 ligase and targeting the bromodomain-containing protein 4 (BRD4), a promising anticancer drug target.

BRD4 is a transcriptional and epigenetic regulator whose inactivation or downregulation inhibits cancer development. Therefore BRD4 is considered as a promising anticancer drug target. However, current inhibitors are limited by their potency or oral bioavailability.

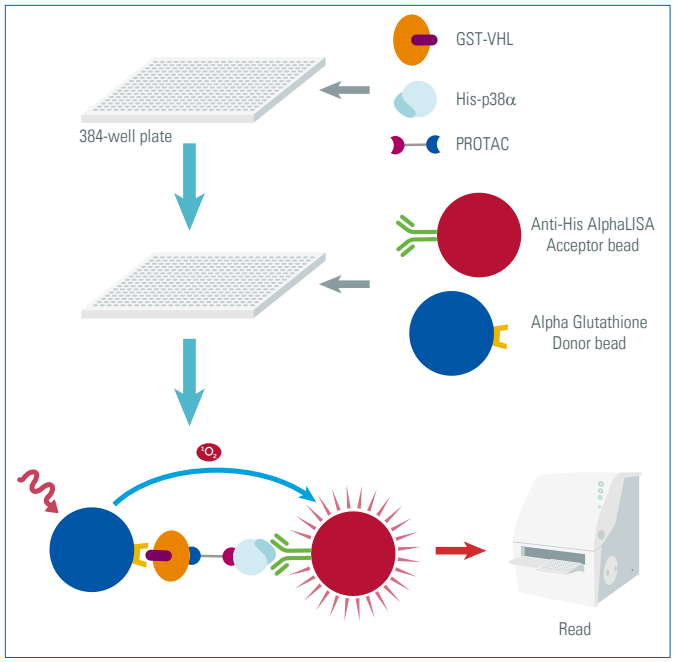


Figure 11: AlphaLISA assay protocol.

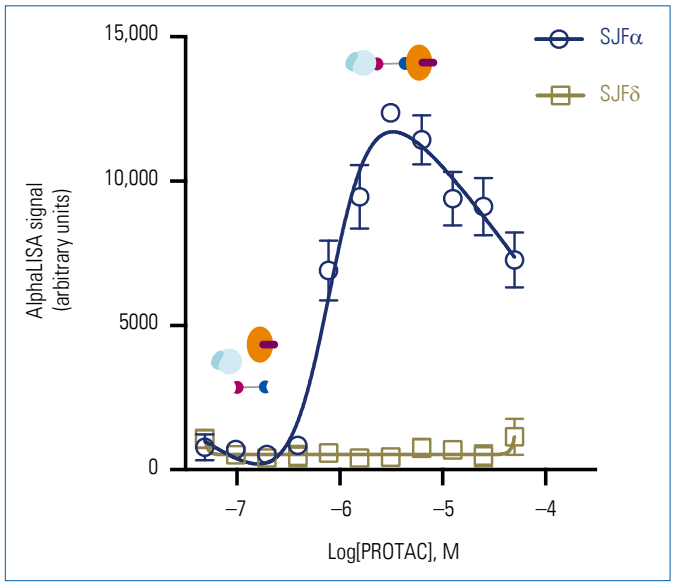


Figure 12: Proximity-based AlphaLISA assay. His-p38α and GST-VHL were incubated in the presence of increasing concentrations of SJFα and SJFδ and the extent of ternary complex formation was assessed by excitation with incident light with λ = 680 nm and capture of the emission light at λ = 615 nm. Error bars represent the s.d. from quadruplicate experiments.

Copyright: Smith, B.E. et al. (2019). Differential PROTAC substrate specificity dictated by orientation of recruited E3 ligase. Nature Communications, 10(1), 131.

The authors sought a linking strategy to facilitate the new PROTAC discovery process and ended with a dozen new PROTACs.

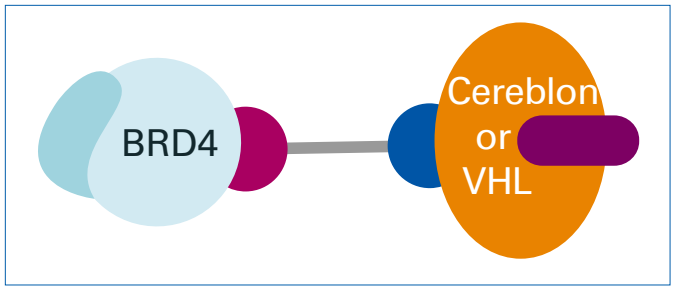


Figure 13: PROTACs specific to BRD4 compound structure. Synthetized with either a ligand specific to VHL or specific to cereblon.

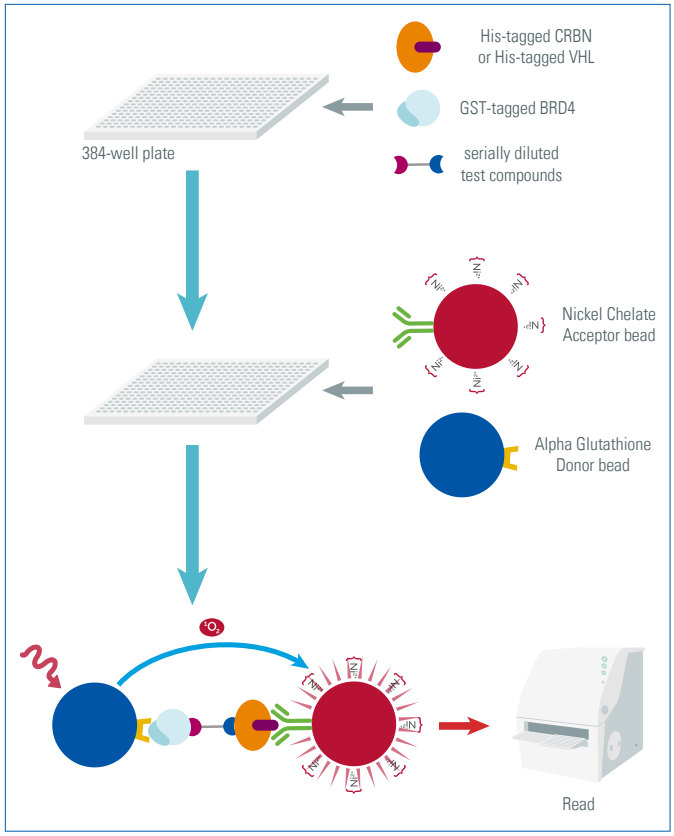


Figure 14: AlphaScreen assay principle.

To assess whether the ternary complex could be formed by the bispecific molecule, an amplified luminescent proximity homogeneous assay utilizing AlphaScreen technology was developed. The principle of the assay is described below.

Glutathione-S-transferase (GST)-tagged BRD4 protein and poly-His-tagged E3 ligase Nickel Chelate were mixed with Glutathione Donor beads and AlphaScreen Acceptor beads to capture the luminescence arising from proximity of E3 ligase-bound acceptor beads and BRD4-bound donor beads through ternary complex formation. The results are presented in figures 15 and 16 below.

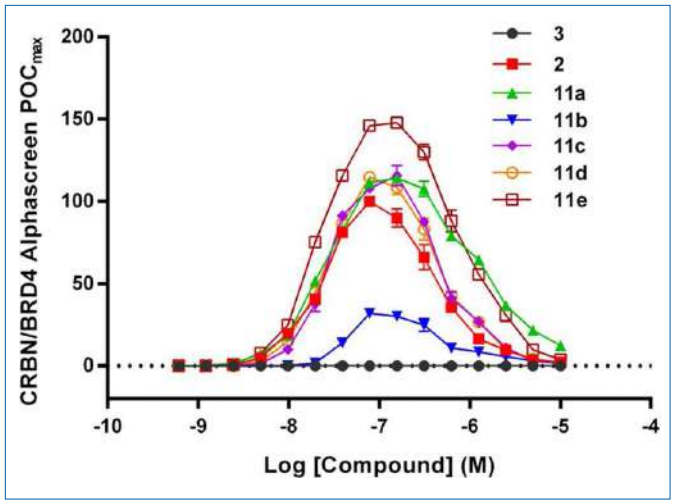


Figure 15: Proximity assay data for CRBN/BRD4 PROTACs.

Reprinted with permission from Wurz, R.P. et al. (2018). A "Click Chemistry Platform" for the Rapid Synthesis of Bispecific Molecules for Inducing Protein Degradation. Journal of Medicinal Chemistry, 61(2), 453–461. Copyright 2020 American Society.

Results are in as a bell-shaped dose–response curve, as given by Zorba, A. *et al.* (Figure 9). As the maximal normalized signal POCmax is directly linked to the maximal amount of ternary complex which can be formed, Wurz *et al.* used it to compare the ability of PROTACs to induce ternary complex formation.

Most of the synthetized PROTACs were able to induce ternary complex formation. Compound 11e was found to be the most effective.

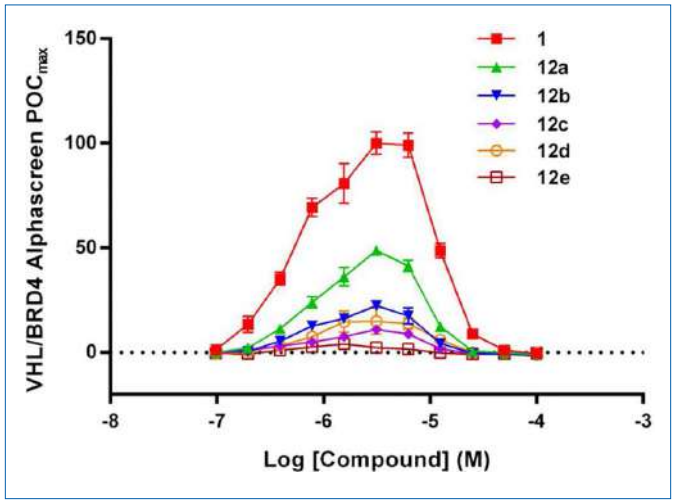


Figure 16: Proximity assay data for JQ-1-VHL PROTACs.

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Secondly, the authors tested a BRD4-PROTAC-VHL compound with an AlphaScreen protocol. Compound 1 was used as the positive control with a POCmax=100. All compounds tested showed moderate to low complex formation compared to the positive control. This can be explained by the presence of the triazole motif in the linker, which could lead to a small decrease in solubility.

Figure 17 recapitulates previous results. Briefly, PROTAC compounds built with a VHL ligand demonstrated a globally more efficient ternary complex formation than CRBN ligand built PROTACs. Moreover, in a cellular assay the authors observed that the compound with the longest linker was the most active (compound 11e).

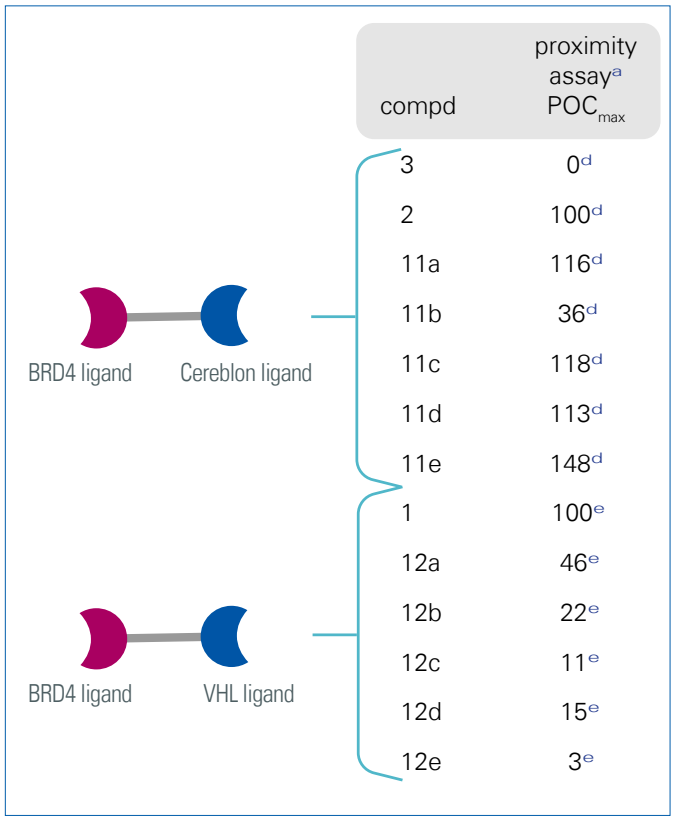

Figure 17: Proximity Assay Results for Cereblon/BRD4 and VHL/BRD4 PROTACs. 2 was used as a positive control. 3 was a BRD4 ligand used as a negative control.

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With these experiments, Wurz *et al.* demonstrated how easily compounds can be discriminated using AlphaScreen assay. They studied linker length and activity relationships.

Conclusion

To conclude, PROTAC compounds offer the possibility to entend the druggable proteome far beyond the current 15% and open many possibilities for the study of previously undruggable human proteins.

The publications featured in this review demonstrate that repurposing existing inhibitors with PROTACs may lead to the destruction of the protein instead of its inhibition. Linker nature, linkage site, and E3 ligase are all crucial for the ternary complex efficiency.

Homogeneous assay technologies enable ternary complex assessment with versatility and robustness. Methods like HTRF or Alpha assays are fast, simple, and robust with no wash for PROTAC compound characterizations at higher throughput.

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