Protein Degradation

A Path to New Therapeutics

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Introduction

Small inhibiting molecules and monoclonal antibodies nowadays constitute the two main classes of drugs for cancer, autoimmune diseases, and neurodegenerative disorders.

However, these drugs have several side effects, such as drug resistance due to mutations or the lack of an active site, and off target effects.

Over the recent decades, research on targeted protein degradation has provided great hopes to overcome these limitations. Indeed, many pharmaceutical companies are currently investing millions to develop new drugs using this new technology. The market is even expected to grow over USD 3.3 billion by 2030.

The mode of action of this technology involves the degradation of the protein of interest instead of its inhibition. The most popular and promising technology is named PROTACs (PROteolysis Targeting Chimeras). PROTACs are hetero bifunctional molecules promoting the ubiquitination of the protein of interest (POI) by forming a ternary complex with an E3 ligase.

However, PROTAC present some limitations and are not the only player in the field. It appears that emerging new degradation technologies could significantly expand the variety of targets that may be selectively degraded. Some of them use a similar mode of action: TRAFTAC and molecular glue degrade proteins through the proteasome as PROTACs. On the other side, some technologies exploit a second major degradation pathway: LYTAC, AUTAC and ATTEC involve the lysosome degradation pathway.

Revvity, a global leader committed to innovating for a healthier world, continues to support these early stage investigations by providing more than a thousand no-wash AlphaLISA® and HTRF® immunoassay kits to generate accurate and highly sensitive data. The company is proud to provide the research community with reliable top-quality assays, as well as expert advice through application notes, webinars, and technical guides.

It is in the spirit of offering the scientific community cutting-edge research reports that Revvity, in collaboration with *GEN*, is sponsoring this eBook, with a focus on the most recent Protein Degradation advances. The ebook summarizes key information about protein homeostasis, its regulations and latest trends. ■

Learn more

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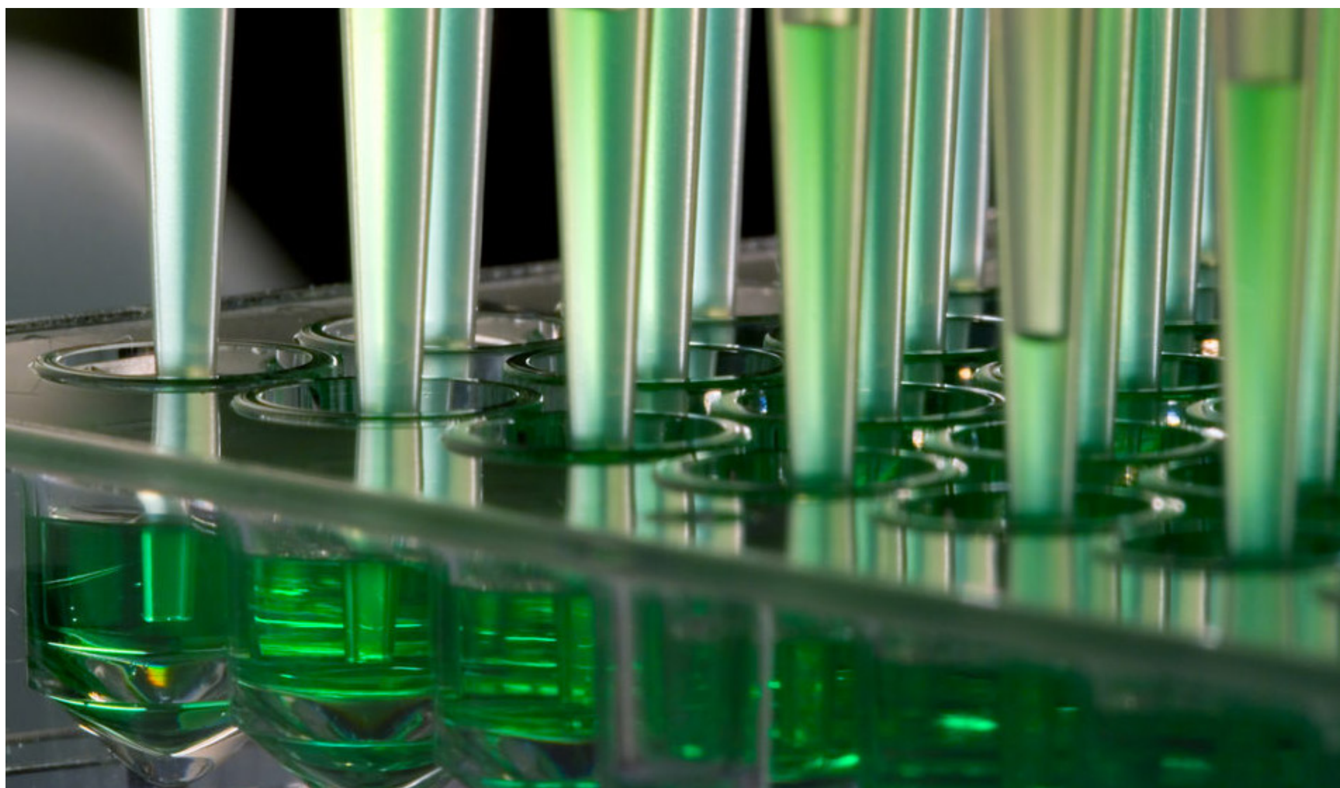
Targeted Protein Degradation Represents a Promising Therapeutic Strategy



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Despite enormous efforts to advance traditional pharmacology approaches, more than three quarters of all human proteins remain beyond the reach of therapeutic development, according to scientists from the CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences in Vienna. They maintain that targeted protein degradation (TPD) is a novel approach that could overcome this and other limitations, and thus represents a promising therapeutic strategy.

TPD is based on small molecules (known as degraders), which can eliminate disease-causing proteins by causing their destabilization. Mechanistically, these degrader drugs repurpose the cellular protein quality control system, tweaking it to recognize and eliminate harmful proteins. More specifically, they re-direct members of the

protein family of E3 ubiquitin ligases (E3s) towards the disease-causing target protein. This leads to a "molecular earmarking" of the harmful protein via ubiquitination, says CeMM principal investigator Georg Winter, PhD, who adds that subsequently, the ubiquitinated protein is recognized and degraded by the proteasome, which serves as the cellular garbage disposal system.

Researchers in Europe, led by Winter and his CeMM team, focused on a subset of degraders called molecular glue degraders. This class of small molecules has been shown to induce the degradation of target proteins that could not be blocked using traditional pharmacology approaches. Consequently, these proteins had been termed undruggable. The best characterized examples are the clinically approved thalidomide analogs, effective for the treatment of different blood cancers. Unfortunately, the discovery of the few described molecular glue degraders has historically been a process entirely driven by serendipity and no rational discovery strategies existed, notes Winter.

To overcome this limitation, Georg Winter's group at CeMM set out to innovate with a scalable strategy towards the discovery of novel molecular glue degraders via phenotypic chemical screening. To do so, first author and CeMM postdoctoral fellow Cristina Mayor-Ruiz, PhD, and colleagues engineered cellular systems widely impaired in E3 activity. Differential viability between these models and E3-proficient cells was used to identify compounds that depend on active E3s and were potential molecular glue degraders.

Researchers integrated functional genomics with proteomics and drug-interaction strategies to characterize the most promising compounds. They validated the approach by discovering a new RBM39 molecular glue degrader, structurally similar to others previously described. Importantly, they discovered a set of novel molecular glues that induce the degradation of the protein cyclin K, known to be essential in many different cancer types. These novel cyclin K degraders function via a molecular mechanism of action that involves the E3 CUL4B:DDB1 and that has never been therapeutically explored before.

The researchers published their study entitled "Rational discovery of molecular glue degraders via scalable chemical profiling" in Nature Chemical Biology.

"Targeted protein degradation is a new therapeutic modality based on drugs that destabilize proteins by inducing their proximity to E3 ubiquitin ligases. Of particular interest are molecular glues that can degrade otherwise unligandable proteins by orchestrating direct interactions between target and ligase. However, their discovery has so far been serendipitous, thus hampering broad translational

efforts. Here, we describe a scalable strategy toward glue degrader discovery that is based on chemical screening in hyponeddylated cells coupled to a multi-omics target deconvolution campaign. This approach led us to identify compounds that induce ubiquitination and degradation of cyclin K by prompting an interaction of CDK12–cyclin K with a CRL4B ligase complex," write the investigators.

"Notably, this interaction is independent of a dedicated substrate receptor, thus functionally segregating this mechanism from all described degraders. Collectively, our data outline a versatile and broadly applicable strategy to identify degraders with nonobvious mechanisms and thus empower future drug discovery efforts."

This study provides the first framework towards the discovery of molecular glue degraders that can be highly scaled, but also strongly diversified," says Winter.

"I truly believe that we are only scratching the surface of possibilities. This study is chapter one of many chapters to follow. We will see a revolution in the way researchers perceive and execute therapeutic strategies for previously incurable diseases by crafting glue degrader strategies that will enable them to eliminate therapeutic targets that could not be explored with traditional pharmacologic approaches," he explains. ■

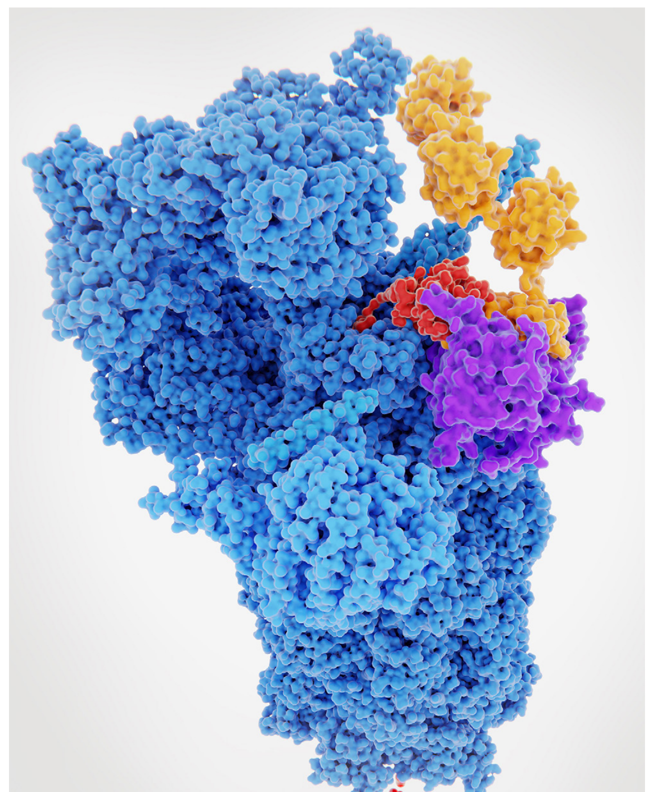
A New Therapeutic Approach Addresses Undruggable Proteins Single-Cell Immune Profiling

Small bifunctional PROteolysis TArgeting Chimeras PROTACs) hijack the cell's protein degradation machinery to irreversibly destroy a target

Author: MaryAnn Labant

Functional protein degradation mechanisms are essential for cellular homeostasis and cell survival. Proteostasis is the biological mechanism that controls a protein's fate from synthesis to degradation. Complex and interconnected pathways ensure maintenance and regulation of protein abundance, turnover, folding, functionality, subcellular localization, and, ultimately, degradation (the breakdown of proteins into smaller peptides or amino acid building blocks).

Impaired proteostasis can lead to the accumulation of normally-degraded or aggregated proteins, damaged organelles, or, conversely, to excessive protein degradation and has been associated with aging as well as clinical indications, such as cancer, autoimmune diseases, and neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington diseases.



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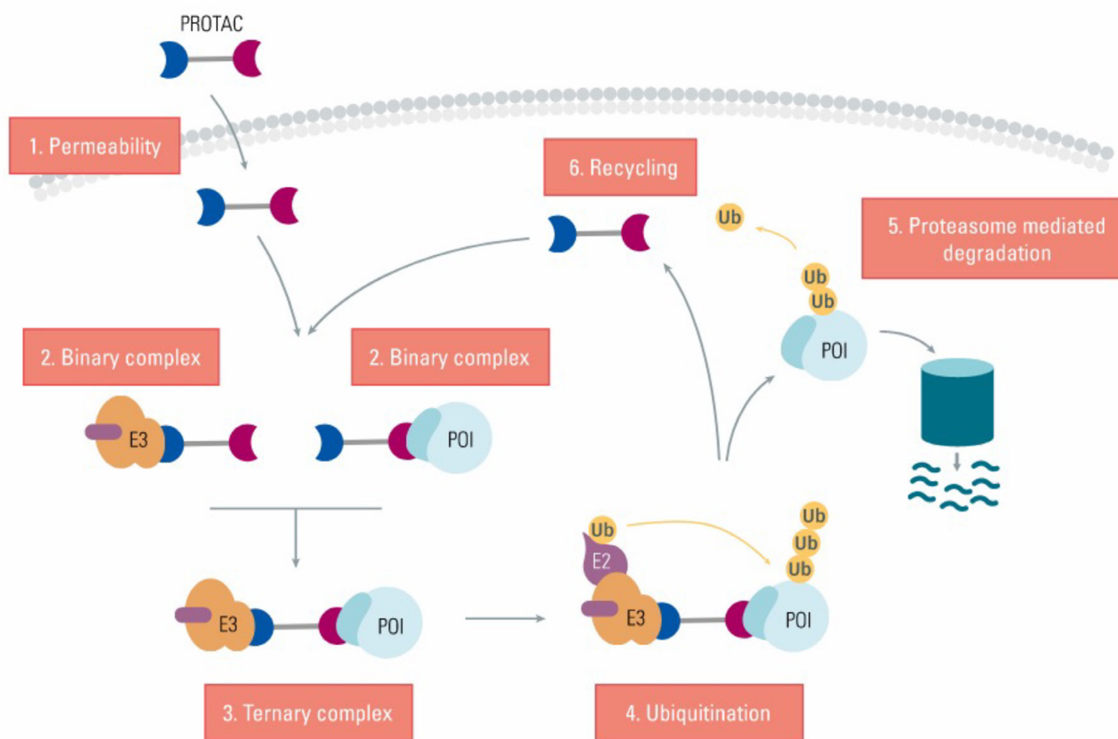
Two pathways control protein degradation: the Ubiquitin Proteasome System (UPS) and the Autophagy-Lysosomal System (ALS). The UPS is the principal proteolytic mechanism responsible for degradation of short-lived proteins as well as damaged and misfolded proteins while the ALS is



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involved in the clearance of long-lived proteins and organelles, and in the recycling of amino acids. Both systems regulate essential cellular functions such as cell growth and apoptosis.

Huge leaps in the understanding of these pathways' molecular mechanisms have benefited the creation of new tools and molecules to facilitate innovative therapeutic approaches to target protein malfunction. The next generation of small molecules will most likely play an important role as advances in structural biology, molecular modeling, computational chemistry, artificial intelligence and machine learning identify novel ways to pursue small molecule drug discovery against historically elusive targets.

Yet compared to small molecule therapies that can induce chemoresistance over time, targeted protein degradation therapies are expected to require lower concentrations for sustained activity and to potentially induce fewer side effects and chemoresistance.

Although still early in the evolution of this field, protein degradation therapies are beginning to enter clinical trials. For example, Arvinas' compounds for prostate and breast cancer are in Phase I and II trials. Arvinas was built on the groundbreaking research of Craig Crews, PhD, of Yale University, one of the pioneers in the protein

degradation field, to address the >80% of proteins that evade inhibition and have been "undruggable" by traditional approaches, such as inhibitors and agonist/antagonists.

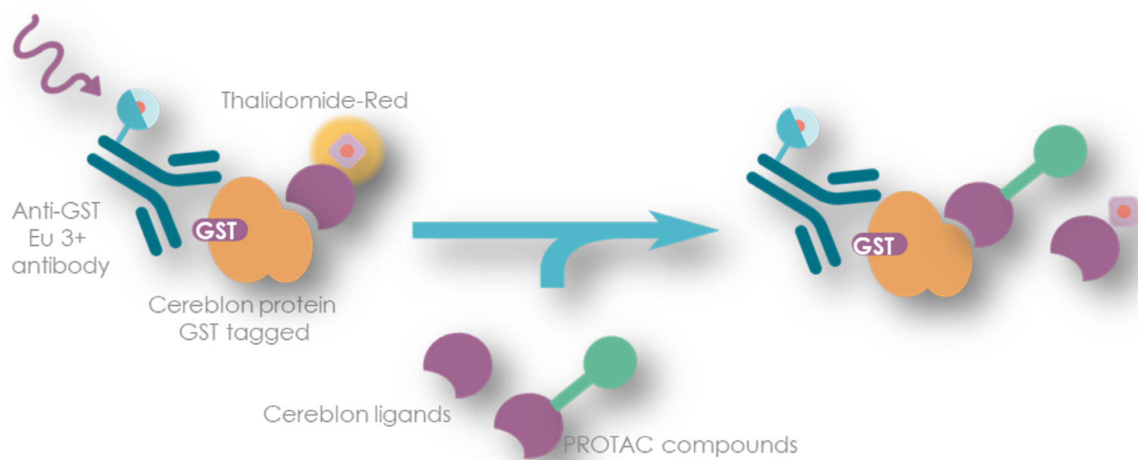
PROTAC Technology

PROteolysis TArgeting Chimeras (PROTACs) are small bifunctional molecules that hijack the cell's protein degradation machinery to irreversibly destroy a target. Each PROTAC consists of a linker with a ligand on one end to confer target specificity and bind to the protein of interest (POI), and another ligand on the other end to engage the cell's ubiquitin system by binding to the enzyme responsible for degrading the POI, E3 ubiquitin ligase, to form a binary complex.

The PROTAC brings the E3 ubiquitin ligase and POI together to create a ternary complex. Next the E3 ubiquitin ligases recruit enzymes that lead to the ubiquitination of the POI. The resulting polyubiquitin chain flags the target protein for recognition and degradation by the proteasome via the UPS (Figure 1).

PROTAC technology is emerging as a new therapeutic method to treat diseases, such as cancer or neurodegenerative disorders, caused by the aberrant expression of a pathogenic protein. In addition to drug discovery, PROTAC technologies can also be used to silence proteins for research applications to manipulate or modulate the expression of the POI instead of using siRNA to silence gene expression and downregulate protein expression.

"One nice thing about the PROTAC approach is that the cell can recycle the compounds to induce a new cycle of protein degradation, enabling sustained protein degradation," says Fabienne Charrier-Savournin, PhD, Product Manager, Revvity. "Our two main homogenous technologies, AlphaLISA® and HTRF®, are perfectly suited to the



targeted protein degradation space with ready-to-use kits available in biochemical and cell-based formats to facilitate both low- and high-throughput screening in the drug discovery process."

AlphaLISA is a bead-based, no-wash luminescent technology. When Alpha Donor and Acceptor beads are brought together through binding to the target, a cascade of chemical reactions is set in motion, creating an amplified signal. HTRF (Homogeneous Time Resolved Fluorescence) is another frequently used technology to measure analytes in a homogeneous format. Both technologies are ideal platforms for drug target studies in high-throughput screening applications.

The Revvity all-in-one E3 ligase binding kits have optimized reagents and detailed protocols making them quick and easy to implement. Washing the sample or performing separation steps is not required to get accurate and reproducible pharmacological results.

E3 Ligase Binding Kits

"During the drug discovery process a series of questions can be raised. For example, how does a PROTAC compound bind to the E3 ligase? How can the affinity of a PROTAC be improved? Can we identify new E3 ligase ligands with improved pharmacological properties? To help address these questions we are continuing to expand our portfolio of AlphaLISA and HTRF E3 ligase binding kits, and recently introduced Cereblon, cIAP1 BIR3 and BIR2 and MDM2 E3 ligase binding kits," continues Charrier-Savournin.

Cereblon (CRBN) is the substrate receptor of the cullin 4-RING E3 ligase complex (Figure 2). cIAP1 is a cellular inhibitor of apoptosis protein 1. The BIR domain interacts and inhibits caspase activity, while the RING domain is an E3 ubiquitin ligase. MDM2 (Mouse double minute 2 homolog), also known as E3 ubiquitin-protein ligase Mdm2, is an important negative regulator of the p53 tumor suppressor.

"The E3 ligase binding assays based on AlphaLISA and HTRF technologies are used to discover, screen, and characterize compounds, and to identify and discover new binders," explains Charrier-Savournin. "After the formation of the binary complex is established, scientists can determine the formation of the ternary complex. Since it is possible to bind but not induce degradation once it is established that the compound can bind the POI within the cell, a total protein assay, either with HTRF or AlphaLISA, enables the monitoring of protein degradation of the POI upon the addition of targeted molecules."

"In addition, our Alpha CETSA® technology monitors binding of the compound of interest in the natural living cellular environment. The kits combine the Cellular Thermal Shift assays (CETSA®)

from Pelago Bioscience with Revvity Alpha technology. These can be helpful to assess the permeance of the compound to enter the cell and bind to the targeted protein."

Custom Applications

Seven hundred different E3 ligases exist in the genome. Currently, very few, about 1%, are used in scientific protein degradation for targeted degradation purposes. Revvity kits are based on the most commonly-used disclosed ligases.

In order to further improve protein degradation efficacy, pharmaceutical and biotech companies routinely search for new ligases that can be used for targeted protein degradation.

"In addition to our catalog listings of extensively validated kits, we perform custom work with companies to build assays with their proprietary ligases at our R&D locations both in the United States and Europe. Our experts are well-versed in assay development and custom labeling to accommodate unique demands," says Charrier-Savournin.

Summary

Protein degradation therapies have the potential to greatly impact the healthcare field by addressing the >80% of proteins that evade inhibition and have been "undruggable" by traditional approaches. Revvity E3 ligases binding kits are an invaluable tool to aid this timely research. ■

Targeted Protein Destruction: Advances in PROTACs Other Degraders

Commercial and academic laboratories are refining their methods for designing and optimizing highly specific small-molecule degraders

Researchers have learned to hijack cells' natural protein turnover mechanisms to degrade disease-causing proteins throughout the human body. Engineered small-molecule drugs recruit the ubiquitin proteasome system (UPS) to the target protein with a high degree of selectivity, providing advantages over other targeted therapies.

A handful of targeted protein degrader drugs have already reached clinical application, with many additional therapies entering the pipeline as companies and academic researchers continue to develop new molecules and mechanisms. So far, the field has primarily focused on cancer therapeutics because aberrant proteins in cancer cells are obvious targets. However, targeted protein degradation technology could eventually be applied to myriad diseases and disorders, including those affecting the central nervous system.

Designing/optimizing degrader molecules

"We're about to translate awesome new modalities that have really incredible pharmacology into drugs that we believe will make a huge difference to patients," says Stewart L. Fisher, PhD, chief scientific officer, ***C4 Therapeutics***.

C4 Therapeutics is one of many biotech companies developing and testing *PRO*teolysis *TA*rgeting Chimeras, known as PROTACs. These are small-molecule drugs that recruit specific E3 ubiquitin ligases to transfer polyubiquitin chains onto target proteins, thereby marking them for degradation by the cell's native proteasome.

Unlike many existing drugs, such as inhibitors, targeted protein degraders need not bind to an active site on a protein. So long as the degrader forms a ternary complex with the E3 ligase, the protein can be ubiquinated, and degradation will occur. "This is opening up space where we can go

Juan Gaertner / Science Photo Library / Getty Images

Rather than neutralize disease-causing proteins by deploying small-molecule inhibitors, some developers would destroy protein targets by ushering them to the proteasome, the cell's garbage disposal system. This alternative approach, which applies tags to proteins in order to consign them to oblivion, could be effective against currently undruggable targets.

after traditionally undruggable proteins," notes Fisher. "We're not limited to just that binding site."

Most existing PROTACs rely on bifunctional degradation activation compounds (biDACs), which are heterobifunctional molecules featuring one site that binds a target protein and another that binds an E3 ligase. However, targeted protein degradation can also be achieved using monofunctional degradation activation compounds (monoDACs), also known as molecular glues or glue degraders. These bind to either the E3 ligase or the target protein and chemically modify the surface, prompting protein-protein interactions that ultimately cause the E3 ligase to bind to the target protein, resulting in degradation.

Fisher says that monoDACs tend to be smaller molecules than biDACs, which could simplify optimization with respect to bioavailability and compliance with drug development guidelines. However, monoDACs' reliance on protein-protein interactions can weaken their ability to select the target of interest.

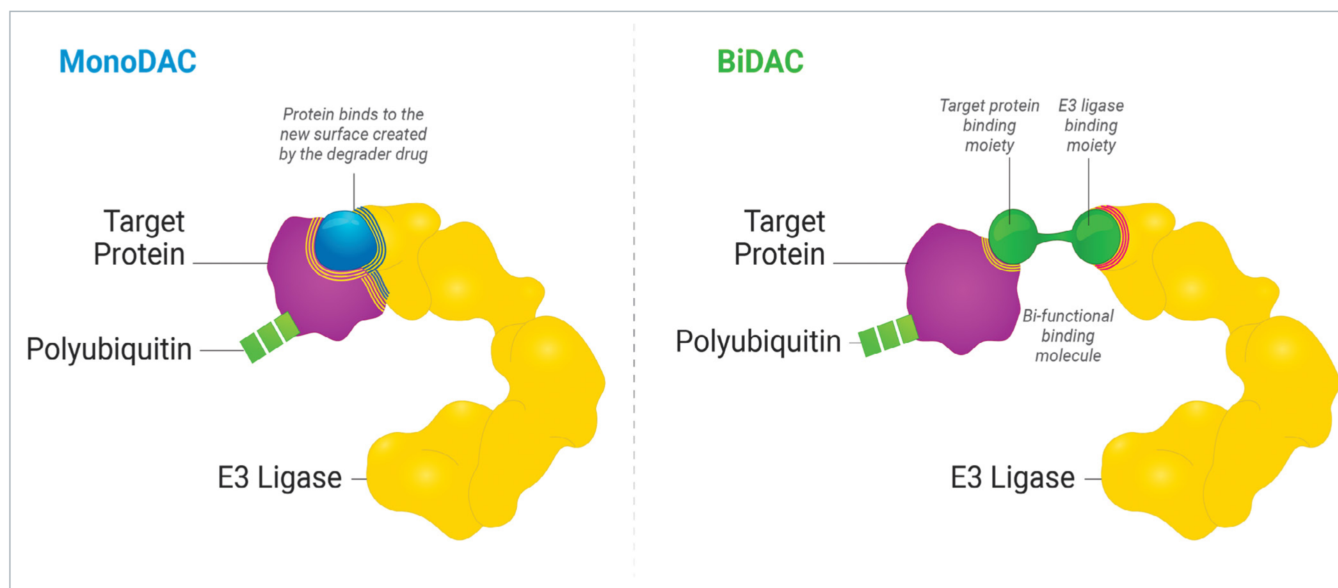
"One of the things that I think differentiates us at C4 Therapeutics is that we use both of these approaches as the targets present opportunities,"

maintains Fisher. "I'm not aware of other biotech firms in this space that have the capability to focus on both monoDACs and biDACs." C4 Therapeutics currently has two PROTACs in preclinical trials: an IKZF1-targeting monoDAC for treating hematologic malignancies, and a BRD9-targeting biDAC for treating sarcoma.

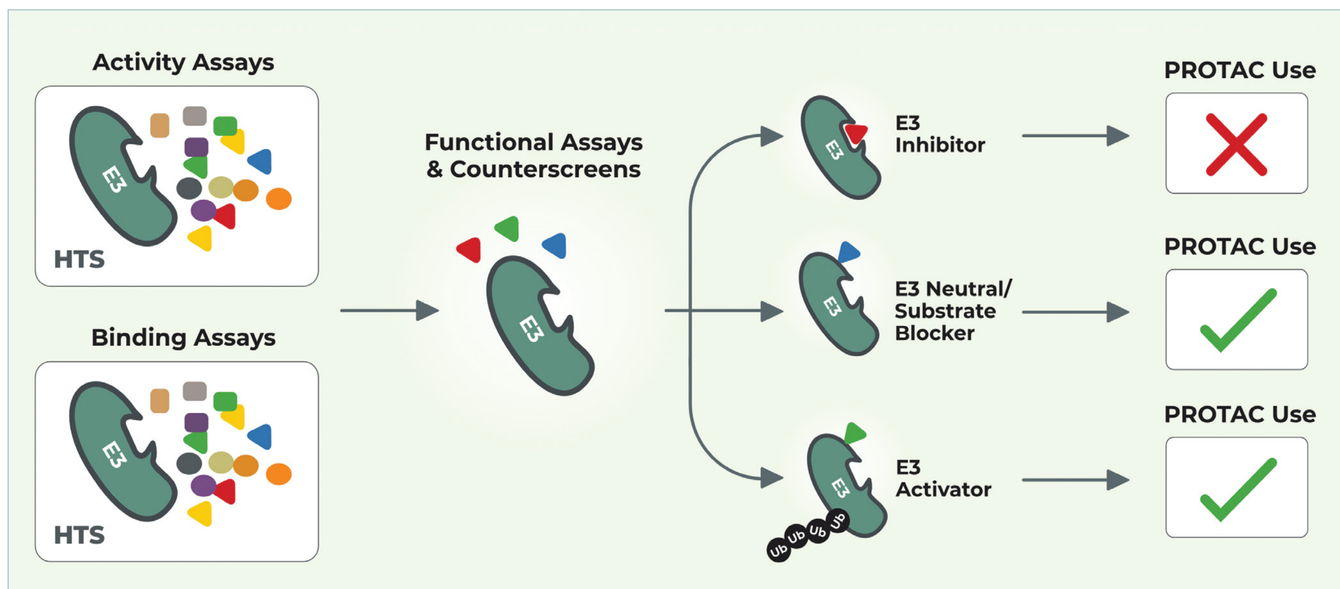
All of C4 Therapeutics' degrader molecules recruit the same E3 ligase, known as cereblon. Along with the Von Hippel–Lindau (VHL) ligase, cereblon is currently one of the most commonly recruited E3 ligases in targeted protein degradation. Fisher says that his company decided to invest deeply in cereblon because it is involved in the molecular action of well-known drugs such as thalidomide. Clinical history indicates that the ligase's action is well tolerated and unlikely to cause severe side effects.

Expanding the E3 ligase toolbox

Although PROTACs have been on the scene for a relatively short time, resistance mechanisms have already cropped up in preclinical trials. Most frequently, cancer cells evolve to downregulate the E3 ligases that the PROTACs depend on for polyubiquination of their target proteins. Furthermore, some proteins of interest are not effectively degraded using cereblon or VHL. Developing PROTACs that recruit E3 ligases other than VHL or cereblon could help bypass resistance mechanisms and expand the range of viable targets. In particular, some scientists



C4 Therapeutics is advancing monofunctional and bifunctional degradation-activating compounds—MonoDACs™ and BiDACs™, respectively. MonoDACs, or "glue degraders," bind to and create a new surface on E3 ligases to enhance the binding of E3 ligases to target proteins. BiDACs, or "heterobifunctional degraders," are designed so that one end binds to the disease-causing target protein and the other end binds to the E3 ligase.



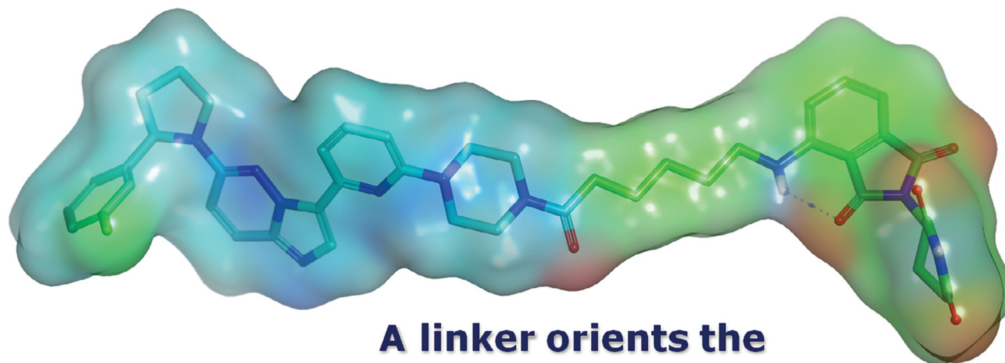
By exploring the ubiquitin pathway, Progenra advances the discovery of multiple classes of therapeutic molecule. For example, the company has developed UbiPro, a platform for the high-throughput screening of selective inhibitors of ubiquitin pathway enzymes such as E3 ligases and deubiquitinases.

have highlighted the potential advantages of focusing on E3 ligases that serve essential pathway roles. Taking this approach could make it harder for cells to downregulate them in response to PROTAC application.

"The creation of novel E3 ligands is the future of targeted protein degradation," says Jing Liu, PhD, executive director of medical chemistry, ***Cullgen***. The company has identified multiple ligands that bind E3 ligases not previously exploited for targeted protein degradation, and has confirmed that these ligands can be incorporated into bifunctional degrader molecules.

Liu says that roughly 50% of Cullgen's research and development efforts are aimed at developing novel E3 ligands, whereas the remaining 50% of these efforts focus on developing the company's existing internal pipeline of targeted protein degraders. Cullgen has built a library of linkers with different chemical and physical properties, allowing for efficient drug optimization.

For example, Cullgen previously reported on its creation of potent and selective degraders for tropomyosin receptor kinase A (TRKA), a key target for cancer treatments. However, the initial degrader molecules—CG416 and CG428—showed low oral bioavailability, so company scientists returned to the laboratory and created second-generation degraders—CG1037 and CG1054—for the same targets. These second-generation molecules showed higher oral bioavailability in mouse models without sacrificing efficacy or causing significant side effects.



Cullgen is developing ubiquitin-mediated, small molecule–induced target elimination (uSMITE) technology. This image shows the chemical structure of one of Cullgen's selective degraders. Notice that the structure consists of three moieties: one for binding the target (in this case, tropomyosin receptor kinase A); one for binding cereblon (which forms part of the E3 ubiquitin ligase complex); and one for linking the other two moieties.

Liu points to this process of developing TRKA degraders as a proof-of-concept example, saying, "We can utilize such pinpoint targeting capabilities to develop degraders with unique selectivity profiles to treat different diseases."

Implementing location specificity

Suresh Kumar, PhD, senior director and head of discovery, ***Progenra***, says that avoiding resistance isn't the only reason to develop PROTACs that recruit E3 ligases other than cereblon and VHL.

"If your target is a membrane protein, and you want to degrade that protein with a PROTAC," Kumar says, "that job is better done with a membrane-targeted E3 ligase than with a nuclear-located ligase."

For example, K-Ras, a well-known yet famously elusive oncological therapeutic target, is a membrane protein. Kumar says that to his knowledge, Progenra is "currently the only company that has a membrane-targeted ligase." At multiple conferences in September and October 2020, Kumar and his colleagues presented experimental results from the development of a potent, membrane-targeted PROTAC capable of degrading K-Ras with high specificity.

Over the past 15 years, Progenra has developed and utilized a proprietary platform that it calls UbiPro,

which consists of a series of enzyme activity assays that "closely replicate physiological milieu." Progenra uses this platform for drug discovery involving both E3 ligases and deubiquitinases, another group of enzymes in the UPS. The platform has the capacity for high-throughput screening, with a panel of over 30 purified E3 ligases that can be applied for profiling and selectivity.

Kumar expects that Progenra and the rest of the PROTAC field will eventually expand far beyond cancer therapeutics. "Our ligases have extremely high relevance to human biology, with implications in diseases ranging from cancer to Parkinson's disease, Alzheimer's disease, and inflammatory disorders," he insists. "All these human diseases have an underlying problem at the fundamental cellular level in terms of degrading proteins—either lack of degradation or excessive degradation." Progenra is currently evaluating novel PROTACs as anti-inflammatory agents but has not yet made further details public.

Bypassing E3 ligases entirely

Even as PROTAC technology continues to advance, there remain limitations. Recruiting specific E3 ligases means relying on a relatively narrow set of chemical structures that bind those ligases. Those molecules can present design challenges and limit target scope. ***Amphista Therapeutics*** decided to let other companies tackle PROTACs, and instead secured funding to pursue novel methods of targeted protein degradation.

"The ubiquitin proteasome system is, in many ways, one of the most rubbish enzyme systems there is, because it's really very poorly selective," says Ian Churcher, PhD, chief scientific officer, Amphista Therapeutics. "If you get substrates close enough for long enough to the ubiquitin proteasome system, they will be degraded. And that's really what we set out to do."

Amphista has designed multiple "magnet" ligands that are believed to recruit multiple UPS proteins and activate multiple parallel degradation pathways that depend on critical cellular components. This approach makes it more difficult for cancers to develop resistance. Researchers incorporate these magnet ligands into bifunctional molecules that bind to target proteins and bring them into proximity with the UPS.

"We don't believe anyone has ever used these molecules before in this way," remarks Churcher. "We often think of it as next- generation targeted protein degradation. It's an amazing field with huge potential, but we want to expand that potential into more drug targets, better profiles, and better dosing routes for patients."

Amphista is currently developing two types of small-molecule drugs that utilize these novel mechanisms. One recruits deubiquitinase enzymes, whereas

At Mount Sinai's Icahn School of Medicine, the laboratory of Jian Jin, PhD, used a hydrophobic tagging approach to generate MS1943, a first-in-class degrader of EZH2, a protein that is overexpressed in multiple types of cancer. Importantly, MS1943 has a profound cytotoxic effect in multiple triple-negative breast cancer cells.

the other recruits the proteasome directly. Amphista plans to bring its first molecule into the clinic in 2023.

Initiating protein self-sabotage

Jian Jin, PhD, director of the Mount Sinai Center for Therapeutics Discovery, is the lead author on a February 2020 paper in *Nature Chemical Biology* describing MS1943, a first-in-class selective degrader for histone methyltransferase EZH2. This degrader relies on a technique known as hydrophobic tagging, which Jin says has been understudied by the biomedical community thus far.

Hydrophobic tagging involves attaching a bulky hydrophobic chemical group to a small-molecule binder of the target protein—in this case, EZH2 inhibitor C24. Jin indicates that the mechanism of degrader action is not yet fully elucidated, but scientists believe that the presence of the large hydrophobic group causes the protein to misfold, which ultimately triggers its degradation via the natural action of the UPS.

MS1943 showed high potency in triple- negative breast cancer cells while sparing normal cells. Furthermore, the compound showed high oral bioavailability, which Jin hypothesizes may be due in part to the molecule's smaller size compared to most PROTACs.

"Now that we have published this approach, I think more and more research groups will explore this technology," predicts Jin. "We are actively optimizing these compounds and will hopefully progress into clinical studies." ■

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 perme-ability, or their undesired off-target effects. The creativity of researchers is unlimited and constantly pushes them to invent new approaches for the devel-opment 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.

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

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.

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

Figure 2: PROTAC compound tridimensional structure.

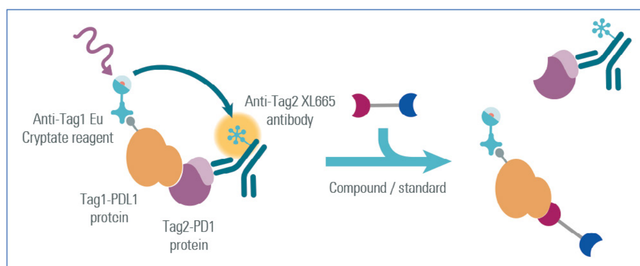


Figure 3: PD1 PD-L1 HTRF assay principle.

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.

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

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 (Revvity #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 series.

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.

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

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

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.

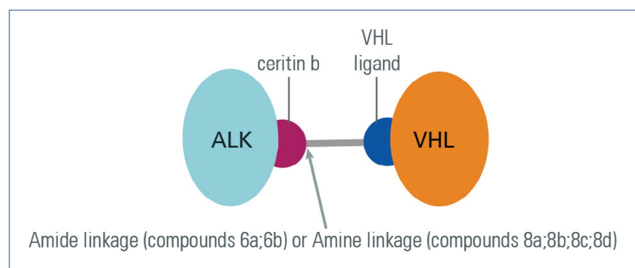


Figure 4: PROTAC specific to ALK compound structure.

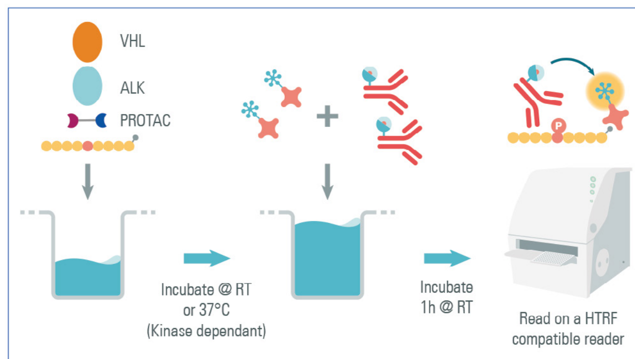


Figure 5: ALK activity inhibition detection assay protocol.

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)

Table 2: *In vitro* evaluation of ALK degraders.

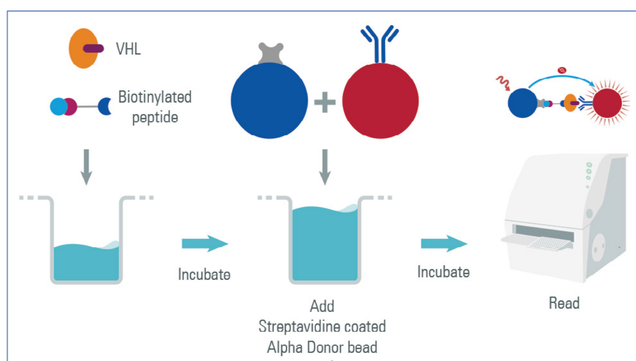


Figure 6: Alpha assay protocol.

toward ALK and VHL.

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

Results of the assay are shown in table 2.

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.

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

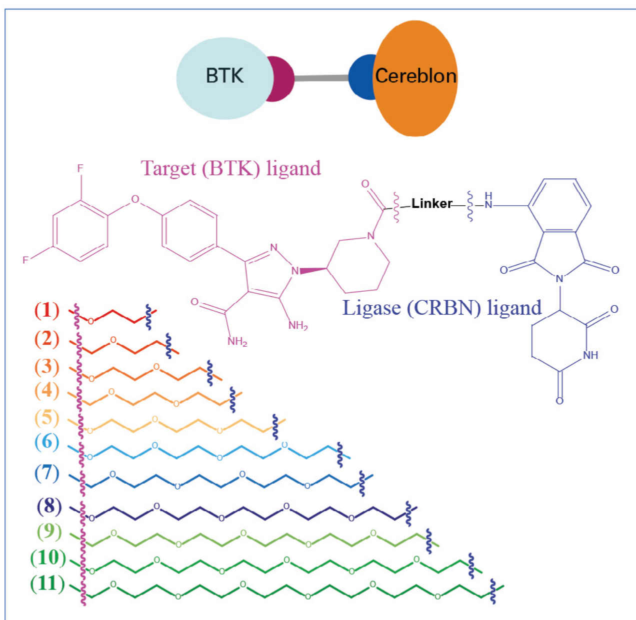


Figure 7: BTK and CRBN parent molecules (Top) from which 11 PROTACs of varying linker lengths were generated (Bottom).

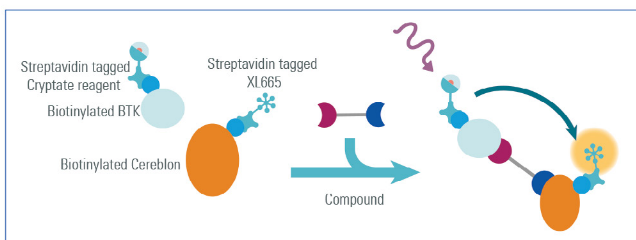


Fig. 8: HTRF assay principle.

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)

To evaluate the linker length effect on {target–PROTAC–ligase} ternary formation and screen for the most potent BTK PROTAC, an *in vitro* HTRF assay was set up. Recombinant biotinlylated BTK and CRBN proteins were incubated with Streptavidin donor

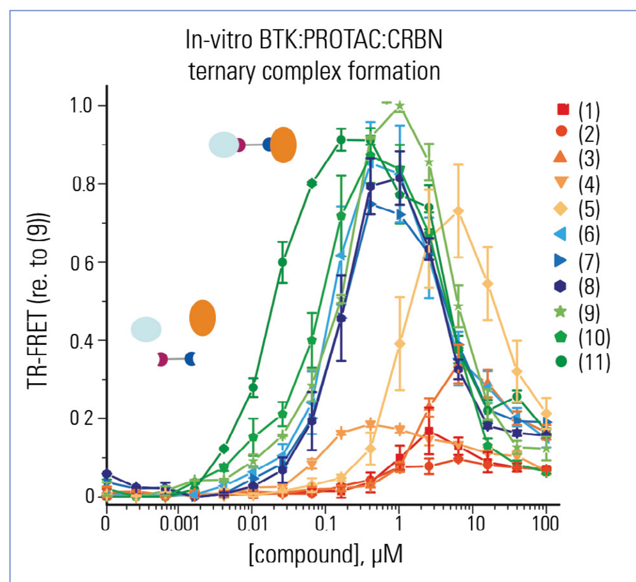


Figure 9: TR-FRET–based assay to evaluate the effect of linker length on {BTK–PROTAC–CRBN} ternary complex formation. 200 nM biotinylated BTK and 500 nM biotinylated CRBN were incubated with varying PROTAC concentrations for 30 min before endpoint data collection at 620 nm (donor) and 665 nm (acceptor).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

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).

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).

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

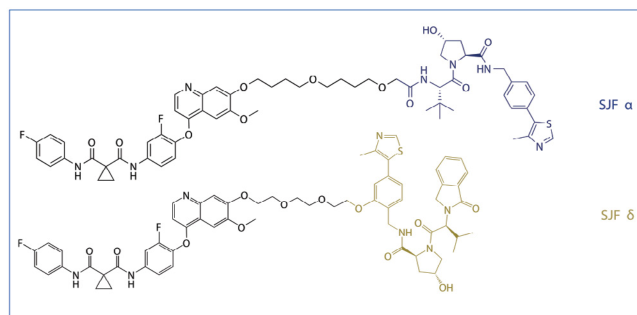


Figure 10: SJFα PROTAC (13-atom linker, amide attachment) and SJFδ PROTAC (10-atom linker, phenyl attachment)

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

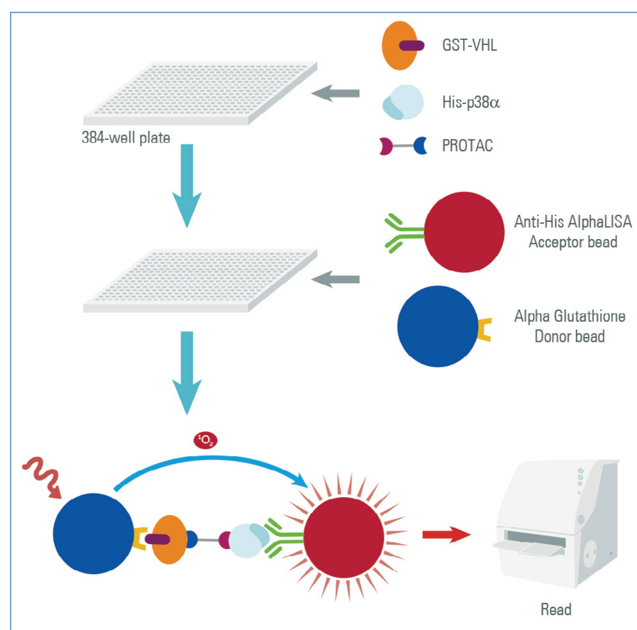


Figure 11: AlphaLISA assay protocol.

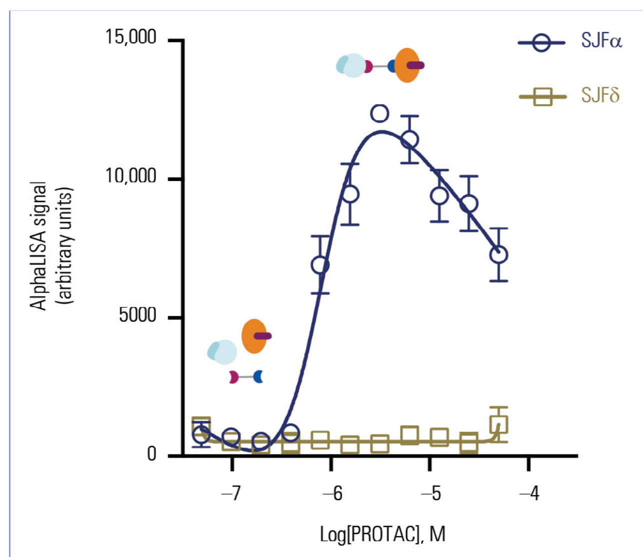


Figure 12: Proximity-based AlphaLISA assay. His-p38α and GST-VHL were incubated in the presence of increasing concentrations of SJFα and SJFδ and the extent of ternary complex formation was assessed by excitation with incident light with λ = 680 nm and capture of the emission light at λ = 615 nm. Error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

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

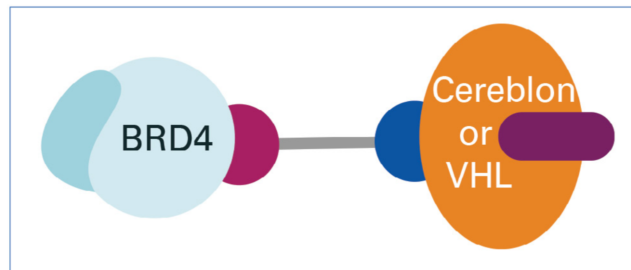


Figure 13: PROTACs specific to BRD4 compound structure. Synthetized with either a ligand specific to VHL or specific to cereblon.

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.

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

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

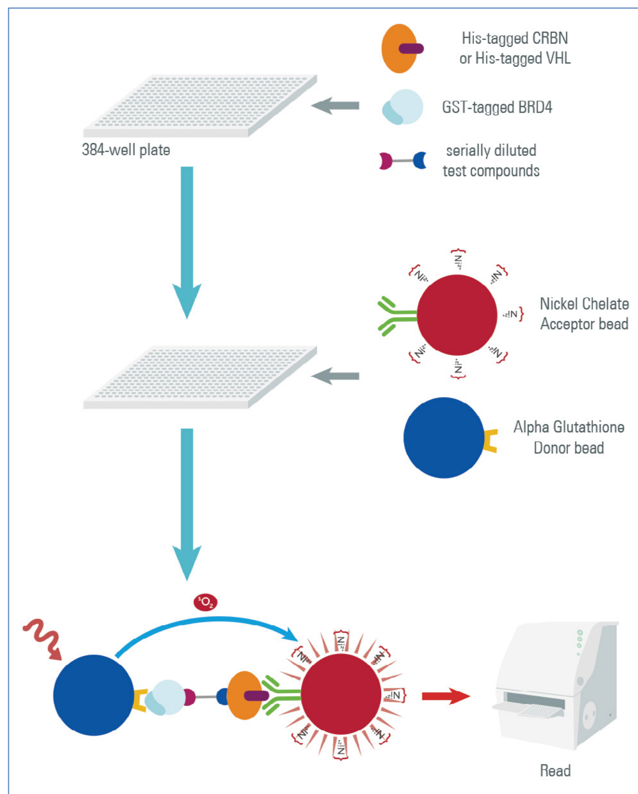


Figure 14: AlphaScreen assay principle.

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.

Results are in as a bell-shaped dose–response

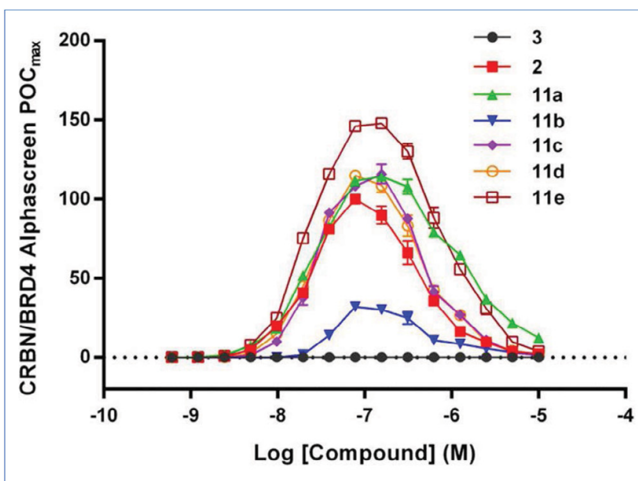


Figure 15: Proximity assay data for CRBN/BRD4 PROTACs.

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

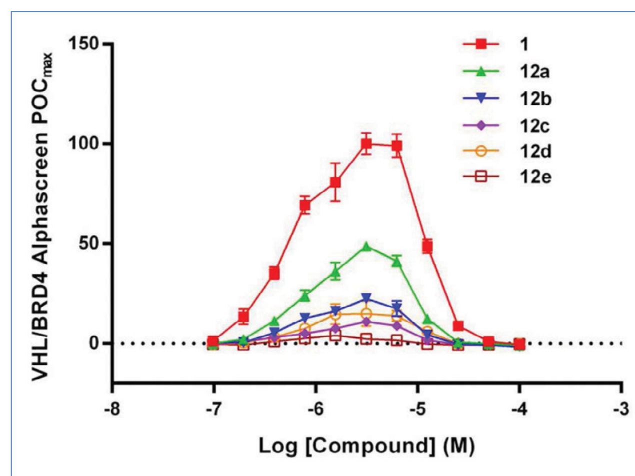
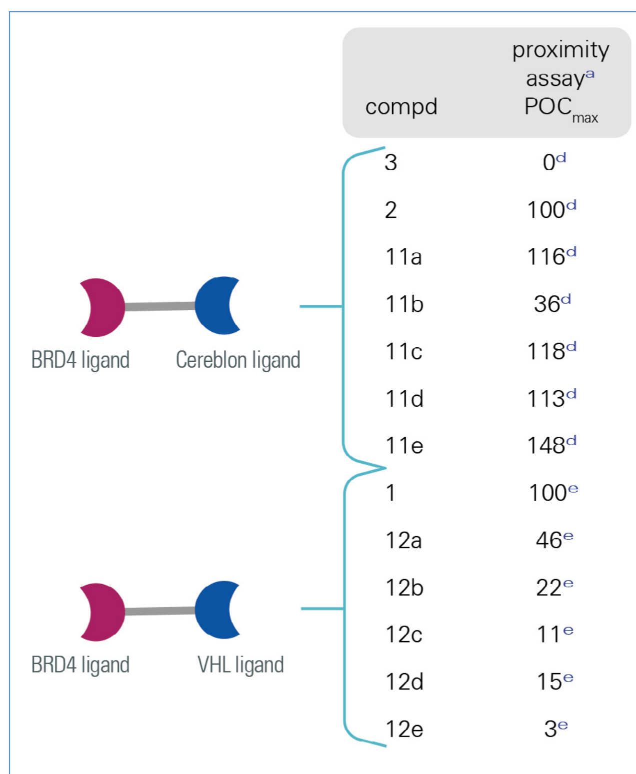


Figure 15: Proximity assay data for CRBN/BRD4 PROTACs.

induce ternary complex formation. Compound 11e was found to be the most effective.

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



compd	proximity assay[a] POC_{max}
3	0[d]
2	100[d]
11a	116[d]
11b	36[d]
11c	118[d]
11d	113[d]
11e	148[d]
1	100[e]
12a	46[e]
12b	22[e]
12c	11[e]
12d	15[e]
12e	3[e]

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.

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).

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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2. Kang, C.H. et al. (2018). Induced protein degradation of anaplastic lymphoma kinase (ALK) by proteolysis targeting chimera (PROTAC). *Biochemical and Biophysical Research Communications*, 505(2), 542–547.

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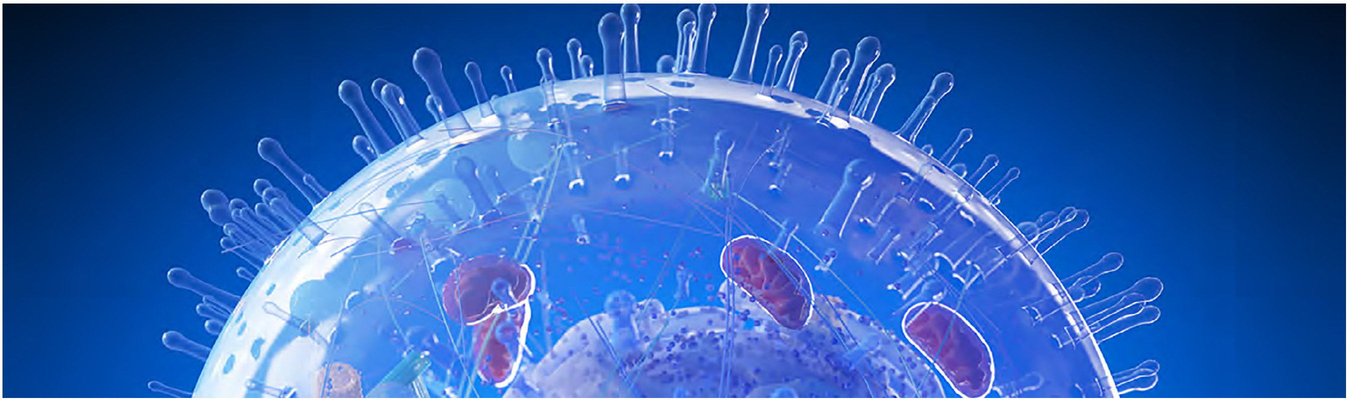
5. Zorba, A. et al. (2018). Delineating the role of cooperativity in the design of potent PROTACs for BTK. Proceedings of the National Academy of Sciences, 115(31), E7285–E7292.

FIGURE CREDITS:
Fig. 1: Adapted from Tinworth et al. (2016) *Med. Chem. Comm.* 7/22/06.
Fig. 2, Table 1: 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.
Fig. 7, 8, 9: 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.
Fig. 10, 11, & 12: Copyright: Smith, B.E. et al. (2019). Differential PROTAC substrate specificity dictated by orientation of recruited E3 ligase. *Nature Communications,* 10(1), 131.
Fig. 15, & 17: 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.



Improve PROTAC Targeting Kinase Discovery with HTRF® Kinase Binding Kits

APPLICATION NOTE: HTRF Technology

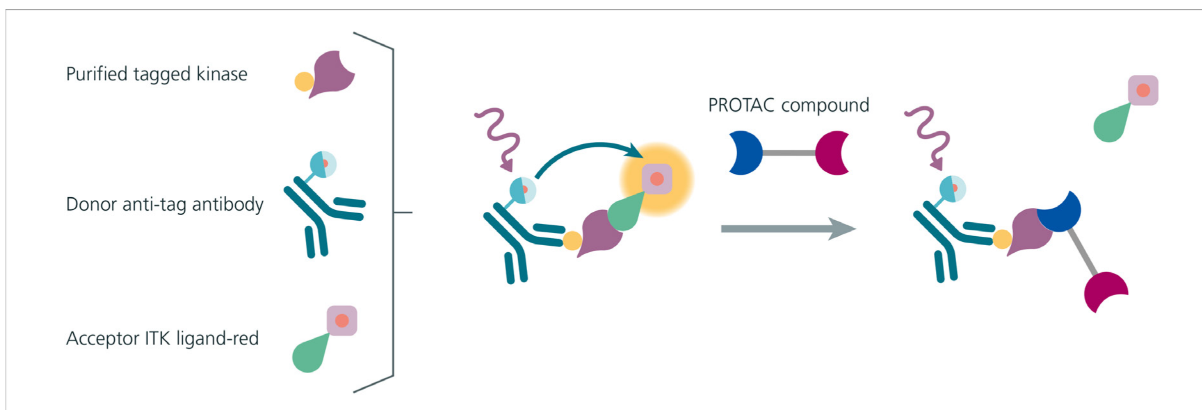
Authors: Fabienne Charrier-Savournin, Julie Vallaghé & Elodie Dupuis

Kinases are essential proteins involved in signal transduction, regulating numerous biological events such as cell growth and proliferation, cell differentiation, and migration. Consequently, kinase dysfunctions due to gene amplification or mutations are associated with numerous diseases, such as cancer, immune and metabolic disorders, and cardiovascular and neurodegenerative diseases. From a therapeutic perspective, kinase inhibitors competing with ATP binding, as well as monoclonal antibodies, have been developed extensively and mainly approved for cancer treatment. However, these drugs suffer from several limitations, such as drug resistance, off-target effects, and other side effects. More recently, promising new approaches relying on induced protein degradation, such as PROTAC, have gained traction because they are expected to overcome most of the limitations inherent to small molecule inhibitors and even target the "undruggable" proteome. Proteolysis-targeting chimera (PROTAC) compounds are hetero-bifunctional molecules that bind both to the targeted protein and to E3 ubiquitin ligase, forming a ternary complex that ultimately leads to POI ubiquitination and its subsequent proteasome degradation. To date, PROTAC technology has been used to induce the degradation of various proteins, including kinases.

Here, we demonstrate how HTRF kinase binding assays are used to efficiently and accurately identify, characterize, and optimize PROTAC targeting kinases on two relevant models: BTK and CDK4.

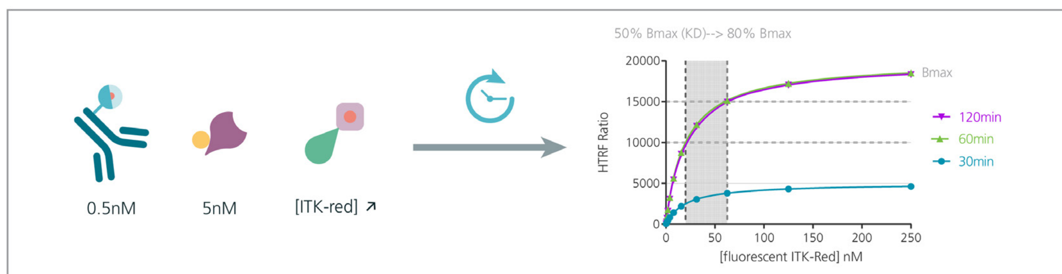
Assay principle and workflow

The assay is based on an HTRF sandwich format relying on HTRF donor anti-tag antibody or streptavidin, which recognize the corresponding tagged kinase and an HTRF acceptor fluorescent derivative of kinase inhibitor. In the presence of competitive inhibitors such as ITK-derived PROTAC compounds, the binding of fluorescent ITK ligand-red is abrogated, leading to HTRF signal decrease.

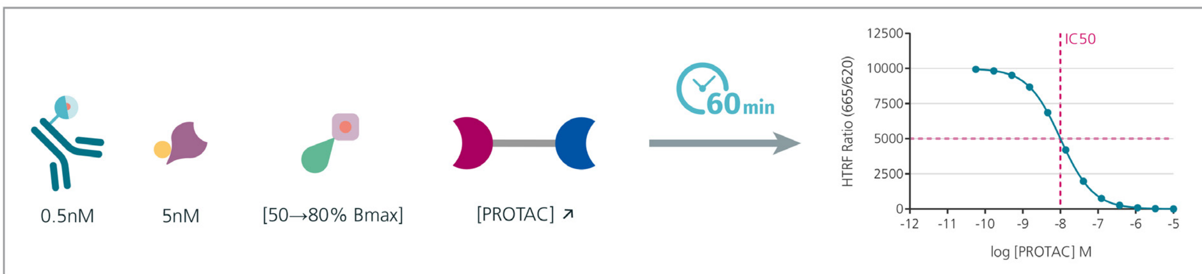


2-step experiment workflow:

1. **HTRF saturation binding curve is evaluated to determine the KD of acceptor fluorescent ligand towards the kinase of interest at equilibrium.**



2. **HTRF competitive binding assay is performed by adding a fixed concentration of fluorescent ITK-red from 50% (KD) to 80% of the Bmax to a dilution series of the compound of interest.**



The measured IC50 values are then used to calculate apparent Ki potency using the Cheng-Prussoff equation:

$$K_i = \frac{IC_{50}}{\left(1+\left(\frac{\text{Dasatinib-Red}}{K_d}\right)\right)}$$

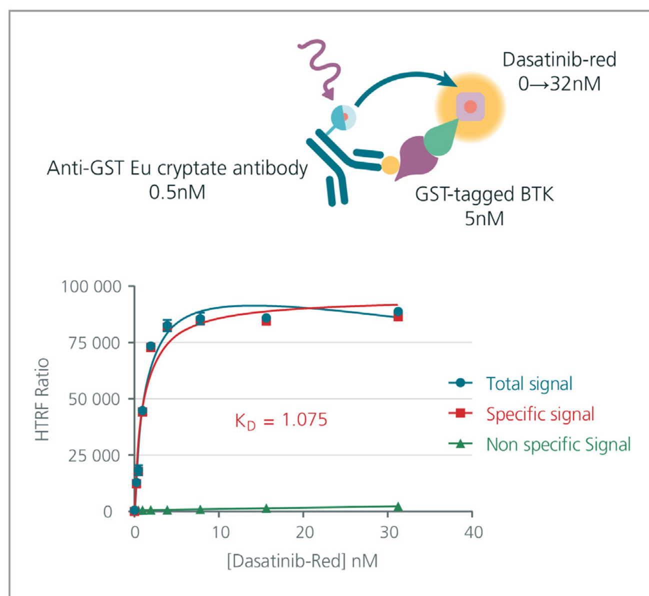
PROTAC compound screening through HTRF BTK kinase binding assay

GST-tagged BTK kinase binding assay

1. Saturation binding experiment: Determination of dasatinib-red Kd

The first step in developing the kinase inhibitor assay is to determine the optimal fluorescent ITK concentration for the kinase of interest, in this case the GST-BTK. The assay's dynamic range depends on the kinase concentration.

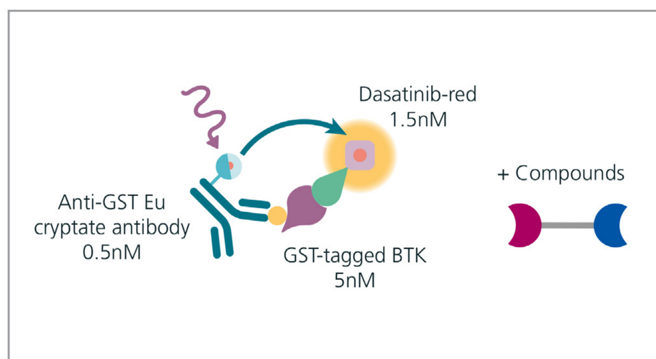
Here, 5 nM of GST-BTK, 0.5nM of anti-GST Eu cryptate anti-body, and a concentration ranging from 0-32 nM of dasat-inib-red were used. nM of dasatinib-red were used.

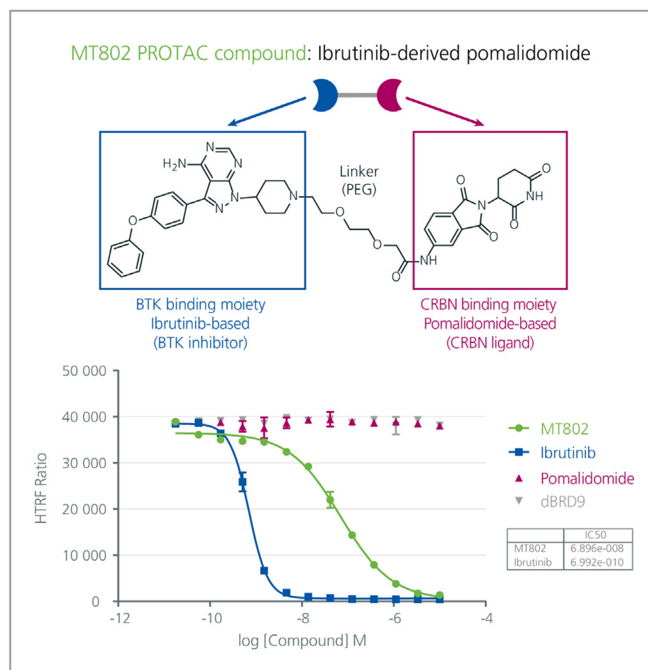


The Kd of dasanitib-red for GST-BTK is 1.075nM

1. Assessment of MT802 PROTAC compound binding through HTRF competitive inhibition experiment

From the above results, dasatinib-red was used at 1.5 nM in a competitive assay where ibrutinib and its PROTAC derivative MT802 were tested. Two irrelevant compounds were also included in the experiment: Pomalidomide, corresponding to the cereblon ligand and comprised in MT802; and dBRD9, another PROTAC compound targeting BRD9 protein.





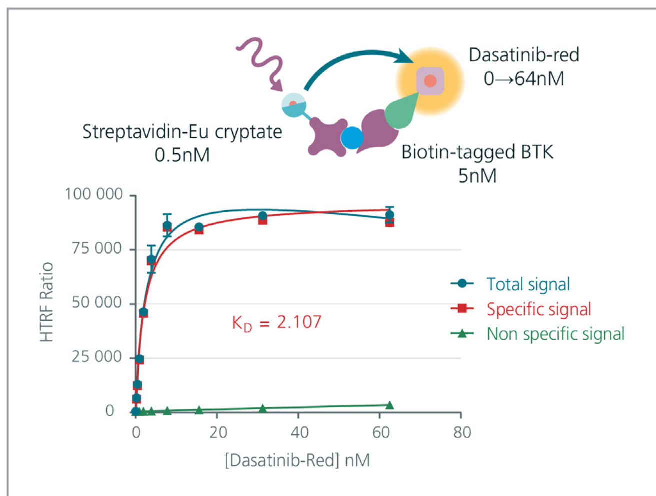
As expected, unlike the irrelevant dBrd9 and pomalidomide compounds, both ibrutinib and MT-802 compounds efficiently compete with the dasatinib-red ligand. This experiment revealed that the potency of the MT802 compound is approximately 100 times lower than ibrutinib, likely explained by the greater size and sterical hindrance from the PROTAC compound.

Biotin-tagged BTK kinase binding assay

1. Saturation binding experiment: Determination of dasat inib-red Kd

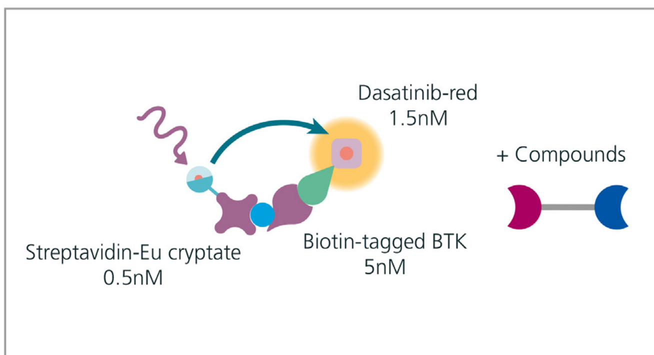
The same principle as that described above was applied to a recombinant biotinylated-BTK.

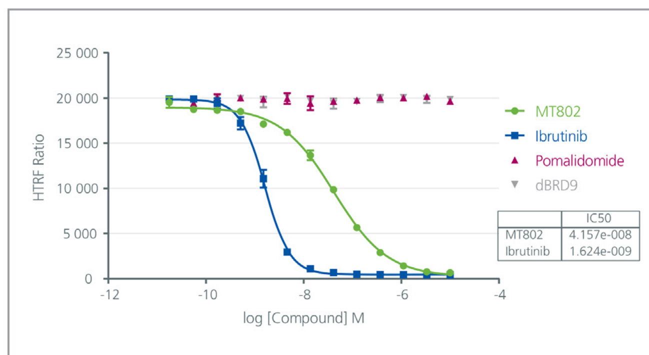
Here, 5 nM of biotin-BTK, 0.5nM of streptavidin-Eu cryptate, and a concentration ranging from 0-64 nM of dasatinib-red were used.



Here, the Kd of dasanitib-red for biotin-BTK is 2.107nM.

2. Assessment of MT802 PROTAC compound binding through HTRF competitive inhibition experiment.





From the above results, dasatinib-red was used at 1.5 nM in a competitive assay where ibrutinib and its PROTAC derivative MT802 were tested. Two irrelevant compounds were also included in the experiment: Pomalidomide, corresponding to the cereblon ligand and comprised in MT802; and dBRD9, another PROTAC compound targeting BRD9 protein.

Comparable results were obtained with the biotinylated-BTK enzyme. In this experiment, MT802 compound potency is approximately 25 times lower than that of the Ibrutinib.

Materials and methods:

- GST-tagged BTK, Carna Biosciences (ref. 08-180)
- MAb Anti GST-Eu kinase binding cryptate, Revvity (ref. 62KBGSTKAF)
- Biotinylated-tagged BTK, Carna Biosciences (ref. 8-480-23N)
- Dasatinib-red, Revvity (ref. 62KB02REDC)
- Streptavidin-Eu cryptate Kinase Binding,
- Revvity (ref. 62KBSAKAF)
- Kinase Binding Buffer, Revvity (ref. 62KBBRDD)
- MT802, Revvity (not commercially available)
- Ibrutinib Selleckchem (ref. S2680)
- Pomalidomide, Tocris (ref. 6302)
- dBRD9, Tocris (ref. 6606)
- ProxiPlate-384 Plus, Revvity (ref. 6008280)

HTRF saturation binding experiment:

+ 5 µL kinase binding buffer (or 5µL of unlabeled dasatinib 3µMf for non-specific signal)
+ 5 µL recombinant GST or biotin-tagged BTK (5 nMf)
+ 5 µL HTRF anti-GST or streptavidin-Eu cryptate
+ 5 µL HTRF dasatinib-red serial dilutions

1 hr incubation at RT before reading HTRF signal

HTRF competitive experiment:

+ 5 µL of serial dilution compounds
+ 5 µL recombinant GST or biotin-tagged BTK (5 nMf)
+ 5 µL HTRF anti-GST or streptavidin-Eu cryptate
+ 5 µL of HTRF dasatinib-red (1.5nMf)

1 hr incubation at RT before reading HTRF signal

Table 1 shows the results obtained using either a GST or biotind etection system. Both assay formats provide correlated results and enable convenient and robust screening of PROTAC targeting BTK compounds, as well as their pharmacological characterization.

Table 1	GST-BTK	Biotin-BTK	Reference values in literature
Dasatinib-red Kd (nM)	1.075	2nM	nd*
MT802 Ki / IC50 / SB	29nM / 69nM / 120	23nM / 41nM / 88	IC50=48.88nM (1)
Ibrutinib Ki / IC50 / SB	0.29nM / 0.69nM / 60	0.9nM / 1.6nM / 43	IC50=0.5nM (2)

*nd: not determined

1. Buhimschi et al. Targeting the C481S Ibrutinib-Resistance Mutation in Bruton's Tyrosine Kinase Using PROTAC-Mediated Degradation. Biochemistry 2018, 57, 26, 3564–3575

2. Akinleye et al. Ibrutinib and novel BTK inhibitors in clinical development. Journal of Hematology & Oncology 2013, 6:59

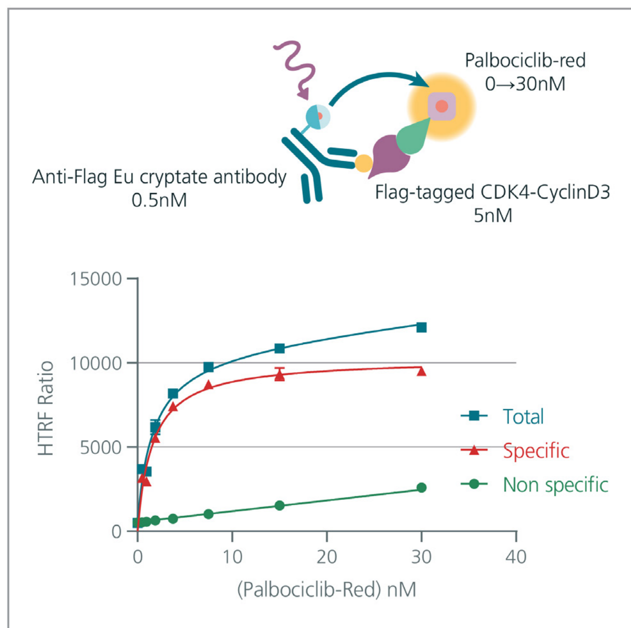
PROTAC compound screening through HTRF CDK4 kinase binding assay

Flag-CDK4 kinase binding assay

1. **Saturation binding experiment: Determination of palbociclib-red Kd**

This experiment was performed with 5 nM of Flag-CDK4, 0.5nM of anti-Flag Eu cryptate antibody, and a concentration ranging from 0-30 nM of palbociclib-red.

The Kd of palbociclib-red for Flag-CDK4 is 1.57nM; the 80% Bmax is 5nM.



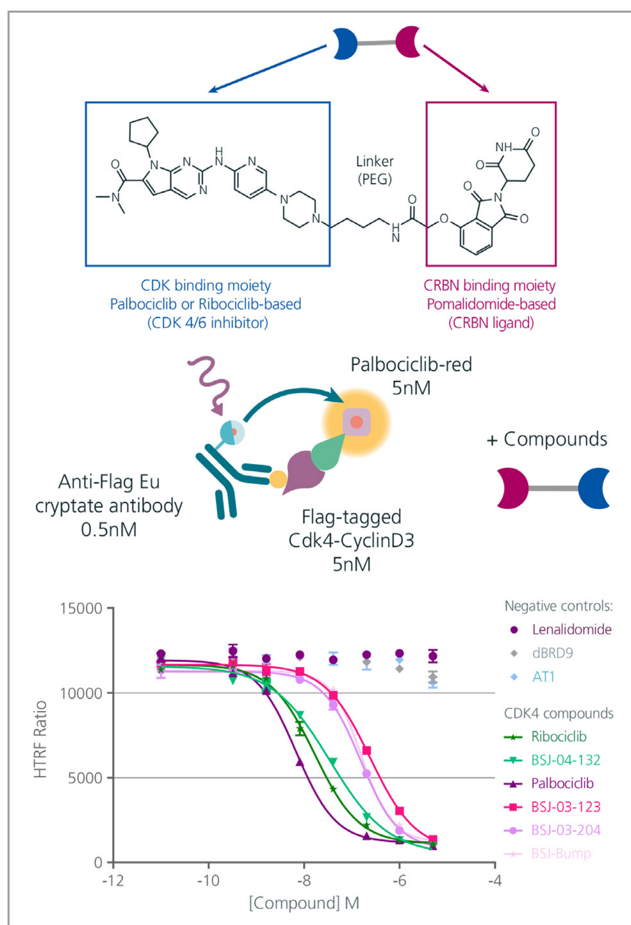
The Kd of palbociclib-red for Flag-CDK4 is 1.57nM; the 80% Bmax is 5nM.

1. **Assessment of CDK4 degrader binding through HTRF competitive inhibition experiment.**

From the above results, a competitive experiment was carried out using palbociclib-red at 5 nM. The following compounds were tested:

- Palbociclib and ribociclib, which are orthosteric inhibitors of CDK4 and CDK4/6 kinases, respectively.
- BSJ-03-123, BSJ-03-204, and BSJ-Bump, which are pal-bociclib derived PROTAC compounds including a thalidomide moiety.
- BSJ-04-132, which is a ribociclib-derived thalido-mide-based PROTAC compound.
- Lenalidomide, dBrd9, and AT1, which are irrelevant compounds.

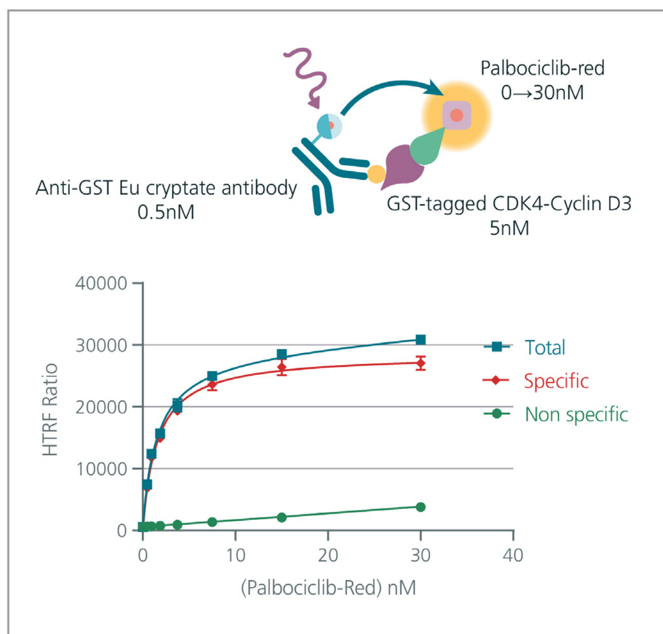
As expected, unlike the irrelevant dBrd9, lenalido-mide or AT1 compounds, palbociclib and ribociclib as well as their PROTAC derivatives efficiently compete with Palbociclib-Red ligand binding. Interestingly, derivatization of palbociclib with thalidomide (BSJ-03-123, BSJ-03-204, BSJ-Bump) significantly impacts its affinity (from 25 to 36-fold), whereas derivatization of ribociclib (BSJ-04-132) shows less impact (2-fold). Pharmacological parameters are reported in Table 2.



GST-CDK4 kinase binding assay

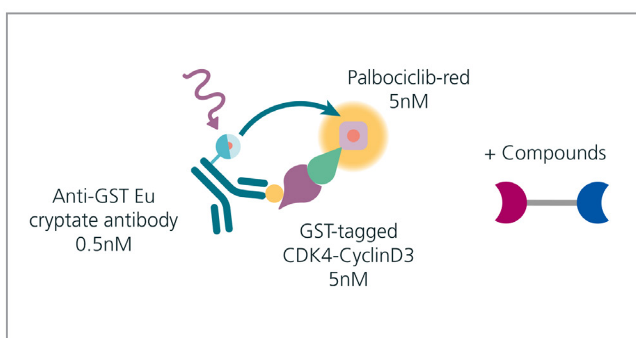
1. Saturation binding experiment: Determination of the Palbociclib-red Kd

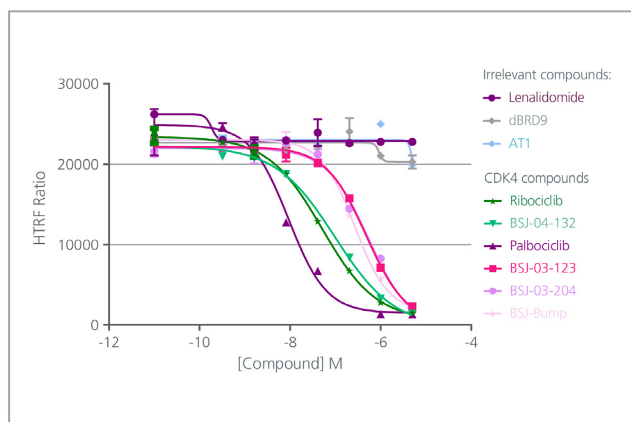
This experiment was performed with 5 nM of GST-CDK4, 0.5nM of anti-GST Eu cryptate antibody, and a concentration ranging from 0-30 nM of palbociclib-red.



The Kd of palbociclib-red for Flag-CDK4 is 1.57nM; the 80% Bmax is 5nM

2. Assessment of CDK4 degrader binding through HTRF competitive inhibition experiment.





From the above results, palbociclib-red was used at 5 nM in a competitive assay using the same panel of compounds as previously described.

Comparable results were obtained using the GST tagged CDK4 enzyme. Palbociclib and ribociclib, as well as their PROTAC derivatives, exhibit efficient competition with palbociclib-red ligand binding.

Again, the derivatization of palbociclib with thalidomide (BSJ-03-123, BSJ-03-204, BSJ-Bump) compounds leads to decreasing their potencies, whereas thalidomide derivatization of ribociclib (BSJ-04-132) does not impair its potency. Pharmacological parameters are reported in Table 2

Materials and methods:

- Flag-tagged CDK4, Carna Biosciences (ref #04-405-20N)
- MAb Anti FLAG M2-Eu cryptate, Revvity (ref. 61FG2KLA)
- GST-tagged CDK4, Carna Biosciences (ref #04-105)
- MAb Anti GST-Eu cryptate, Revvity (ref. 61GSTKLA)
- Palbociclib-red, Revvity (not commercially available)
- Kinase Binding Buffer, Revvity (ref. 62KBBRDD)
- ProxiPlate-384 Plus, Revvity (ref. 6008280)

HTRF saturation binding experiment:

+ 5 µL kinase binding buffer
(or 5µL of unlabeled palbociclib 3µMf for non-specific signal)
+ 5 µL recombinant GST-tagged CDK4 (5 nMf)
+ 5 µL HTRF Mab anti GST-Eu cryptate (0.5nMf)
+ 5 µL of HTRF palbociclib-red serial dilutions

2 hrs incubation at RT before reading HTRF signal

HTRF competitive experiment:

+ 5 µL of serial dilution compounds
+ 5 µL recombinant GST-tagged CDK4 (5 nMf)
+ 5 µL HTRF anti GST-Eu cryptate
+ 5 µL of HTRF palbociclib-red (5nMf)

2 hrs incubation at RT before reading HTRF signal

Table 2 shows the results obtained using either a Flag or GST detection system. Despite the imperfect match between experimental and reported pharmacological values, both assay formats provide results consistent with literature, with palbociclib being twice as potent as ribociclib. They both enable convenient and robust screening of PROTAC targeting CDK4 compounds, as well as their pharmacological characterization.

Table 2	Flag-CDK4	GST-CDK4	Reference values in literature
Palbociclib-red Kd (nM)	1.55	1.57	nd*
Palbociclib Ki / IC50 / SB	1.56nM / 6.76nM / 10	2.1nM / 9.23nM / 17	IC50 5-20 nM (1, 2) Ki 0.26nM (3)
BSJ-04-123 Ki / IC50 / SB	55.7nM / 241 nM /17	109.7nM / 475 nM /119	IC50 31.2nM (1)
BSJ-04-204 Ki / IC50 / SB	35.1nM /152nM / 14	125.9nM / 1.6nM / 13	nd*
BSJ-Bump Ki / IC50 / SB	37nM / 160nM / 9	61.4nM / 266nM / 14	IC50 41.1nM (1)
Ribociclib Ki / IC50 / SB	3.8 nM / 16.7nM / 11	11.5nM / 50nM / 42	IC50 30nM (2) Ki 0.53nM (3)
BSJ-04-132 Ki / IC50 / SB	7.9nM / 34.3 / 24	22.6nM / 98.1nM/ 16	

*nd: not determined

1. Brand M. et al., Homolog-Selective Degradation as a Strategy to Probe the Function of CDK6 in AML. Cell Chemical Biology; 26, 300–306

2. O'Leary B. et al., Treating cancer with selective CDK4/6 inhibitors. Nature Reviews Clinical Oncology; 13,417–430

3. Chen et al., Spectrum and Degree of CDK Drug Interactions Predicts Clinical Performance. Molecular Cancer Therapeutics; 15(10); 2273–81

Conclusions

This application note clearly demonstrates the applicability of HTRF kinase binding kits in a PROTAC context. By combining different anti-tag antibodies and fluorescent ligands, as well as different sources of recombinant protein kinases, it is possible to easily screen and characterize new PROTAC compound targeting kinases. The highly versatile kinase binding kits offer an accurate and straightforward method for exploring and expanding the possibilities of targeted protein degradation strategies.