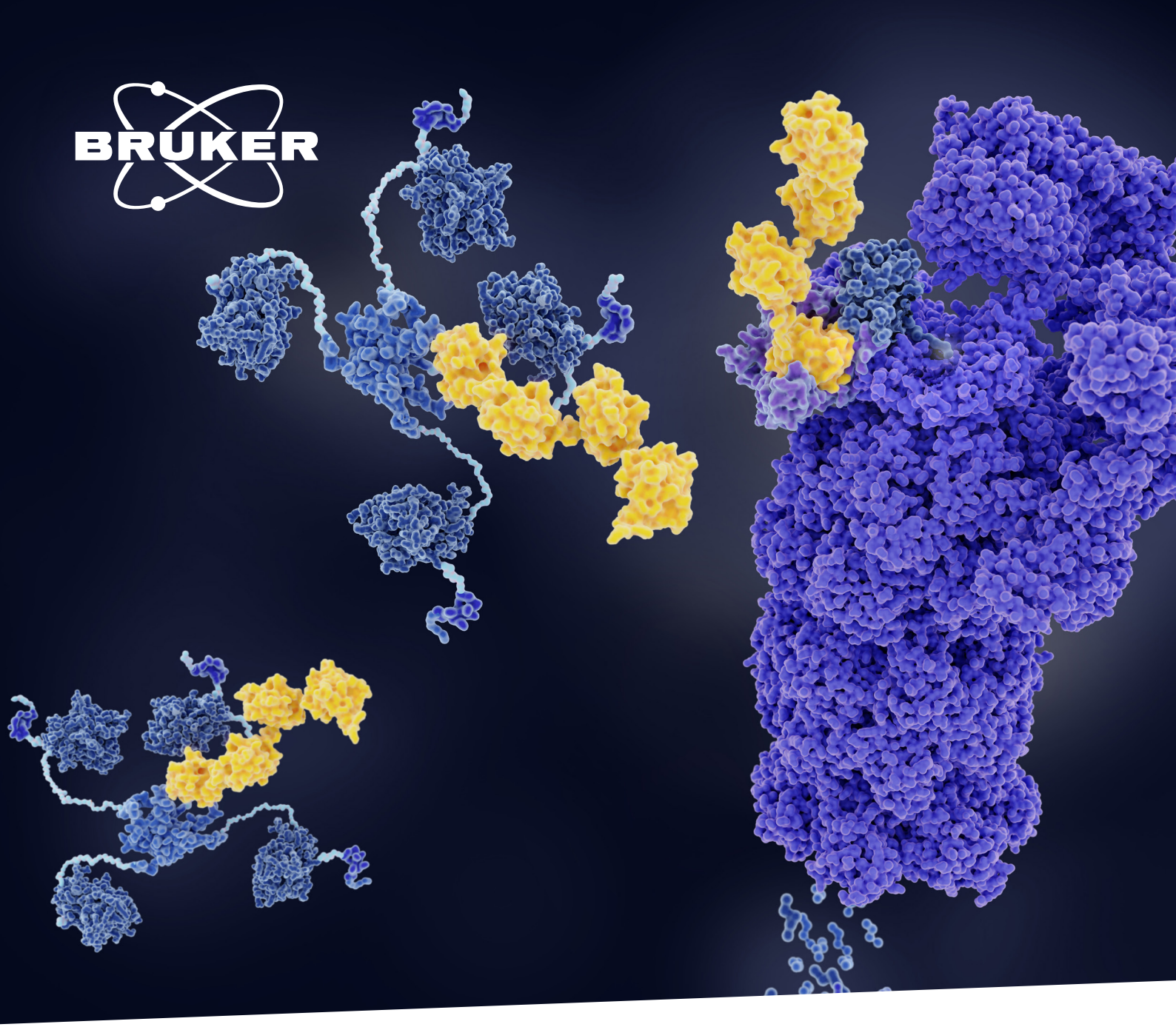


# Navigating Targeted Protein Degraders

## Advanced Analytical Tools for High-Throughput Proteomics

Innovation with Integrity

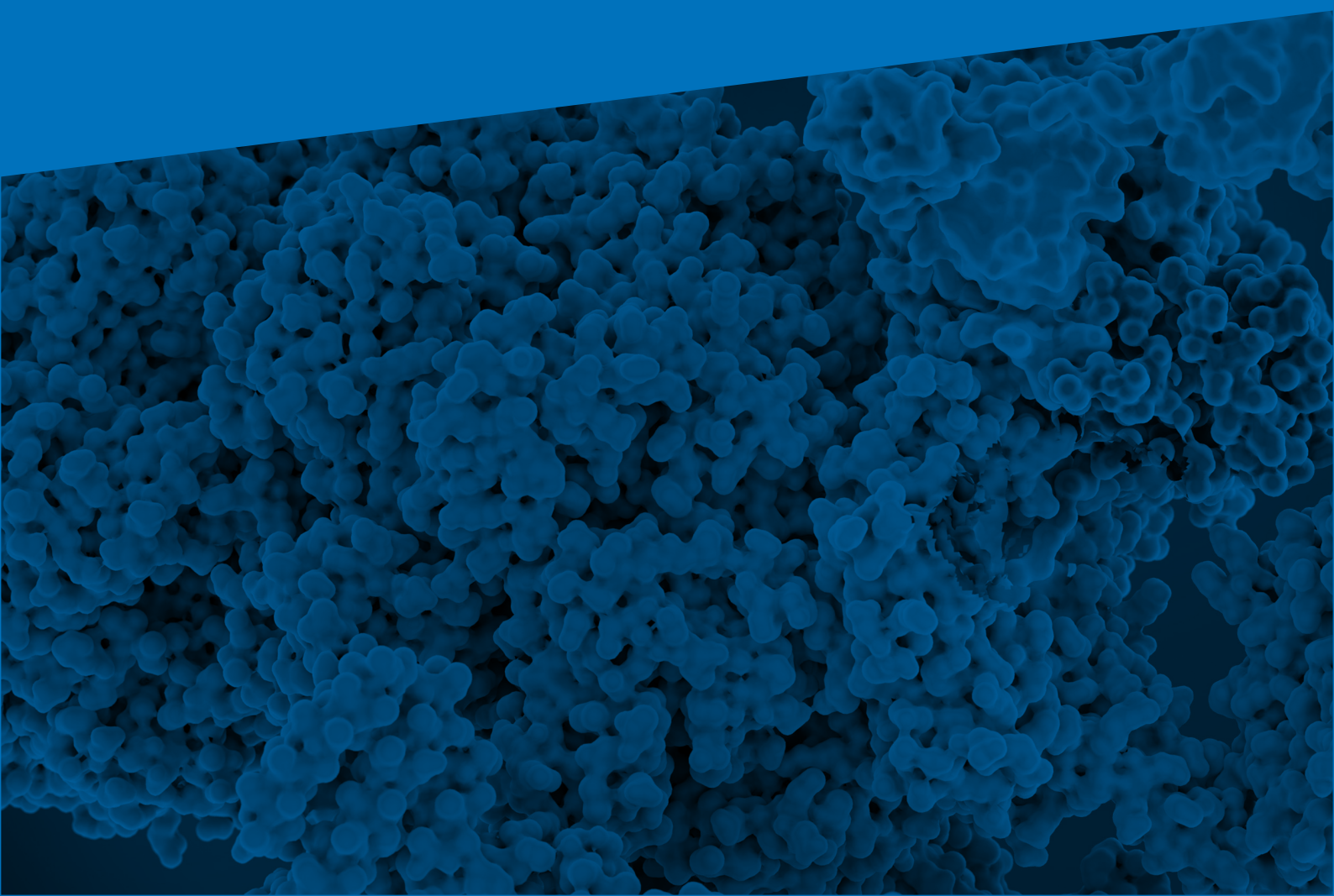
# Foreword

Targeted protein degradation (TPD) involves the selective degradation of disease-causing proteins using small molecule compounds and has shown promise in overcoming the limitations of traditional small molecule inhibitors and antibody-based therapies.

Recent advancements in mass spectrometry technologies have enabled more sensitive and accurate analysis of complex protein samples, and one such advancement is trapped ion mobility spectrometry (TIMS). TIMS is a high-resolution separation technique that can improve the performance of mass spectrometry instruments by separating ions based on their size, shape and charge.

When applied to TPD development, TIMS offers a powerful tool for characterizing the activity of a degrader drug on a protein of interest and the surrounding proteins in the cell. By quantifying proteins and related modifications, researchers can confirm the drug candidate's mechanism of action and make early predictions about possible side effects.

In this eBook, we will explore the potential of TIMS applied to the discovery of novel TPD drugs and development. We will delve into the principles and applications of these techniques and explore the latest advancements in mass spectrometry technology that make TIMS an invaluable tool in proteomic analysis. Ultimately, our goal is to provide a comprehensive guide for researchers and scientists interested in leveraging TIMS to develop more effective TPD therapeutics.



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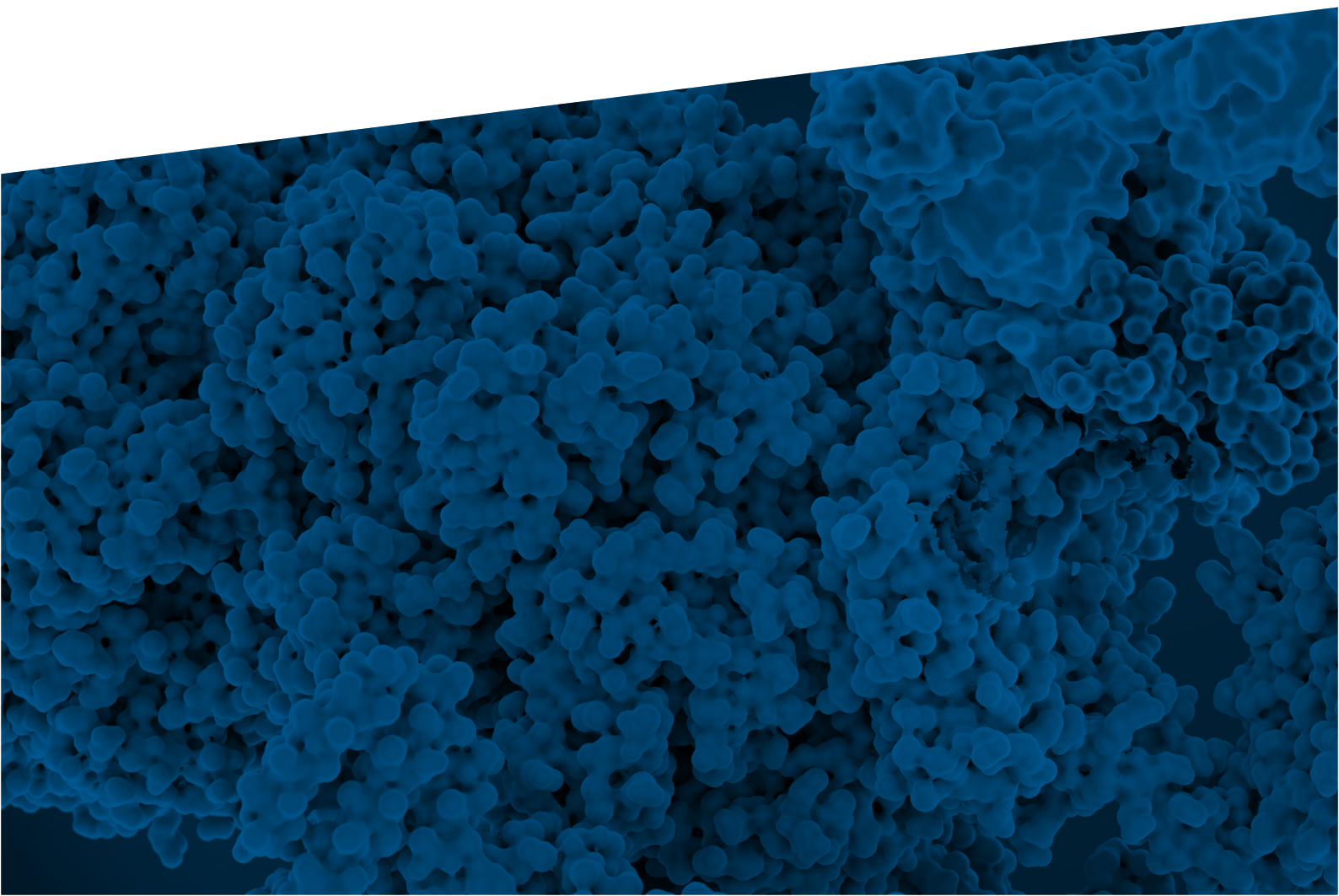
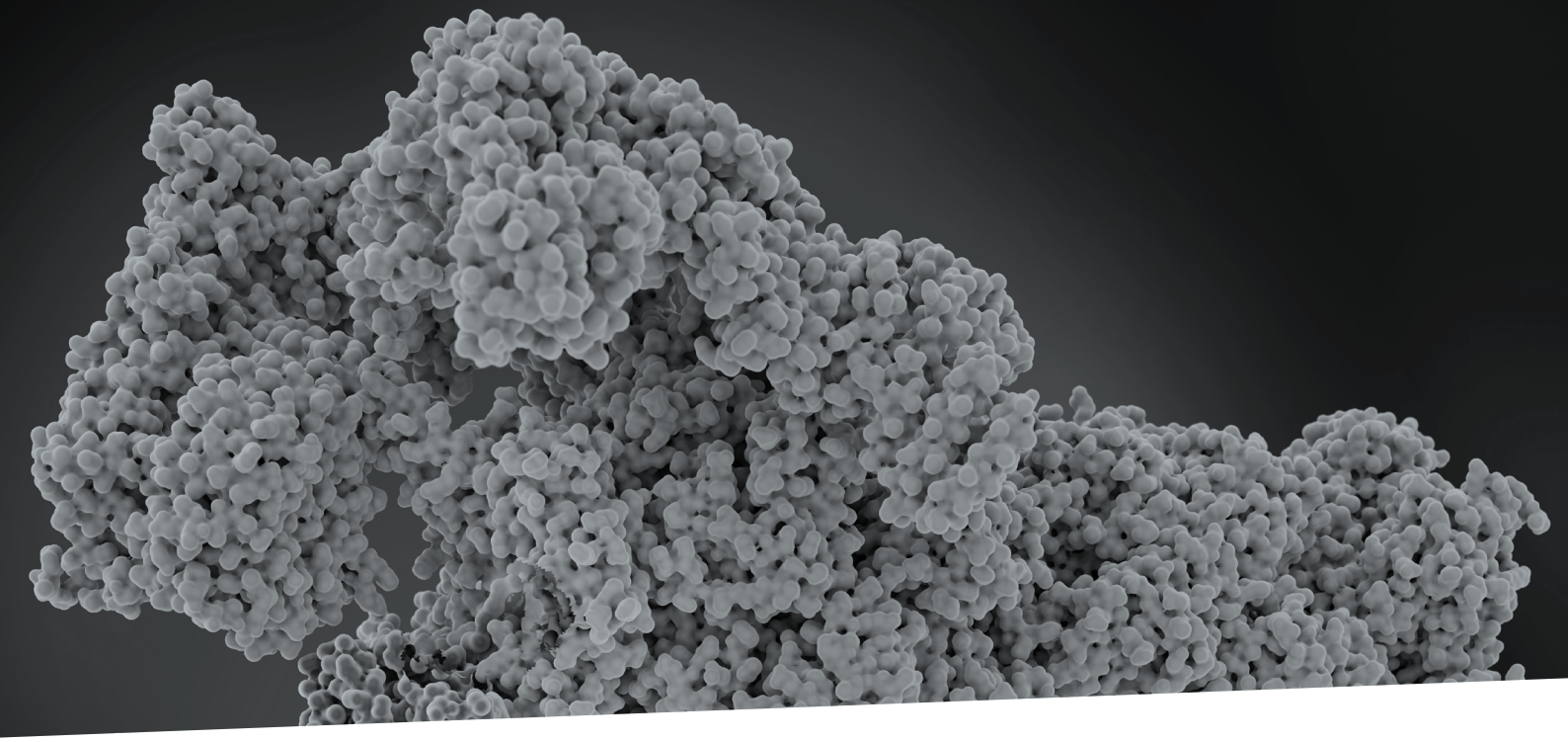


# Exploring Targeted Protein Degradation



## Introduction

The field of drug discovery has grown rapidly in the last few decades, with a worldwide market worth over $70 billion.[1] Despite this, there are on average only 50 new drugs approved each year.[2] Thus, a multitude of diseases are still lacking effective therapeutics. Drug discovery and development face many challenges, including low rates of translation from animal models, heterogeneity of patient populations and unknown pathophysiology.[3] Yet, one of the major challenges is tackling the undruggable proteome. Over 85% of the human proteome is considered "undruggable", meaning that these proteins lack clear binding pockets or enzymatic sites that can be targeted by standard pharmacological agents.[4,5] Many of these undruggable proteins play key roles in human disease, making them of great therapeutic interest and driving the search for innovative technologies. One such innovation is targeted protein degradation (TPD). In TPD, small molecule ligands are used to directly regulate the cell's protein homeostasis and target specific proteins for degradation.[6] Since the concept was first proposed in 1999, TPD-based technologies have become a powerful tool to explore cellular mechanisms and target formerly undruggable proteins.[7]

## Targeted protein degradation

In order to maintain protein homeostasis – or proteostasis – in the cell, both the production and degradation of proteins must be regulated. Protein degradation can occur in response to specific internal and external signals or to remove faulty or damaged proteins from the cellular environment.[8] In mammalian cells, there are two pathways that degrade proteins to their constituent amino acids: the Ubiquitin-proteasome system (UPS) and the autophagy lysosome

**In TPD, small molecule ligands are used to directly regulate the cell's protein homeostasis and target specific proteins for degradation**

pathway (ALP). In the UPS, cytosolic and nuclear proteins are marked for degradation by the repeated attachment of the small polypeptide, ubiquitin. The resulting multi-ubiquitin chain targets the protein for degradation by a large protease complex known as the proteasome.[8,9]

Susceptibility to UPS-mediated degradation can be increased by particular protein elements called degrons (e.g., short amino acid sequences, structural motifs, exposed amino acids). Degrons can be introduced to proteins of interest (POIs) to deliberately induce their controlled downregulation – also known as targeted protein degradation.[10] Conditional degrons, i.e., those activated or inhibited by stimuli such as temperature or the expression of another protein, enable a far more rapid, specific and reversible method of protein knockdown than genetic techniques such as CRISPR-Cas9 or RNA interference.[11]

The development of small molecule degrader systems has opened up degrons to therapeutic applications, whereby specific disease-associated proteins can be removed by the cell's own machinery. Several types of these degrader systems have been established, including proteolysis targeting chimeras (PROTACs), lysosome-targeting chimeras (LYTACs), antibody-based PROTACs (AbTACs) and molecular glues. Of these, PROTACs and molecular glues have seen the most success so far.[7] Both systems rely on the recruitment of an E3 ligase to the POI, forming a ternary complex (Figure 1). The E3 ligase then catalyzes the transfer of ubiquitin (Ub) molecules from the Ub-conjugating enzyme (E2) to the POI. This action is repeated, leading to the polyubiquitination of the POI and its subsequent targeting to the proteasome for destruction.[7,12] Although they both exploit the same process, there are clear differences between PROTACs and molecular glues. PROTACs are heterobifunctional molecules, consisting of an E3 recruiting ligand and a POI targeting warhead, connected by a flexible linker. The PROTAC makes two distinct small molecule-protein interactions to bring the POI and E3 ligase together.[13] In comparison, molecular glues are a single molecule that facilitate ubiquitination by turning the POI into neo-substrate for the E3 ligase, thereby enhancing protein–protein interactions between the two.[14]

In 2019, ARV-110 became the first PROTAC to enter clinical trial and subsequently gave the first clinical proof of concept for PROTACs against cancer targets.[14,15] Since then, several more protein degraders have entered clinical testing, with 18 different molecules in either phase I or phase II trials.[16] However, the full scope of potential TPD therapies is yet to be discovered. For example, from a family of 600 E3 ubiquitin ligases, less than 10 have so far been exploited for TPD.[17,18]

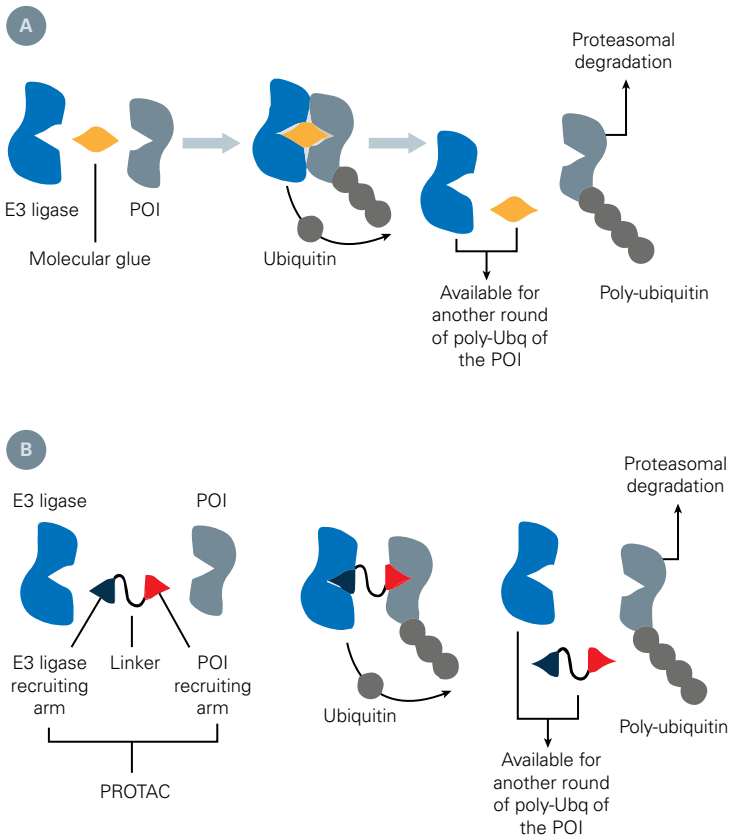



**Figure 1**
A) The mode of action of molecular glues for TPD. B) The mode of action of PROTACs.

**In 2019, ARV-110 became the first PROTAC to enter clinical trial and subsequently gave the first clinical proof of concept for PROTACs against cancer targets**

## Optimizing TPD discovery and design

Until recently, the discovery of many novel degraders has either been serendipitous (such as the discovery that thalidomide functions as a molecular glue), or through screenings of extensive small-molecule libraries.[19] This is particularly inefficient for PROTACs as the structural complexities of the ternary complex make linkers difficult to design.[20] PROTACs also display properties that lie outside the well-established standards of drug properties (e.g., molecular weight), resulting in further development challenges and limited routes of administration.[21] Rational design methods implementing deep learning models and simulations are currently being used to discover new PROTACs by predicting function and ligand binding properties.[20] However, screening and development methods must still be improved if TPD is to become a key player in the sphere of drug discovery.

Current approaches to characterization and validation of protein degraders focus on a stepwise assessment of multiple criteria including cell permeability, target and E3 ligase engagement, polyubiquination and target degradation.[22] Assessment includes phenotypic assays for target engagement (e.g., cellular thermal shift assay or CETSA), end-point assays (e.g., fluorescence-based protocols) and mass spectrometry. Following a phenotypic screen, techniques such as CETSA are used to determine target engagement and deconvolution. CETSA detects changes in the thermal stability of a protein induced by ligand binding, which confirms the engagement of the degrader with the POI and ligase.[23] Traditionally, the detection method in CETSA was a western blot, which limited throughput and demanded high-quality antibodies – which are not always available for the POI.[23] CETSA is now routinely coupled to mass spectrometry (MS). This enables opportunities for higher-throughput methods of detection, though still requires extensive optimization and provides little detail beyond target engagement.[24,25] Analysis of the formation and structure of the ternary complex provides valuable information for degrader validation. This can be assessed using end-point methods such as time-resolved fluorescence energy transfer (TR-FRET), but these methods again result in little data beyond target quantification.[26] Real-time, live-cell methods – such as the recently developed Nanoluc bioluminescence resonance energy transfer (NanoBRET) assay – enable kinetic measurements of target interactions throughout the proteasomal pathway to a greater degree of accuracy.[12,26,27] However, even these methods fail to account for whole-cell, off-target effects of degraders – a major stumbling block in the TPD development pathway.[28]

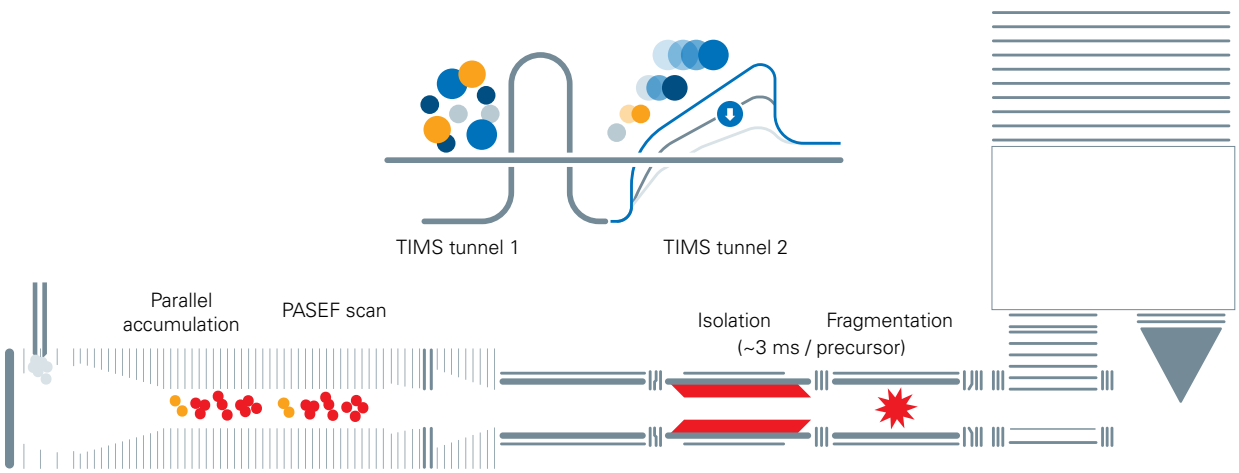
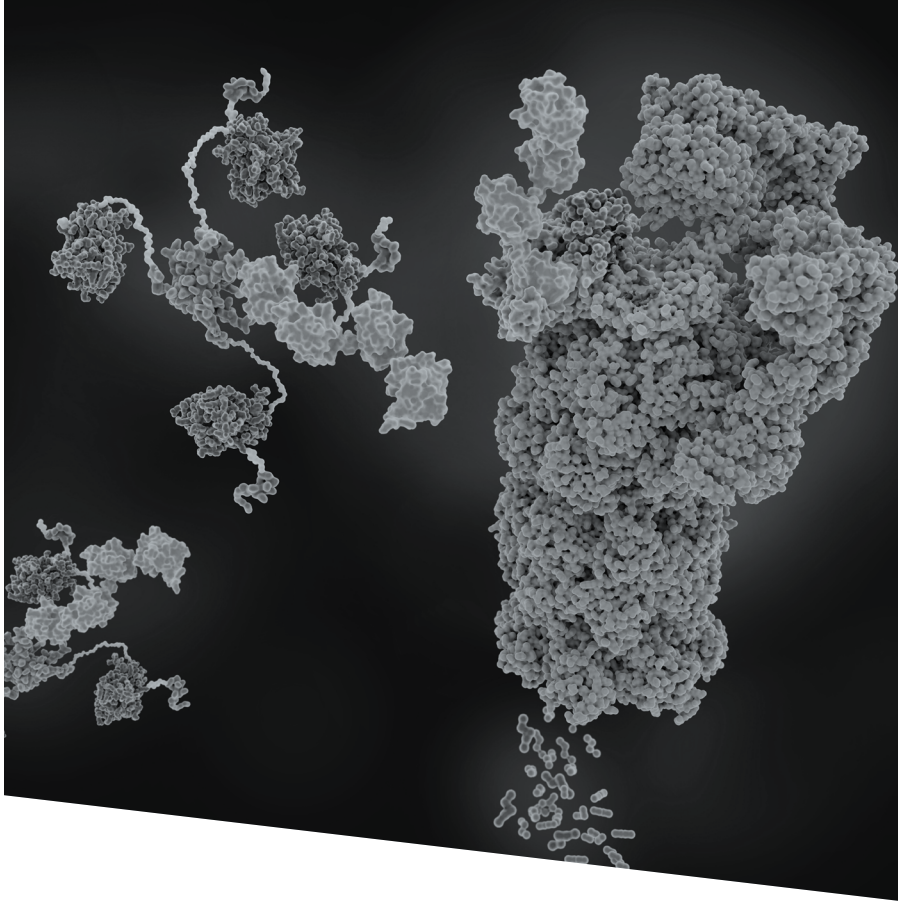



**Figure 2**
Trapped ion mobility spectrometry and the PASEF method.

Assessment of off-target effects is crucial in any drug development workflow. Failure to address this early in the process can result in high levels of drug attrition at later stages. Off-target effects in TPD are often reflected as changes in protein levels, which is poorly assessed by the standard *in vitro* binding assays and transcriptomics analyses used in classic drug development.[29,30] Hence, a different approach is essential to ensure safety for targeted protein degraders. Mass spectrometry (MS) has long been used in the screening and optimization of protein degraders, as it offers sensitive detection without requiring specific antibodies or tags. MS is also the preferred detection method for CETSA assays but can be difficult to precisely deconvolute on- and off-target effects.[31] Recently, however, the use of quantitative MS has enabled a proteomic-based approach to TPD development. Global proteomics analysis allows for the examination of POI abundance and validation of degrader selectivity. Furthermore, it enables the simultaneous assessment of any off-target or cell-wide effects, and therefore a more complete approach to degrader characterization.[26]



## Expanding the capabilities of proteomics approaches

MS-based proteomics approaches are rapidly gaining traction in the TPD field for analysis of on- and off-target effects. These methods can significantly increase the efficiency of optimizing TPD therapeutics, and have already been successfully used in several studies to this effect.[30,32,33] However, the comprehensive detection and quantification of proteomes by MS alone still presents multiple challenges, due to the complexity of samples and the breadth of potential peptides.[34] Many MS techniques are also still limited by time-consuming sample preparation, detection sensitivity and, most importantly, throughput.[34,35,36] In response, recent developments in this area have focused on increasing fragmentation speed, without losing sensitivity.[34]

Trapped ion mobility spectrometry (TIMS) is a gas phase ion separation technique that has been shown to tackle these challenges in TPD development. In TIMS, ions are propelled into a tunnel by a gas flow. The drag force of the gas flow is opposed by an electrical field, which traps ions in place at certain points in the tunnel according to their mobility (Figure 2). Changing the intensity of the electrical field allows the selective release of the ions for analysis based on *m/z* and Collisional Cross Section (CCS) of the molecules.[34,36,37] Coupling TIMS with a quadrupole time of flight (Q-TOF) mass spectrometer increases sensitivity, speed and characterization confidence in proteomics workflows.[34,35,38] TIMS also enables the parallel accumulation–serial fragmentation (PASEF®) method. In PASEF, ion packets are accumulated in the front of the TIMS, then separated by both their *m/z* and CCS using trapped ion mobility. Signal-to-noise is improved from the added dimension of gas-phase separation, enabling the separation of peptides that would otherwise co-elute.[36,39,40]

In standard MS/MS experiments, large amounts of ions are discarded, as only a small proportion of the ion beam is selected for analysis. However, in PASEF, storage in the TIMS cell and rapid quadrupole switching times enable the selection and identification of a far wider range of precur-

**MS-based proteomics approaches are rapidly gaining traction in the TPD field for analysis of on- and off-target effects. These methods can significantly increase the efficiency of optimizing TPD therapeutics**

sors, resulting in up to a 10-fold increase in sequencing speed and increased sensitivity.[40] The combination of PASEF with data-independent acquisition methods (dia-PASEF) gives a quantitative result for every analyte in a sample. This reduces the risk that the same precursors won't be selected in multiple samples and ensures that identification and quantitation results are highly reproducible between samples, even in large cohorts.[34]

A complete proteomics workflow that incorporates TIMS and dia-PASEF results in a range of potential advantages for TPD development. High sensitivity coupled with the reduced co-fragmentation rates offered by TIMS and dia-PASEF can decrease spectral complexity and contribute to higher and more accurate identification rates than traditional methods.[41] The increased speed and high efficiency of PASEF technology makes it the ideal method for deep proteome profiling and quantitation, with previous experiments showing successful profiling of at least 50 to 100 human cell lysate samples per day.[42] Overall, this workflow offers an unparalleled insight into the on- and off-target effects of targeted protein degraders and promises to put an end to the "undruggable" proteome.

## Revolutionize your TPD workflow today

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# Targeted Protein Degradation

## The Future of Drug Development

Despite continuous advances in the drug development industry, around 85% of the human proteome is still considered "undruggable".[1]

Targeted protein degradation (TPD) is rapidly gaining traction as a new therapeutic strategy to target formally undruggable proteins. However, only a small number of degraders have been explored in clinical trials. This infographic discusses the main challenges in TPD workflows and presents innovative solutions to accelerate drug development.

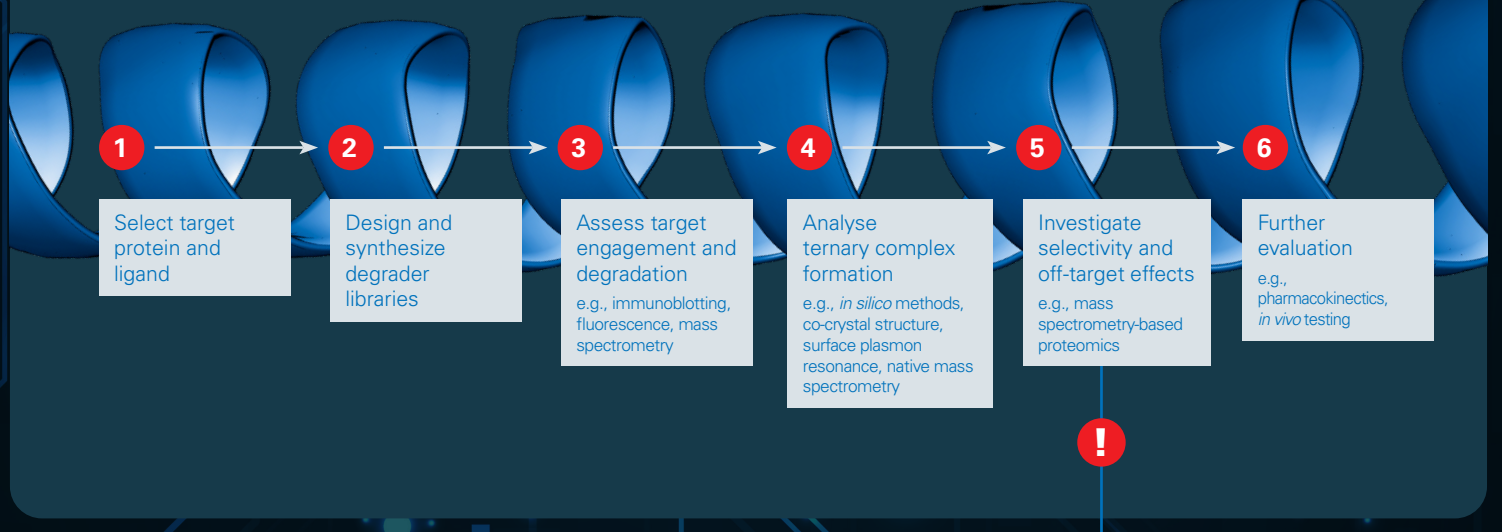
## What is TPD?

In TPD, small molecule ligands such as PROTACs and molecular glues are designed to link a specific protein of interest (POI) to an E3 ligase. This redirects the POI towards the cellular degradation machinery.


1. **Degrader design**
   Constructed to bind both target and E3 ligase, either through a linker (PROTAC) or directly (molecular glue)
2. **Ternary complex**
   Degrader recruits target to E3 ligase
3. **Polyubiquitination**
   Ubiquitin (Ub) added to target's Lys residues
4. **Target degradation**
   Ub-marked target is degraded by the proteasome

## How are targeted protein degraders developed?

Most degraders are discovered through the screening of vast molecular libraries, in a complex, multi-step process. Therefore, the need for rapid high-throughput capabilities is paramount in order to not only screen these large libraries, but to attain statistically relevant data and confidently select promising candidates.



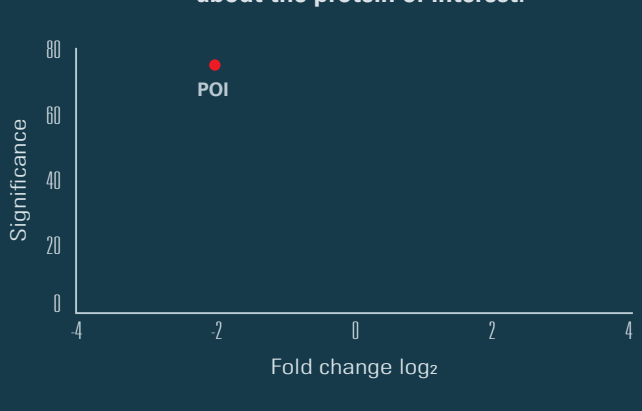
1. **Select target protein and ligand**
2. **Design and synthesize degrader libraries**
3. **Assess target engagement and degradation**
   e.g., immunoblotting, fluorescence, mass spectrometry
4. **Analyse ternary complex formation**
   e.g., *in silico* methods, co-crystal structure, surface plasmon resonance, native mass spectrometry
5. **Investigate selectivity and off-target effects**
   e.g., mass spectrometry-based proteomics
6. **Further evaluation**
   e.g., pharmacokinetics, *in vivo* testing

**Requires a high-throughput, proteome-wide readout in order to accurately assess cell-wide effects.**

## See the bigger picture with high throughput proteomics

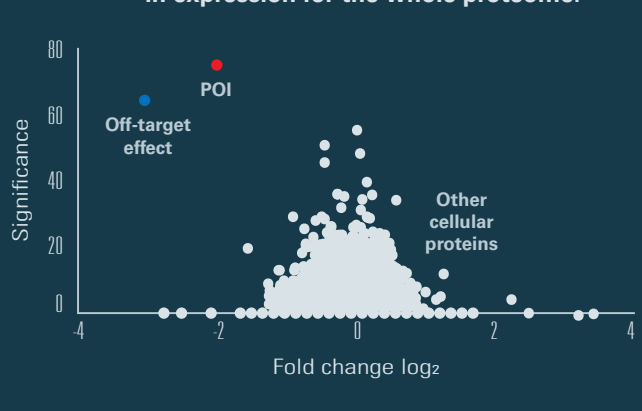
Assessing off-target effects is crucial for drug development. Failure to address these early on can result in high levels of attrition due to safety issues. While end-point assays can produce accurate data, they often lack the scope to assess off-target effects. Proteomic approaches give a complete picture of TPD effects in the cell during novel degrader screens.

**End-point assays show specific data about the protein of interest.**




- Hypothesis driven
- Time-consuming
- Can be low-throughput

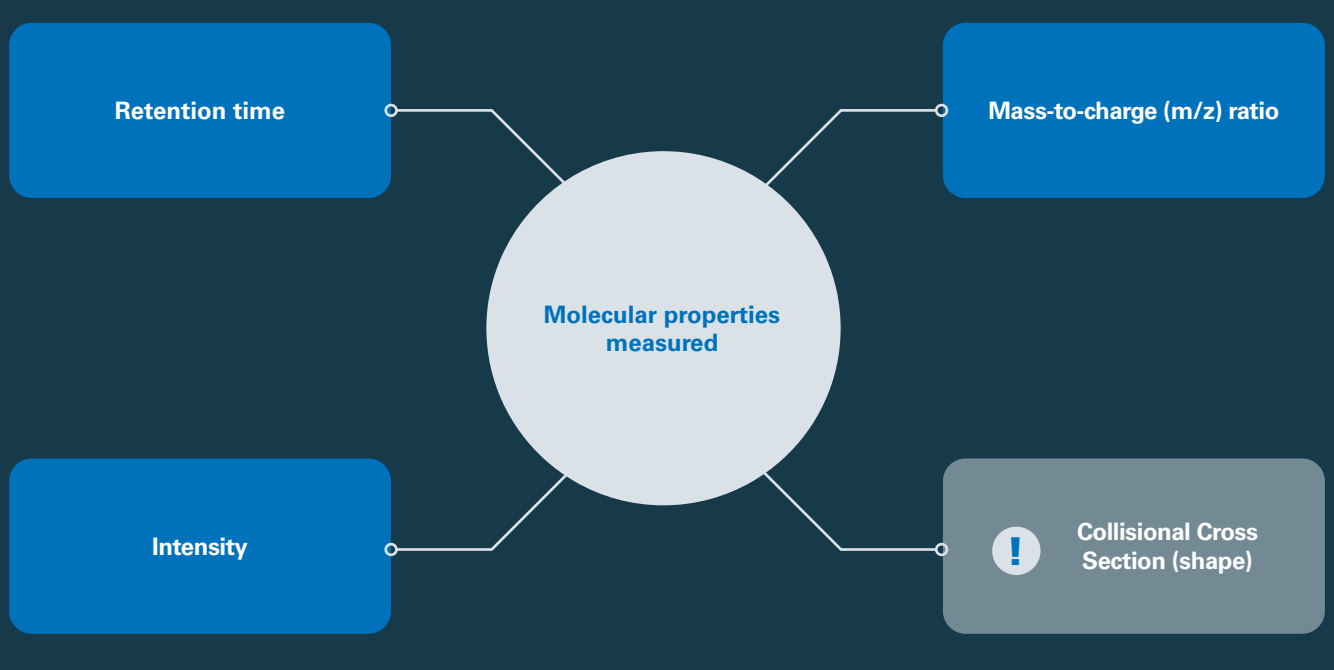
**Proteomics approaches show change in expression for the whole proteome.**




- Rapidly examines changes in abundance for thousands of proteins
- Identifies on- and off-target effects
- Supports characterization of mechanism of action

## Pushing the boundaries of degrader development

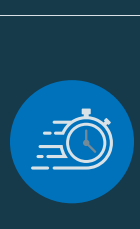
Parallel accumulation serial fragmentation (PASEF) enabled by trapped ion mobility spectrometry (TIMS) improves sequencing speed, accuracy and sensitivity by accumulating and then separating ions in the gas-phase. The Collisional Cross Section (CCS) of the molecule, an inherent physical property, is used to separate isobaric peptides, improving the accuracy of identification.




When coupled with time of flight (TOF) mass spectrometry and PASEF, these methods provide unparalleled confidence and accuracy in your protein identification and quantitation.

Increases selectivity for complex samples in short run times

Increases sensitivity by reducing background noise



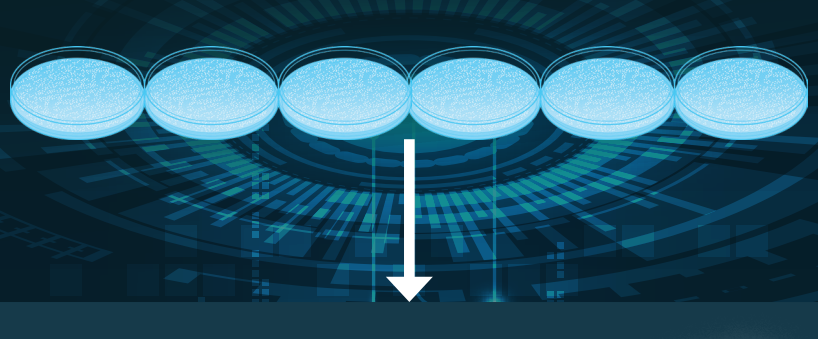
Increases run speed, enabling high-throughput

Increases accuracy for highly confident identification

Enables deep sequencing methods for comprehensive proteome analysis

## A complete solution for TPD analysis

From PreOmics sample preparation kits to the timsTOF HT and choices of data processing software, Bruker offers a range of solutions to support your TPD quantitative proteomics workflow at every stage.

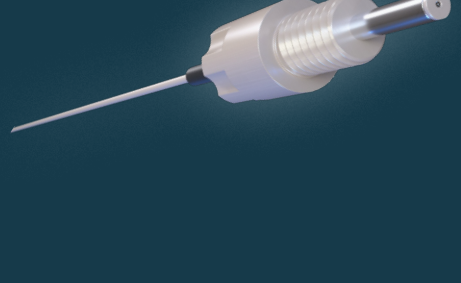


**PreOmics iST Kits**

- Versatile and simple: All-in-one and ready-to-use proteomics sample preparation kits for a variety of applications.
- Fast: The kits only require 1 hour of hands-on time, with a total time of less than 2.5 hours.

**Bruker PepSep™ Columns**

- Robust, high performance nanoLC columns



**timsTOF HT**

- Uniquely enables rapid dia-PASEF experiments, with unparalleled depth of coverage and quantitation in large cohorts

**Bruker ProteoScape™**

- Real-time data processing with TIMS DIA-NN

BRUKER ProteoScape™

**Spectronaut® by Biognosys**

- Comprehensive peptide identification with industry standard

Spectronaut®

**Discover the future of TPD drug development.**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1 Spradlin, Jessica N., Erika Zhang, and Daniel K. Nomura. "Reimagining Druggability Using Chemoproteomic Platforms." *Accounts of Chemical Research* 54, no. 7 (2021): 1801–13. https://doi.org/10.1021/acs.accounts.1c00095.

# NEOsphere Biotechnologies: Breaking the Barriers of TPD Drug Discovery

Developing novel TPD drug candidates is a complex process owing to the lack of easily druggable pockets on the protein of interest. Mass spectrometry (MS)-based proteomics is essential in revealing the complete target spectrum of degrader molecules, confirming on-target degradation, highlighting off-target regulation and identifying potential novel targets. Here, high throughput and rapid turnaround are key to accelerating drug development and avoiding significant bottlenecks. NEOsphere Biotechnologies specializes in deep proteomic screening and MS-based mechanistic validation of potential novel targets to advance the development of degrader drugs for previously undruggable targets. This interview explores the technology of NEOsphere Biotechnology and its impacts on TPD drug discovery.

## Dr. Jutta Fritz

CBO and co-founder
NEOsphere Biotechnologies

Dr. Fritz is a business development expert with more than 15 years of management experience within the life sciences and diagnostics industries. Prior to joining NEOsphere Biotechnologies, she was co-founder and CBO of the cancer diagnostics company NEO New Oncology, VP of Business Development for Proteomics Services at Evotec and Head of Business Development at the proteomics company Kinaxo Biotechnologies. Dr. Fritz has a PhD in molecular biology from the University of Vienna and an MBA in financial management from the University of Wales.

## Dr. Uli Ohmayer

Head of Mass Spectrometry and co-founder
NEOsphere Biotechnologies

Dr. Ohmayer is a leading expert in industrial-scale, deep proteomic screening. He has over a decade of experience in MS-based proteomics and broad expertise in laboratory automation and rapid scaling of proteomics infrastructure. In his previous position at Evotec, he was instrumental in the development of data-independent acquisition MS for single-shot proteomics with unprecedented throughput, depth and sensitivity. Dr. Ohmayer has a PhD in biochemistry from the University of Regensburg and was a postdoctoral researcher in the Mass Spectrometry Core Unit of Helmholtz Zentrum Munich.

## Q: Can you give us a brief overview of NEOsphere Biotechnologies? What sets you apart from other companies in the field?

**Jutta Fritz (JF):** NEOsphere Biotechnologies is a leader in the field of MS-based proteomics for drug discovery, with a focus on targeted protein degradation. Founded in 2022 and located in Munich, we work with pharmaceutical and biotechnology companies to systematically evaluate the true target scope of their degrader compounds in a proteome-wide context.

Our technology combines the highest data quality and proteome coverage with high throughput and fast turnaround time, making it ideal for supporting drug discovery and optimization. To this end, all our laboratory and data analysis processes are automated and scalable.

Our deep proteomic screening platform reveals changes in protein regulation upon compound treatment. The analysis is performed on intact, unmodified cells to monitor compound selectivity in endogenous environments. We routinely quantify up to 11,000 proteins in a single experiment, allowing us to comprehensively evaluate degrader efficacy, assess off-target effects and identify potential new target proteins for degraders. In this regard, our deep proteome coverage combined with reliable protein quantification is critical for the identification of low-abundance proteins that may be of great interest for drug discovery, such as transcription factors. It is also possible to analyze the effect of a degrader on the whole proteome at different time points or concentrations to determine how quickly and strongly it acts and at what point secondary effects may occur. Thanks to our high-throughput capabilities, we can screen degrader libraries of thousands of compounds (for an example of how deep proteomic screening data is presented, see Figure 1).

In addition to deep proteomic screening, we offer MS-based technologies to mechanistically validate potential degrader targets, e.g., by ubiquitinomics or high throughput interactomics. In this way, potentially interesting hits from the deep proteomic screen can be further investigated immediately.

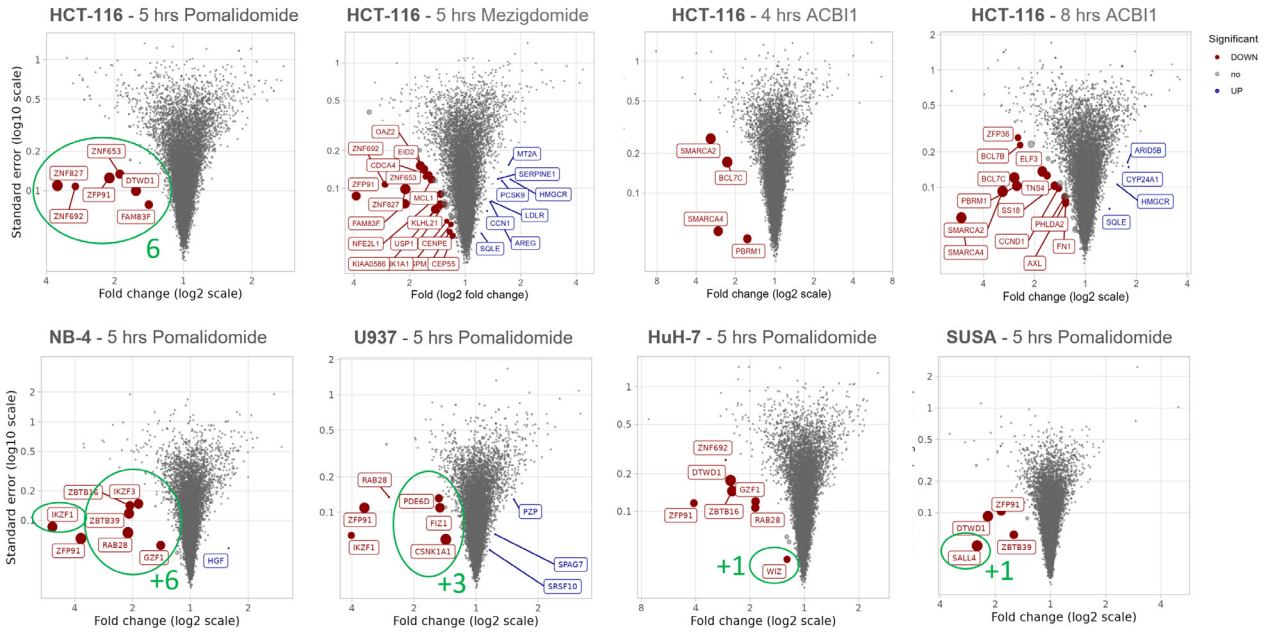


**Figure 1.** Comprehensive detection of compound-specific and cell line-specific degradation events. HCT-116 cells were treated with two immunomodulatory imides (IMiDs) and one PROTAC as indicated (upper panel) and analyzed by single-shot MS analysis. The volcano plots illustrate significant up- (in blue) and downregulations (in red) induced by the different compounds. The x-axis depicts the fold change (log2) of proteins in compound vs DMSO-treated cells and the y-axis depicts the standard error. Known cellular targets such as zinc finger proteins were detected for pomalidomide and mezigdomide. SMARCA2 and SMARCA4 were downregulated upon 4 hours treatment with the VHL based PROTAC ACBI1, together with two interacting BAF complex members, while secondary regulation was seen at a later 8 hours time point. Additional cell lines were treated with pomalidomide for 5 hours (lower panel) and compared to HCT-116. ZFP91 was significantly down-regulated in all cell lines, other neosubstrates exhibited varying degrees of regulation, reflecting cell type-specific expression (e.g., IKZF1 in U937 and NB-4, or SALL4 in SUSA) or different levels of IMiD responsiveness to commonly expressed neosubstrates. In total, 17 different neosubstrates were significantly downregulated in at least one cell line.

## Q: What is your process for screening potential TPD drug candidates?

**Uli Ohmayer (UO):** To evaluate and quantify the effect of a compound on protein degradation while maintaining very short turnaround times, we have developed scalable and robust laboratory processes and statistical methods. We routinely work in a 96-well plate format and test every compound in triplicate, which enables very powerful statistical analyses. Like compound treatment, sample preparation is largely automated. First, we perform a tryptic digest followed by peptide purification so that the complete cellular proteome is cleaved into peptides, which are then separated by liquid chromatography (LC) and analyzed by MS.

To minimize technical variation, maximize data completeness and significantly reduce measurement time for mass spectrometry, we don't use chemical labeling followed by off-line fractionation, but measure samples in a label-free single-shot approach. For MS analysis, we use dia-PASEF (data-independent acquisition and parallel accumulation and serial fragmentation) on timsTOF instruments from Bruker. Here, ion mobility separation is used to reduce signal interference and increase the sensitivity of proteomics analysis. This results in very deep proteome coverage and detection of more than 200,000 precursor ions in one sample, corresponding to approximately 11,000 proteins. Each mass spectrometry run generates a large amount of highly complex raw data. We use DIA-NN software developed by one of our scientific advisors, Dr. Vadim Demichev, as well as proprietary data analysis and statistical tools developed at NEOsphere for the analysis. To detect statistically significant protein regulation upon compound treatment, a comparison is made with untreated controls.

To determine whether regulation is due to protein degradation or other effects, we can then perform mechanistic validation of all potential hits identified in the screen using additional MS-based tools such as interactomics or ubiquitinomics. Degrader compounds induce proximity between the E3 ligase and the protein of interest (POI) and initiate ubiquitination of the POI, marking it for degradation by the proteasome. Our ubiquitination assay quantifies up to 50,000 ubiquitination sites in a single experiment. Comparing the regulation of ubiquitination sites in treated and untreated cells provides clear clues as to whether the protein regulation observed in the proteomic screen is indeed due to degradation.  NEOsphere's ubiquitinomics platform stands out in the field for its depth, speed, precision and throughput.

## Q: How do you optimize the assays in your pipeline to ensure maximum efficiency?

**OU:** We have systematically tested and optimized a variety of parameters for all the

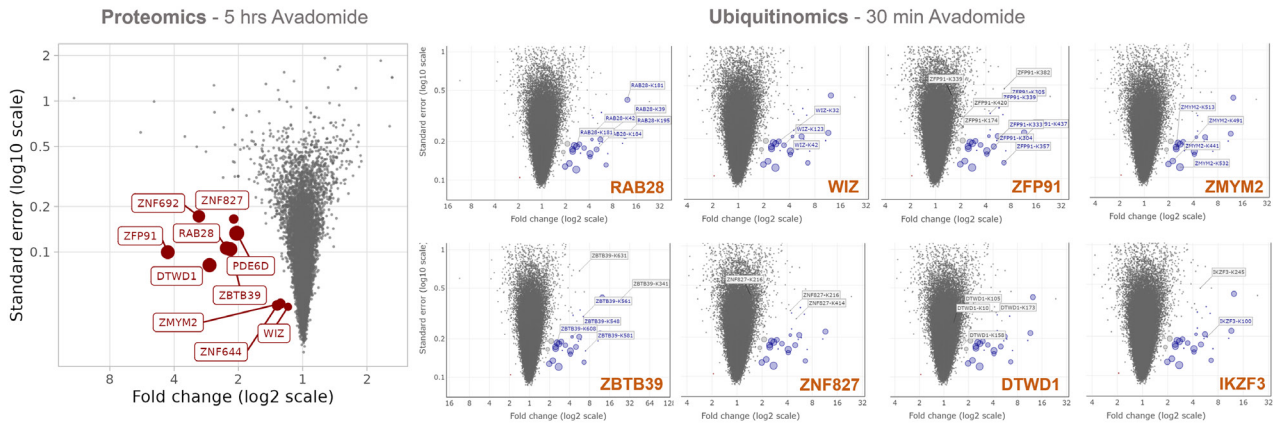


**Figure 2.** Combined analysis of proteomics and ubiquitinomics reveals and validates primary degrader targets. HEK293 cells were treated with the cereblon modulator avadomide. Ubiquitinomics was performed using MS-based K-GG remnant profiling, enabling the quantification of up to 50,000 ubiquitination sites in single DIA-MS runs without cellular proteasome inhibition. Ubiquitinomics revealed induced ubiquitination sites for almost all neosubstrates found to be degraded upon 5 hrs treatment of HEK293 cells. These included sites on IKZF3, a protein not detected in the proteomics experiment due to its extremely low expression in HEK293 cells. Ubiquitinomics allows the rapid validation of cellular downregulations due to E3 ligase-neosubstrate relationships, by analyzing degrader drugs in endogenous cellular systems without the need for pharmacological intervention or genetic modification.

steps of our workflow, from cell treatment and sample preparation to data acquisition, data analysis and statistical evaluation. We have also adjusted all instrument settings to simultaneously achieve very deep proteome coverage and precise protein quantification. At each stage there are many details that can be optimized, and although each of these individual changes may have a rather small effect, they multiply and eventually lead to a highly efficient process.

**JF:** Continuous optimization is very important – our workflows are state-of-the-art, but it's a constant process to maintain them as such and to keep pushing the technological boundaries. All our processes are designed to best support the requirements of drug discovery. We can analyze all types of degraders such as molecular glues, PROTACs, DUB-inhibitors and monovalent degraders. The technology can also be used to measure the effect of protein stabilizers on the proteome. In terms of material, we are very flexible and can use, for example, adhesive and suspension cell lines or primary cells.

### Q: How do you validate your results? What kind of measures do you take to ensure that they are reliable and reproducible?

**UO:** Our technology is quite sophisticated and complex, so strict quality control must be performed on every sample. For example, we continuously check parameters such as digestion efficiency, mass accuracy or peak widths in near real-time to ensure that the performance is always at the required level. By testing samples in triplicate, we can calculate a coefficient of variation and determine reproducibility and consistency between replicates. Our automated data processing includes numerous stringent control mechanisms to ensure the highest data quality. For example, filtering is applied to further enhance data completeness and allow precise quantification of even low abundance proteins, and numerous biostatistical tests are routinely performed to improve statistical power.

### Q: What are some of the biggest challenges you face when screening degraders and how do you overcome these challenges?

**UO:** Apart from turnaround time, one of the biggest challenges in deep proteomic screening is throughput, especially when testing large libraries of tens of thousands of compounds. Screening therefore requires a platform that can measure multiple samples in parallel while keeping the time to analyze a set of samples as short as possible. To meet this requirement, we have built our platform so that each step is scalable. If a project requires screening of large number of compounds, we can thus meet that demand very quickly.

### Q: How do you see the field of TPD evolving over the next few years? What role do you think MS will play in shaping the future of this industry?

**UO:** The structure–activity relationship is very steep particularly for molecular glues – even changes by one atom can cause significant changes in compound potency and cellular target selectivity. MS is the key technology to identify these changes and potential off-target effects. The proteomic data we generate is also very useful for developing the chemistry of degrader drug candidates. Chemists can use our data to further optimize the chemical structure of the degraders and implement a more rational approach to degrader design, making drug discovery much faster and more reliable than current methods.

**JF:** Targeted protein degradation holds enormous potential to address many urgent clinical needs. It's still a young but rapidly growing field, with several promising compounds currently in clinical trials. In our opinion, MS-based proteomics will become one of the key factors for successful development of degrader compounds and thus will gain much importance in the future.

# Plexium's Next-Generation TPD

Current drug discovery processes have barely scratched the surface of targeted protein degradation. Only a handful of E3 ligases have been clinically evaluated until now, and many discovery processes have relied on the serendipitous identification of degraders. To discover the full potential of TPD for the undruggable proteome, high-throughput, rational design approaches are required. Plexium uses a comprehensive platform to develop monovalent protein degraders across a range of modalities. This interview explores Plexium's technologies and their potential to develop next-generation TPD drugs.


## Dr. Alex Campos

Head of Proteomics
Plexium


Alex Campos joined Plexium in 2022 as senior director in the drug discovery department and head of the proteomics department, bringing more than 20 years of experience in proteomics and data science. Prior to Plexium, Alex was at Sanford-Burnham-Prebys (SBP) Medical Discovery Institute where he conducted his postdoc in proteomics as part of a collaboration between SBP and MedImmune and continued on into varying roles of increasing responsibility in the proteomics core. From 2016–2021, Alex served as director of the proteomics core and member of the NCI-designated Cancer Center and initiated important collaborations within emerging TPD companies from San Diego, Boston and San Francisco. He received his PhD in molecular pathology with a focus in proteomics technology in biomedicine from the University of Barcelona where he worked at the Barcelona Science Park.

### Q: Can you give us a brief overview of Plexium? What sets you apart from other companies in the field?

**Alex Campos (AC):** Plexium is the premier, next-generation targeted protein degradation (TPD) company focused on pursuing a new class of selective TPD drugs called "direct degraders". These are small molecules designed to bind to a pathogenic protein to induce selective degradation of the protein by the cell's natural protein quality control machinery. In addition to direct degraders, Plexium is pursuing molecular glues, an approach that also leverages the cell's natural quality control machinery. However, in this case, small molecules bind to an E3 ligase, then redirect the ligase to selectively engage and degrade pathogenic proteins for degradation.

Our company has developed a comprehensive approach toward TPD which is powered by our proprietary best-in-class platform that enables us to discover a wide variety of TPD modalities, from molecular glues to monovalent degraders, while also identifying novel E3 ligases beyond cereblon and the von Hippel–Lindau complex (VHL). We are working toward the discovery of next-generation TPD drugs across multiple therapeutic areas.

As a senior director in the drug discovery department, my role is to lead the proteomics platform, overseeing projects, operations and technologies within the proteomics realm. In addition, I'm actively involved in proteomics data processing, analysis and interpretation to help downstream project decisions. Given the highly collaborative and innovative nature of Plexium as an organization, I work closely with other drug discovery team leaders to further optimize our platform and deliver cutting-edge technologies for TPD.

### Q: What is your process for designing targeted protein degraders? How do you identify potential targets and design degraders specific to those targets?

**AC:** Plexium is pursuing drug targets that have previously been undruggable or inadequately drugged within the oncology and neuroscience disease areas. We believe that we have a rich and diverse portfolio including known valuable targets such as IKZF2 and SMARCA2, among other important cancer-related proteins.

The company is powered by its proprietary TPD drug discovery platform. This integrates degrader chemistry design principles and screening libraries with cell-based, target-specific degradation assays, including an ultra-high-throughput screening platform, to enable the identification of drug-like, cell-permeable degrader molecules. This integrated TPD drug discovery approach has led to the discovery of novel, selective direct degraders, molecular glue drug candidates and two unprecedented E3 ligases.

Plexium has established validated chemistry design principles for target-selective protein degraders and deploys multiple chemistry approaches to generate molecules for medicinal chemistry optimization. Bespoke libraries are constructed for each target. Once target binders are identified, structure-based, ligand-based and diversity-based approaches are deployed for library design within a drug-like space. Our DNA-encoded "one-bead, one-compound" libraries enable the sampling of diverse chemical space. The compounds are tethered to a bead with a photocleavable linker. The bead is then transferred to an assay device well and ultraviolet light is utilized to release the compounds from the bead. Our novel approach overcomes drug screening limitations associated with traditional DNA-encoded libraries and enables cell-based screening of DNA-encoded libraries in a one compound per well configuration.

### Q: How do you screen TPD candidates and evaluate their efficacy *in vivo*? How do you optimize these assays for maximum efficiency?

**AC:** Plexium's proprietary miniaturized assay devices contain up to 88,000 isolated screening wells in a device with a footprint similar to a conventional 96-well plate device, with 20 to 50 cells per well. On-bead libraries enable single compound per well assays. Plexium's tabletop cell-based µHTS instrument supports high content, multiplexed assays. Our assay endpoint is the degradation of one or more proteins of interest in a disease representative cell. Upon assay completion, images are acquired and analyzed to support "hit" calling. The beads associated with hit compounds contain a DNA barcode that uniquely identifies the single compound on that bead, which can be read for compound identification. At this stage, we start a cascade of assays to validate our cellularly-active degrader hits using mainly cell models relevant to the target of interest.

Medicinal chemistry optimization of degradation, potency, selectivity and ADME properties is an important part of this process. In addition, our Biology department (in particular the *in-vivo* pharmacology team), will conduct good laboratory practice (GLP)-toxicology/pharmacology studies and early human clinical trials to evaluate response and safety of lead compounds. Importantly, we identify biomarkers to guide patient selection for human clinical trials as well.

### Q: How do you validate your results? What measures do you take to ensure that they are reliable and reproducible?

**AC:** We rely on different techniques to validate our screening results. Target engagement is usually confirmed with HiBiT or surface plasmon resonance (SPR). Degradation of the target and dependency of the 26S proteasome is commonly confirmed with Western blots with or without co-treatment of cells with neddylation or proteasome inhibitors. However, mass spectrometry (MS) quantitative proteomics is the ultimate technique to evaluate on- and off-target degradation across different cell lines and compound concentrations. At this point, we can also use neddylation or proteasome inhibitors to evaluate the proteasome-dependency of the protein degradation.

Reproducibility and confidence are important aspects of our work. Compliance to standard operating procedures is crucial to obtain reliable results. In addition, robust data analysis processes and proper experimental design are essential; for example, using controls and running replicates for the different tested conditions helps to power our analysis, particularly for proteomics technologies.

### Q: What are some of the biggest challenges you face when developing and screening TPDs, and how do you overcome these challenges?

**AC:** A deep understanding of the target protein is crucial for successful screening campaigns. Protein-level information on target expression distribution in tissues/cell lines, protein turnover, protein-protein interactions and subcellular compartment localization help us to design the screening conditions and assay cascade. Despite the large volume of genomic data in public repositories, we still lack a thorough protein-level database. For example, protein-level data in TCGA is very sparse compared to genomics data. At Plexium, we use MS proteomics to map proteins in cells and tissues and create a cell-specific atlas of the proteome to help design successful TPD screenings.

There are also several challenges associated with the chemistry of designing and screening monovalent degraders. The cellular degradation machinery may have unintended effects on non-target proteins, leading to off-target toxicity. Designing ligands with high selectivity and minimizing off-target effects is crucial to ensure the safety and efficacy of targeted protein degradation therapies. In addition, designing ligands with optimal properties, such as appropriate binding affinity, selectivity and cell permeability, is a complex task. To date, most monovalent degraders have been found serendipitously, and there are no clear rational chemical design principles for converting protein-targeting ligands into monovalent degraders. However, Plexium has already established validated chemistry design principles for target selective protein degraders. The combination of such design principles and cutting-edge AI methods promise to deliver a more rational design of degraders.

From a proteomics perspective, the main challenge is throughput, including MS and sample preparation. The proteomics department at Plexium has heavily invested in sample prep automation, increased throughput of our LC-MS runs and implemented a robust and fast data analysis pipeline.

### Q: How do you see the field of TPD evolving in the next few years? What role do you think MS will play in shaping the future of this industry?

**AC:** I believe that recent developments in proteomics sample preparation technologies and the combination with other fields such as biophysics are revolutionizing the TPD toolbox. Furthermore, advances in LC-MS technology have significantly improved the throughput, sensitivity, precision and robustness of proteomics workflows. The MS-based TPD toolbox has evolved from global proteome profiling to more functional approaches designed to elucidate the mechanism of action of TPD.

An arsenal of approaches has been put forward to elucidate compound-driven proteomic changes. In recent years, we have seen a boom of chemoproteomics and activity-based probe profiling (ABPP) approaches, heavily inspired by the work coming from Ben Cravatt and Dan Nomura's groups. I think we will continue to see more of these methods. For example, the streamline cysteine activity-based protein profiling (SLC-ABPP) method implemented by the Cravatt and Gygi groups enables proteome-wide screening of large fragment-based libraries in a high-throughput fashion.

Although very powerful, chemoproteomics and ABPP typically require the functionalization of small molecules which can be challenging and sometimes unwanted. The combination of MS with other biochemical or biophysical procedures has emerged as a powerful technology to investigate the impact of compound binding on protein structure. Methods such as CETSA-MS, DARTS, LIP-MS, SPROX, HDX and FPOP have been used in the MS-based proteomics field for years to study ligand-protein engagement, and we are now slowly seeing its application in the TPD field. I hope to see more of this to help to elucidate the mechanisms of action of protein degradation.

Characterization of protein structure and protein-protein interactions is essential for understanding TPD. In this niche, I believe that affinity purification followed by MS (APMS) will continue to play an important role in untangling the mechanism of action of degradation. In particular, we are seeing an increasing application of proximity-dependent labelling such as BioID and APEX in TPD.

Finally, crosslink (XL)-MS complements the arsenal of available MS-based techniques for studying interaction, structure and dynamics of proteins involved in degradation. XL-MS data can help to elucidate 3D structures of small molecule-inducible proteins and map protein interfaces. I strongly believe that integrating XL-MS with other techniques of structural biology, such as cryo-electron microscopy, will help us to address important questions in the TPD field.

# TPD as a Powerful Research Tool in Basic Biology and Drug Target Discovery

Tao Wu, Hojong Yoon, Yuan Xiong, Sarah E. Dixon-Clarke, Radosław P. Nowak, Eric S. Fisch
*Nat. Struct. Mol. Biol.* 2020, 27, 605–614

DOI: 10.1038/s41594-020-0438-0

**Summary:**
In recent years, scientific research has capitalized upon naturally occurring ubiquitin-proteasome (UPS) and autophagy-lysosome pathways to develop novel protein activity inhibitors. These inhibitors, also known as "degraders", selectively target proteins for destruction and hence perturb molecular activity. Targeted protein degradation (TPD) methods have a number of benefits over genetic knockdown and chemical inhibition approaches, as summarized in Figure 1.

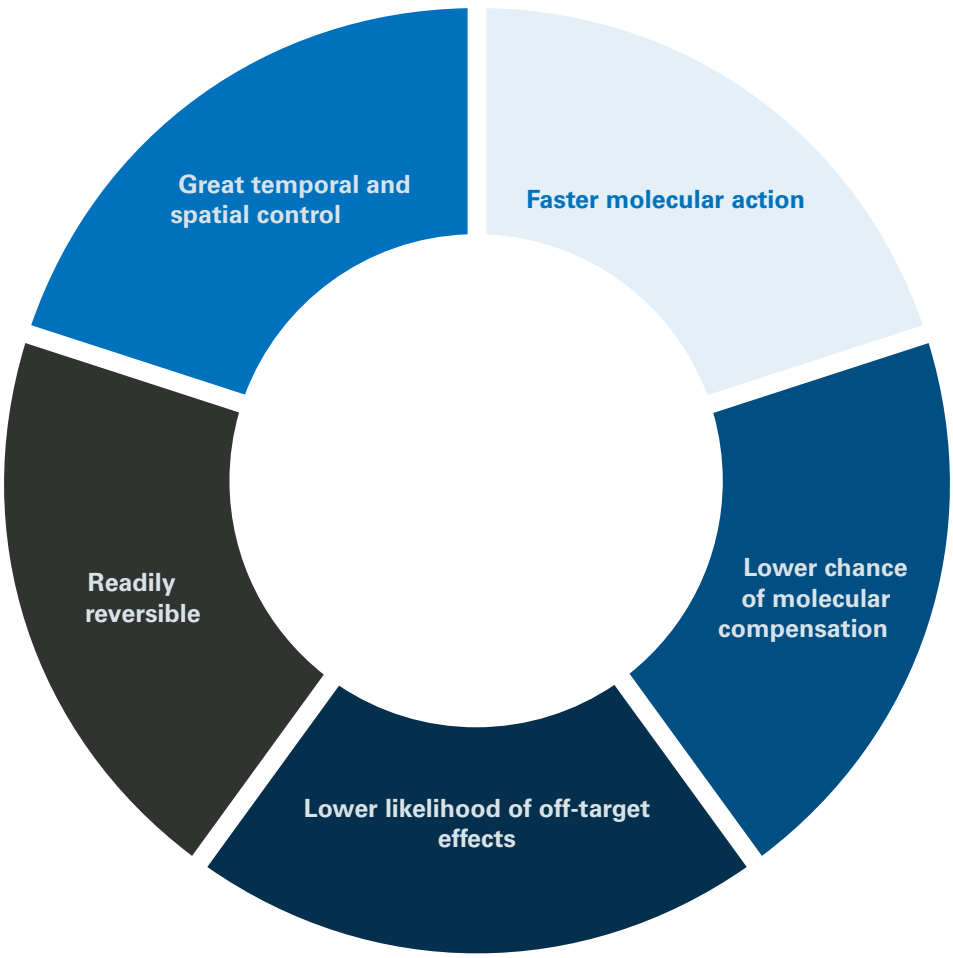


**Figure 1**
Key benefits of TPD approaches to controlled protein perturbation when compared to genetic knockdown (CRISPR) or chemical inhibition methods.

Most current TPD methods work via the UPS. Hence, validation techniques for the characterization and quality control of UPS pathways are essential in the assessment of selectivity and degradation efficiency of potential TPD therapeutics. By interrogating the UPS pathway with known inhibitors (see Figure 2) the effects of TPD modalities can be explored using a variety of molecular biology techniques (see Table 1).

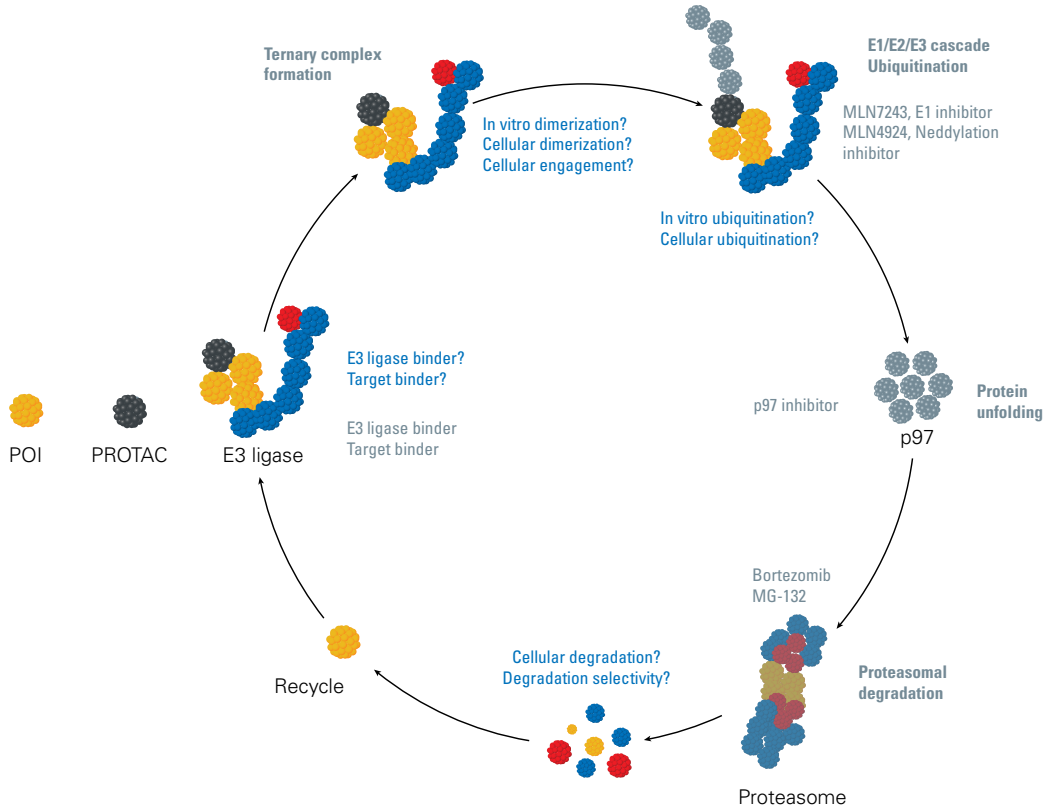


**Figure 2**
Validation methods for targeted protein degradation at different stages of the UPS pathway.

| Steps | Purpose | Example assays and chemical tools |
|---|---|---|
| Dimerization—*in vitro* | Ternary complex formation *in vitro* | TR-FRET4043, SPR/BLITS |
| Dimerization—cellular | Ternary complex formation in cells | NanoBit, NanoBret |
| Cellular engagement | Cellular permeability and cellular engagement of the E3 ligase | Degradation-based engagement assay<br>NanoBret-based fluoro-ligand displacement |
| Ubiquitination—*in vitro* | Verify ubiquitination | *In vitro* ubiquitination assay |
| Ubiquitination—cellular | Ubiquitination quantification and type<br>Identify ubiquitination sites | Western blot, NanoBret, TUBES<br>Proteomics |
| Cellular degradation—targeted approach | Verify ubiquitination in cells | Western blot, GFP-fusion, mCherry reporter lines<br>Endogenous CRISPR fusions (HiBit Tag, split GFP) |
| Cellular degradation—selectivity profiling by proteomics | Verify degradation selectivity in cells | Proteomics approaches, library-based screens |
| UPS inhibitors | Inhibitors of the cullin-RING family of ligases | MLN494-a specific inhibitor of the NAE1/UBA3 Nedd8-activating enzyme<br>CSN5i-3-inhibitor of COP9 signalosome |
| | p97 inhibitor | CB-5083 |
| | Ubiquitin E1 (UBA1) inhibitor | MLN7243 |
| | Proteasome inhibitors | Bortezomib, carfilzomib and MG132 |

**Table 1:** Validation methods for TPD.

Understanding UPS pathways makes it possible to engineer TPD systems that control protein turnover and facilitate the study of proteins of interest (POI) in human health and disease. Hence, by focusing on UPS pathway and small-molecule degrader research, it is possible to gain insights into the mechanisms driving cellular processes and potential novel therapeutics.

# Development of NanoLuc-Targeting Protein Degraders and a Universal Reporter System To Benchmark Tag-Targeted Degradation platforms

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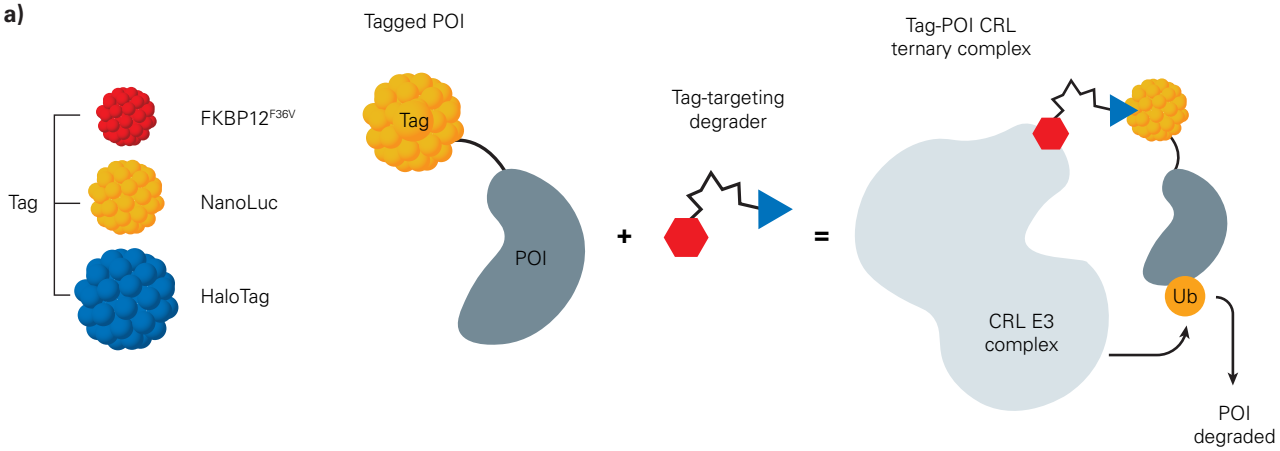
*Nat commun.* 2022, 13 (2073)
DOI: 10.1038/s41467-022-29670-1

Tag-targeted protein degrader (tTPD) systems direct degrader compounds to protein tags using targeted heterobifunctional molecules that allow rapid, reversible degradation of tagged substrate proteins of interest (POIs).

tTPD systems targeting FKBP12F36V (dTAGs) or HaloTag7 (HaloPROTACs) have shown promise as preclinical validation systems, but both have limitations and side-by-side comparisons have not been performed.

**a)**




**b)**

**FKBP12$^{F36V}$ HaloTag and NanoLuc Tag Comparison**

| Tag | MW (kDA) | CRBN degrader | VHL degrader | IAP degrader | Catalytic degrader | Antibodies | Lumines-cence properties | *In vivo* activity |
|---|---|---|---|---|---|---|---|---|
| FKBP12$^{F36V}$ | 12 | Yes | Yes | X | Yes | Yes | X | Yes |
| Nanoluc | 19 | Yes | X | X | Yes | Yes | Yes | NT |
| HaloTag | 33 | X | Yes | Yes | X | Yes | X | Yes |

**Figure 1.** Development of NanoTACs: a NanoLuc-targeting degrader system. a) Schematic depicting the tTPD systems. FKBP12F36V, NanoLuc or Halo epitope tags are fused to POI and tag-targeting heterobifunctional degrader compounds are employed to hijack cullin-RING ligase (CRL) complexes to trigger proteasomal degradation of the tagged POI. b) Comparison of tools and properties of each tag for tag-targeted protein degradation. CRBN, cerebelon; VHL, Von Hippel-Lindau; IAP, inhibitors of apoptosis protein; NT, not tested.

NanoLuc is a bioluminescent protein tag with multiple advantages over other tags such as stability, catalytic properties, small size and ease of availability. In this work, NanoLuc-targeting PROTACs (NanoTACs) were developed to expand the tTPD repertoire and trigger proteasomal degradation of NanoLuc tagged substrates. The properties of the NanoLuc reporter system were assessed in comparison with dTag and HaloPROTACS (Figure 1a, 1b).

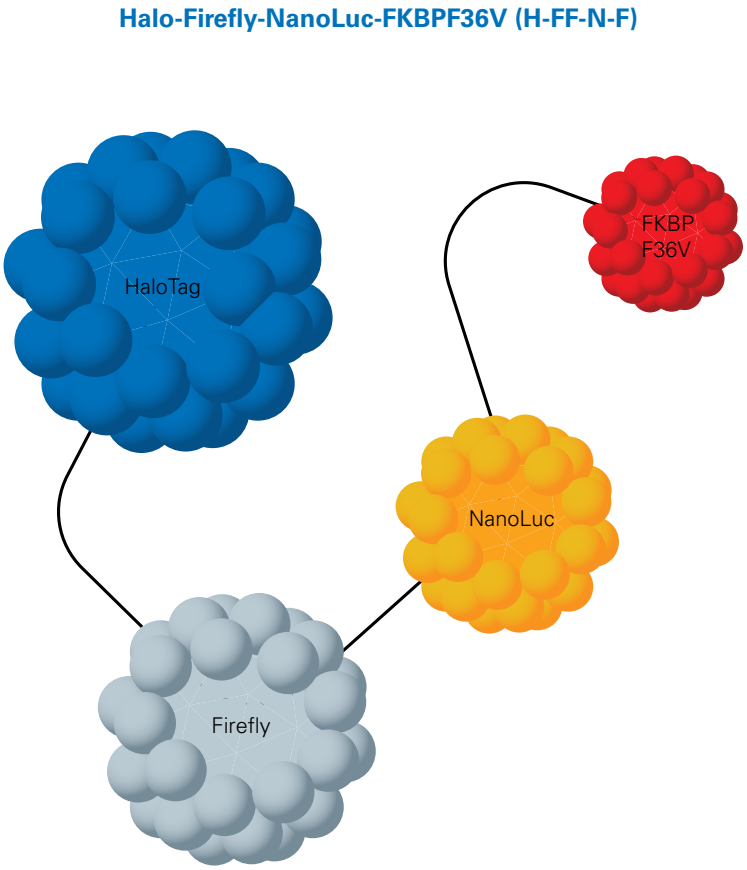
A stable, universal tTPD reporter protein containing all three protein tags (Halo, FKBP12$^{F36V}$ and NanoLuc) was synthesized to enable comparative studies (Figure 2). The addition of a Firefly luciferase enabled direct comparison of all tTPD systems by luminescence.

Using the H-FF-N-F reporter system, multiple HaloPROTACs, NanoTACs and FKBP12$^{F36CV}$-targeting degraders were assessed for degradation capabilities. Although the NanoLuc-CRBN targeting NanoTac, NC4, could trigger efficient degradation, the FKBP$^{F36V}$- targeting (dTag) system was seen to trigger the most efficient substrate degradation.

Additionally, the ability of the tTPD system to degrade a biologically relevant, tagged protein was examined. Both the NanoTAC NC4 and the FKBP$^{F36V}$-targeting degrader FV1 induced degradation of pro-necroptotic pseudokinase MLKL, to a level sufficient to prevent necroptotic cell death. Importantly, the global proteomic analysis demonstrated that NC4 triggers specific degradation of the target substrate with no significant off-target degradation observed when assayed against 5591 proteins

Overall, FKBPF36V tTPD systems are the most efficient and fastest platforms for degrading tagged substrates. However, the addition of NanoTACs to the tTPD repertoire adds further flexibility to tTPD studies. Together, NanoLuc and other tTPD systems will be a crucial part of future validation studies on prospective therapeutic targets.

**Figure 2**
Schematic depicting the Halo-Firefly-NanoLuc-FKBPF36V (H-FF-N-F) fusion protein.



## Notes

## Notes

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