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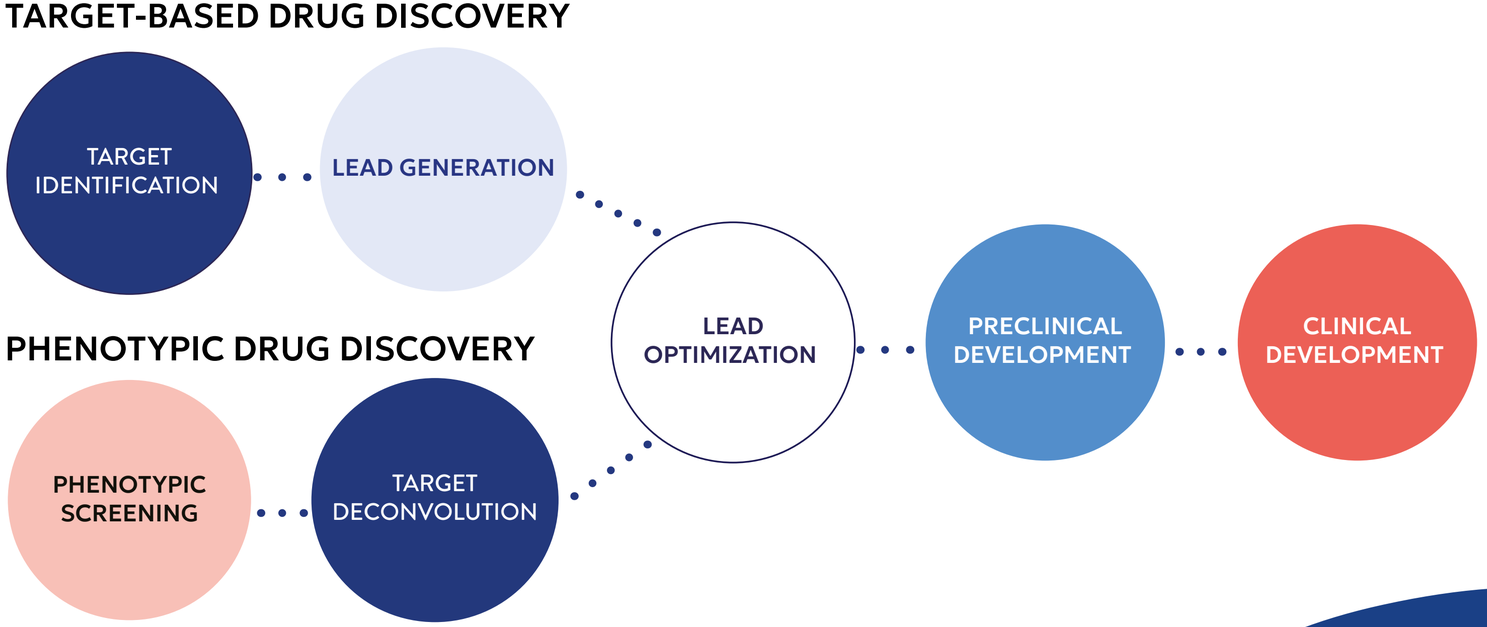
RIGHT TOOLS, RIGHT TIME: BOOST YOUR DRUG DISCOVERY SUCCESS BY ADOPTING PROTEOME-WIDE CETSA PROFILING EARLIER

Contents

Chapter 1: Rethinking your approach to target identification

In drug discovery, achieving high efficacy and good safety requires a deep understanding of the pharmacologically active compound's mechanism of action and the therapeutic target. To develop the most effective drug, scientists must determine how the target is modulated by the compound and its role in the disease of interest. It's also essential to obtain insightful knowledge about how the compound interacts with unintended protein targets and the consequences of these interactions.

The two key strategies in drug discovery include target-based (hypothesis-driven) and phenotypic-based (empirically driven) approaches. Target-based drug discovery begins with the search for a target, usually a protein, that has a presumed or validated role in the disease (target identification). Once the target is identified, compound libraries are screened to find a compound or 'hit' that selectively binds to the target and elicits the desired therapeutic effect.



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The historically more common phenotypic-based approach has regained popularity in recent years. This approach relies on phenotypic measures of response and starts with identifying a suitable assay that determines a cellular response readout related to the therapeutic effect, such as changes in proliferation, expression levels, or cytokine release. The established assay is then used to screen a compound library effectively. As the targets of any hits are initially unknown, they are identified retrospectively through target deconvolution and validation and investigations into how the compound exerts its pharmacological effect (the mechanism of action, or MoA).

Irrespective of which drug discovery approach you use in your research, it's crucial to fully understand the complexity of the MoA, including the effect on the primary target and any off-targets. Choosing the most efficient and reliable target identification method from the outset can minimize risks to your drug discovery pipeline and help protect your investments. Much like exploring uncharted territory, if we don't use the most suitable tools from the start, we might take much longer to gather the information we need and even miss essential data that other more effective methods may have detected.

In this eBook, we outline how applying proteome-wide CETSA profiling earlier in your research can avoid the limitations of more traditional approaches, including insights from our drug discovery expert, Stina Lundgren, and MS CETSA expert, Alexey Chernobrovkin. We outline examples of where research groups have adopted the method to great effect, such as helping to minimize the risk of late-stage failure, improving translational models, and providing a richer understanding of a compound's MoA and pathway effects. Now widely recognized as a highly valuable tool for drug discovery, we discuss how this method is excitingly impacting the development of new and better medicines for patients.

> Choosing the most efficient and reliable target identification method from the outset can minimize risks to your drug discovery pipeline and therefore help protect your investments
>
> **ALEXEY CHERNOBROVKIN, PROTEOMICS EXPERT**

Chapter 2: Choosing the most efficient target identification approach

Unbiased methods for identifying therapeutic targets typically fall into one of two broad categories: functional genomics and mass spectrometry (MS)-based proteomics tools. Functional genomics uses our growing knowledge of the human genome to modify genes through downregulation or knockout, which can help to identify genetic targets involved in pathological cellular function. For example, genome-wide CRISPR-Cas9 methods are showing great promise in discovering therapeutic targets in different cancers with high specificity (Liu et al., 2019).

Despite the value of functional genomics in target identification, gene downregulation/knockout does not recapitulate the action of a compound blocking a specific part of a protein target, which could make results highly misleading. For example, a compound could block the active site of an enzyme, leaving the enzyme scaffold function unaffected, yet gene downregulation/knockout would not fully recapitulate these functional consequences of the compound-protein binding.

Moreover, gene downregulation/knockout can affect more than one protein in the disease pathway, and achieving complete downregulation can be challenging. This makes the selectivity and activity of the genetic modification difficult to predict. Genetic modification also creates a highly artificial cell system.

Alternatively, MS-based proteomics methods enable the direct measurement of compound-protein interactions in unmodified systems to produce more relevant and reliable insights. These methods have so far had three main applications (Schirle et al., 2012):

1. Characterizing direct or indirect drug-target interactions for target deconvolution and selectivity profiling

2. Elucidating the MoA by which a drug exerts its pharmacological effect, target characterization, and validation

3. Identifying biomarkers that can be used for monitoring the effect of target modulation in an in vivo setting

Chemoproteomics and the widely acknowledged method of thermal proteome profiling are the two main MS-based approaches used to directly measure proteome-wide drug-target binding, which will be the focus of the rest of this eBook. In the following sections, we discuss some important considerations about each of these methods that could help maximize the efficiency and productivity of your target identification research, reduce risk, and increase success rates in your discovery efforts.

Chemoproteomics

Chemoproteomics is used in target identification and deconvolution to verify the direct binding of small molecules with protein targets. Typically, the method relies on a chemical probe replicating the parent compound to capture proteins. The probe is linked to an analytically detectable bead (often through covalent immobilization) or to another agent for separation, followed by MS-based detection of the protein targets the probe has bound.

After substantial advances in recent years, including improvements to MS technology, chemoproteomic methods now enable the detection of direct compound-protein interactions (Meissner et al., 2022). However, despite being a highly valuable technique, chemoproteomics methods have several limitations that have yet to be fully addressed. One major issue is that most human protein targets still lack proof-of-relevance probes—even for so-called 'druggable' proteins (i.e., targets presumed or known to bind to a drug). As chemoproteomics is mainly only practical in lysates, the method cannot identify downstream pathway hits or work with compounds that need metabolic modification after administration (so-called prodrugs). The approach also involves artificial ligand or protein target modification and cannot quantify drug-target binding in living cells. This limits its physiological relevance and, thus, its predictivity regarding how a drug will act within the patient.

The limitations of chemoproteomics mean you could miss therapeutic targets that might otherwise offer valuable opportunities for generating hits or elucidating the MoA. The risk of obtaining limited insights is further exacerbated by the fact that highly selective probes can only identify direct drug binding with the primary target and cannot reveal the drug's impact on the rest of the disease pathway. Moreover, building fit-for-purpose, highly selective probes can take 6–12 months, involving a rigorous probe optimization and validation process. Even after investing substantial time and funds into developing your probe, it's uncertain you will have one that sufficiently replicates the function of the parent compound. There is also a risk that the probe labels proteins non-specifically and identifies probe-specific hits rather than targets of the parent compound.

Proteome-wide CETSA profiling

Successfully applied in both targeted drug discovery and phenotypic approaches, proteome-wide CETSA profiling (also referred to as ‘thermal proteome profiling’ or TPP, and more recently as ‘protein integral solubility alteration’ (PISA)) is a label-free and physiologically relevant method for measuring drug-target binding in live cells and tissue that combines quantitative proteomics with the Cellular Thermal Shift Assay (CETSA®). The technique offers several advantages over chemoproteomics, including greater efficiency and richer insights into the disease pathway (not just the primary target).

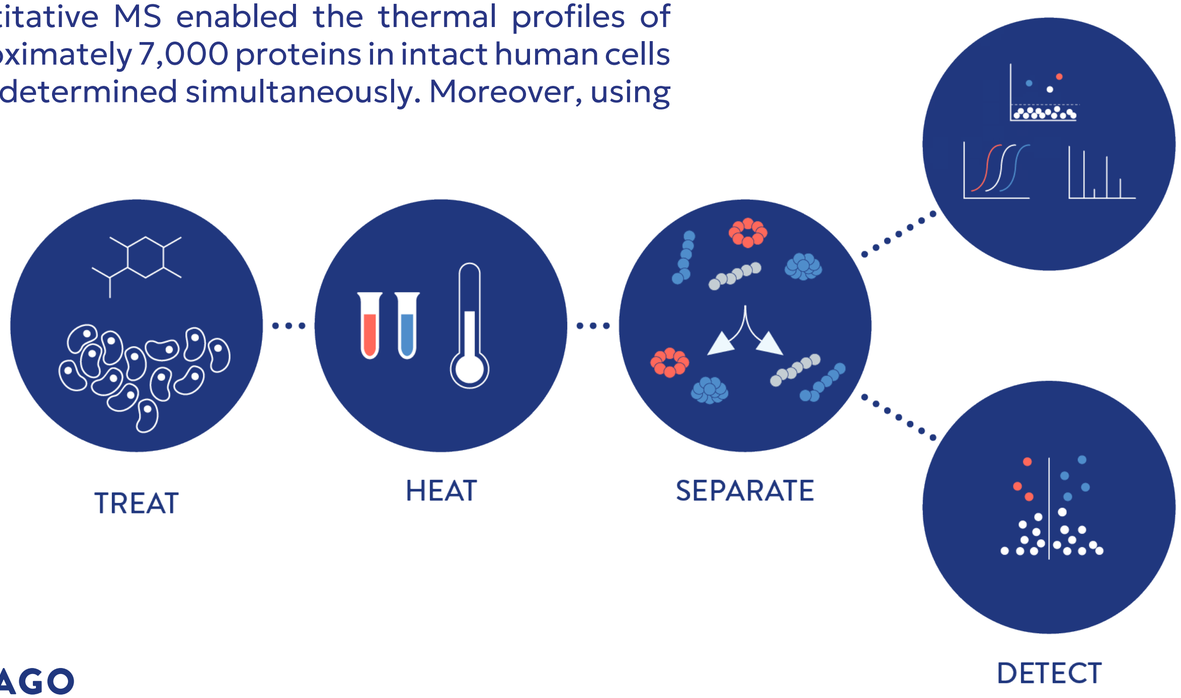
As discussed in the following chapters, using this approach right from the start of your research can be highly beneficial. As in the exploration of unknown territory, throwing your net as wide as possible from the beginning not only offers you the highest chance of success in obtaining the information you need but also allows you to get this valuable data far faster than more traditional methods while avoiding potential obstacles along the way.

> “Chemoproteomics is like taking a watch to pieces and counting the cogs—it doesn’t tell you which cogs drive which process and how the wider system works. This is why CETSA and Mass Spec can add value to your target identification studies.
>
> **PÄR NORDLUND, PROFESSOR AT KAROLINSKA INSTITUTE AND THE INVENTOR OF CETSA**

Chapter 3: The value of proteome-wide CETSA profiling in drug discovery

Before discussing the benefits of proteome-wide CETSA profiling, let's look at the method in more detail. CETSA is a technique that measures target engagement in live, intact cells, helping to generate more physiologically relevant insights into drug-target binding (for a brief description, see Box 1 or refer to our other literature on how CETSA works and how it enables lead generation and optimization).

Shortly after Martinez Molina et al., (2013) introduced the CETSA method, Savitski et al., (2014) showed how combining CETSA with multiplexed quantitative MS enabled the thermal profiles of approximately 7,000 proteins in intact human cells to be determined simultaneously. Moreover, using proteome-wide CETSA profiling, the researchers revealed new insights into important anticancer drugs, including the molecular causes of side effects observed in patients taking vemurafenib and alectinib (see Chapter 4) as well as the identification of more than 50 targets for the kinase inhibitor staurosporine.



Box 1: A brief description of the Cellular Thermal Shift Assay (CETSA)

CETSA is based on well-established thermodynamic principles: when a compound binds to a protein, it often changes the thermodynamic properties of that protein, which can be detected by a shift in its melting temperature. First developed by Martinez Molina et al., (2013), the CETSA method involves these basic steps:

1. The cells or lysates are treated with the compound of interest
2. The sample aliquots are subjected to a range of temperatures to induce denaturation of the proteins
3. Denatured and aggregated protein is removed
4. A protein-detection method is used to quantify the remaining soluble protein and compared to an untreated control to quantify the relative change in the amount of protein that has stayed folded because of the compound interactions

Proteome-wide CETSA profiling is label-free and physiologically relevant and can be used to analyze the entire proteome for drug-target engagement in live cells. Therefore, this tool offers unbiased and highly efficient verification of drug-target binding as well as off-target monitoring, elucidation of the molecular MoA, pathway effects, and biomarker discovery (see Figure 1 for the different compound-induced biomolecular changes that proteome-wide CETSA profiling can detect in the disease pathway).

Proteome-wide CETSA profiling has now become widely recognized as a powerful tool for discovering targets of orphan clinical drugs and hits from phenotypic screens, identifying off-targets, and explaining polypharmacology and drug toxicity (see Chapter 4). Pelago Bioscience's most used and versatile format of proteome-wide CETSA profiling is the compressed (CR) format. This format involves integrating the individual protein melt curves across the experimental temperatures, which yields concentration-response curves that reflect the compound-induced changes in the integral thermal stability of the proteins.

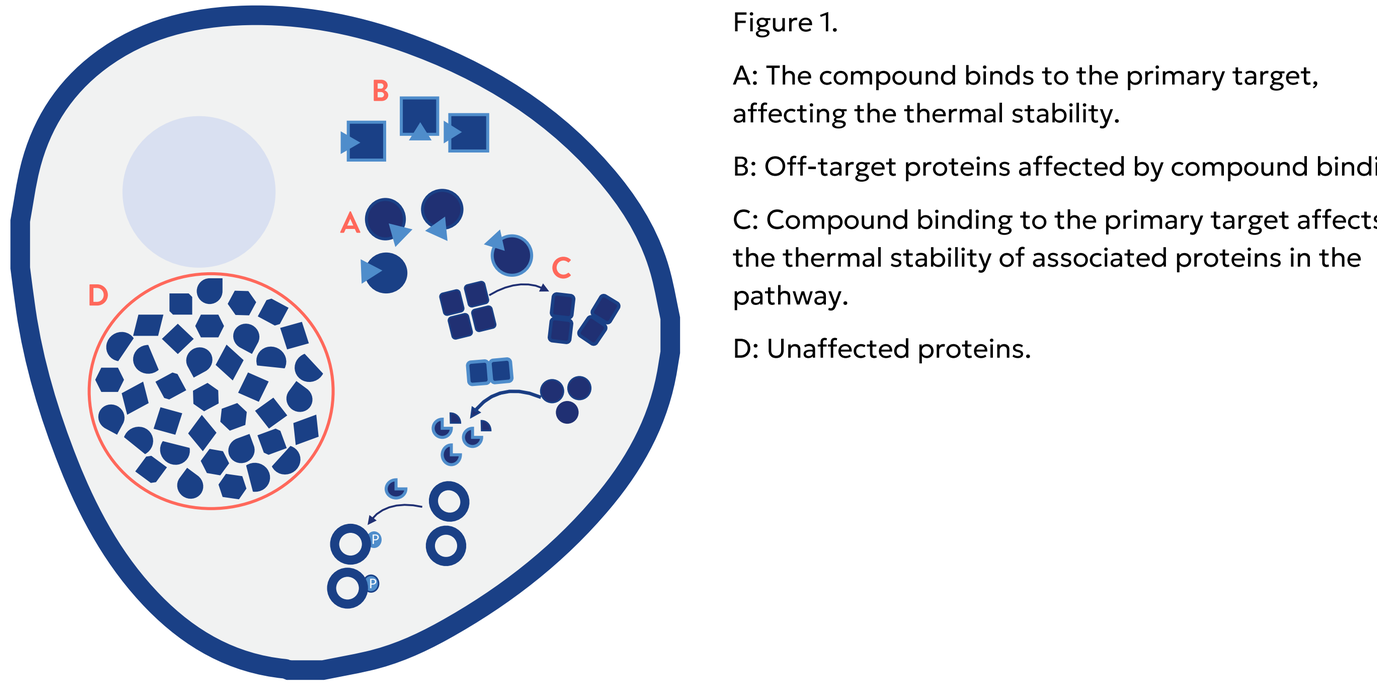


Figure 1.

A: The compound binds to the primary target, affecting the thermal stability.

B: Off-target proteins affected by compound binding.

C: Compound binding to the primary target affects the thermal stability of associated proteins in the pathway.

D: Unaffected proteins.

Exploring the target landscape using CETSA

To make the most of our exploration, we need to efficiently collect information about the unknown that may be relevant for generating future hypotheses. In the same way, using a tool like proteome-wide CETSA profiling from the start of your target identification studies allows you to collect the data you need as quickly as possible, which can benefit your research in numerous ways.

One key benefit is that you won't have to build a chemical probe or library of probes, which can save you 6–12 months (or more) of preparation. This means you can expedite the research process and potentially increase the number of hit compounds you progress down the pipeline. Using the unmodified compound directly avoids the potential bias a probe can induce.

As only a small proportion of targets have a proof-of-relevance probe, using proteome-wide CETSA profiling also allows you to interrogate a much larger portion of the human proteome than chemoproteomics methods. Additionally, unlike chemoproteomics, CETSA allows you to monitor the effects of your compound on both the primary target and associated proteins of the entire disease pathway in the cellular matrix of your choice. These richer insights can help you better understand the complexity of your compound's MoA, including validating novel disease-relevant targets and quantifying drug-target selectivity for off-target monitoring. Consequently, proteome-wide CETSA profiling can help ensure the efficacy and safety of your compounds to bolster and de-risk your discovery efforts.

What's more, it allows you to immediately obtain the robust data you need when you need it, saving you time and avoiding the additional costs you may incur when using more inefficient methods. Adopting proteome-wide CETSA profiling right from the start of your research, therefore, helps minimize risks to your project compared to using chemical probes, which have more uncertain timelines, costs, and data outputs.

> "Proteome-wide CETSA profiling is a more predictable method than chemoproteomics, so it can help keep your project on track by helping you meet key milestones and stay within budget.
>
> **STINA LUNDGREN, DRUG DISCOVERY EXPERT**

Chapter 4: How proteome-wide CETSA profiling enables drug discovery — Case studies

Many studies have already demonstrated the enormous value proteome-wide CETSA profiling can add to drug discovery. In this chapter, we showcase some of these studies and reveal five ways CETSA has had the biggest impact on drug discovery so far (see Table 1).

Table 1: Sumary of the key impact areas of unbiased CETSA profiling in drug discovery

1. Understanding the wider biological impact of your drug on your model system
2. Getting actionable results on MoA when other methods fail
3. Off-target monitoring for safety assessments
4. Protecting against unknown liabilities
5. Improving translational models

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1. Understanding the wider biological impact of a drug on your model system

As we have briefly touched upon, unbiased CETSA profiling can uncover the biological effects of your compound on both the primary target and the entire disease pathway. This section highlights two case studies in which proteome-wide CETSA profiling has successfully identified novel pharmaceutical targets for hits from phenotypic screens.

Kitagawa et al., (2017) examined the targets of a novel compound (a131). This drug selectively kills cancer cells (activated by the Ras-signaling pathway) through mitotic catastrophe but protects normal cells by allowing them to retain their proliferative capacity. Using CETSA, Kitagawa et al., (2017) uncovered how a131 could induce these potent effects. The researchers identified the pharmacological targets as two PIP4K lipid kinases (PIP4K2A and PIP4K2C), which are known to be involved in tumorigenesis (see Figure 2). Follow-up experiments supported this by showing that PIP4Ks regulate the cell cycle entry between normal and Ras-activated cancer cells.

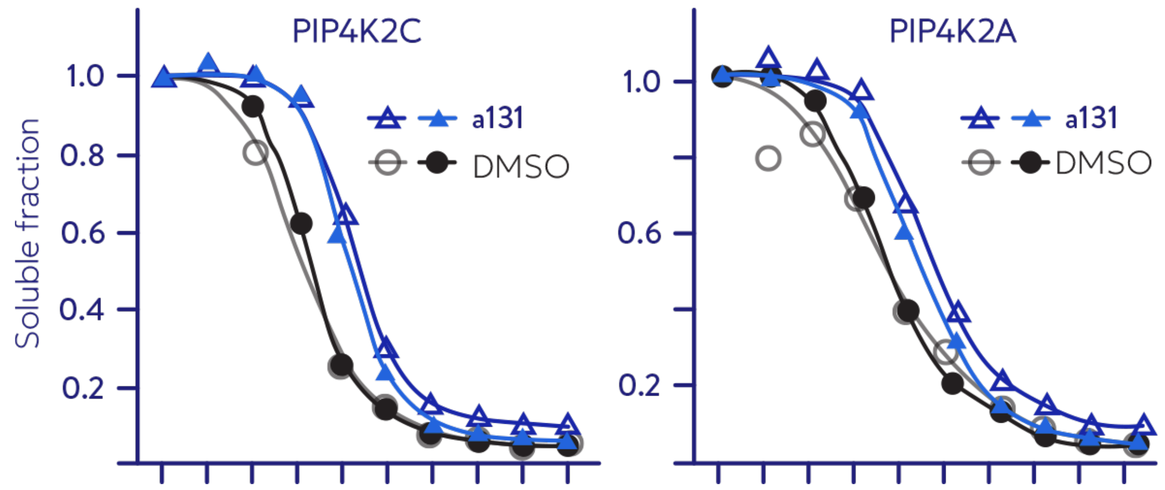
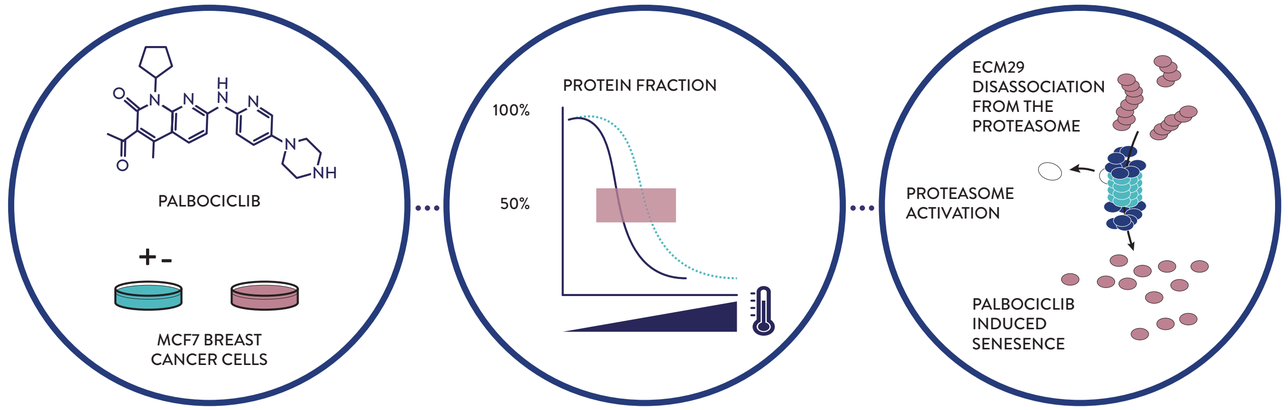


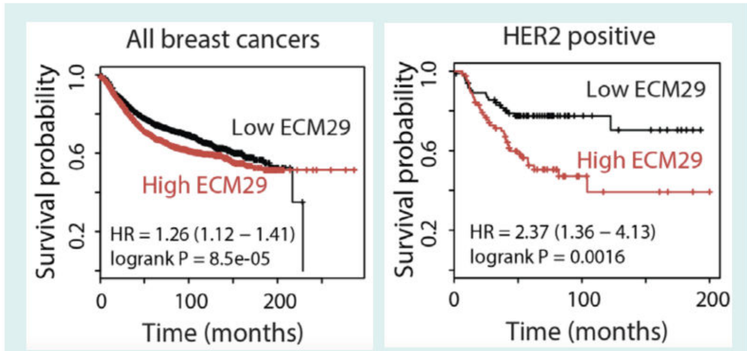
Figure 2: CETSA MS melt curves for PIP4K isoforms in duplicated experiments of a131 and DMSO treatment.

Another study by Miettinen et al. (2018) applied unbiased CETSA profiling to investigate the molecular effects of palbociclib (Ibrance®, a CDK4/6 inhibitor approved for metastatic breast cancer), which is known to cause cell cycle arrest and cellular senescence.

As well as identifying known CDK4/6 targets, Miettinen et al. (2018) identified a novel downstream target of palbociclib: the 20S proteasome (a protein complex that degrades unneeded or damaged proteins via proteolysis). Specifically, the analysis found that palbociclib induced the ECM29 protein to dissociate from the proteasome, which affected the thermal stability of the 20S proteasomal subunit detected by CETSA. The ECM29 dissociation caused proteasomal activation, which induced cellular senescence and blocked cell proliferation.

In a follow-up study, Miettinen et al. (2018) investigated whether ECM29 levels were linked to patient survivability, given that patients with low levels of ECM29 are more susceptible to cell senescence in general. In ground-breaking work, the researchers revealed that ECM29 levels are predictive of relapse-free survival in breast cancer patients treated with endocrine therapy. This demonstrates how unbiased CETSA profiling can efficiently and cost-effectively reveal cause-effect biomarkers that inform optimal treatment strategies for specific patient populations to improve survival rates.





> It would have been almost impossible to identify the ECM29 predictive biomarker of breast cancer without CETSA Mass Spec.
>
> **TEEMU P MIETTINEN, RESEARCH FELLOW AT MIT**

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2. Getting actionable results on MoA when other methods fail

Another area where proteome-wide CETSA profiling generates more insights than other methods is clarifying the MoA of existing and candidate drugs in a disease-relevant setting (Friman 2019). This is demonstrated in a study by Dziekan et al. (2019), which used CETSA to profile two leading antimalarial drugs (quinine and mefloquine), which have been used for hundreds of years, and identify their direct targets. Specifically, the researchers identified purine nucleoside phosphorylase (PNP) as a common binding target for both drugs (Figure 3), which they supported by further biophysical and structural studies.

This is the first time the target of these drugs has been identified, demonstrating how unbiased CETSA profiling can reveal valuable insights undetectable by traditional methods. Uncovering the MoA of antimalarial drugs and other therapies in this way could help to better understand decreasing responsiveness to treatments and inform the development of new therapies.

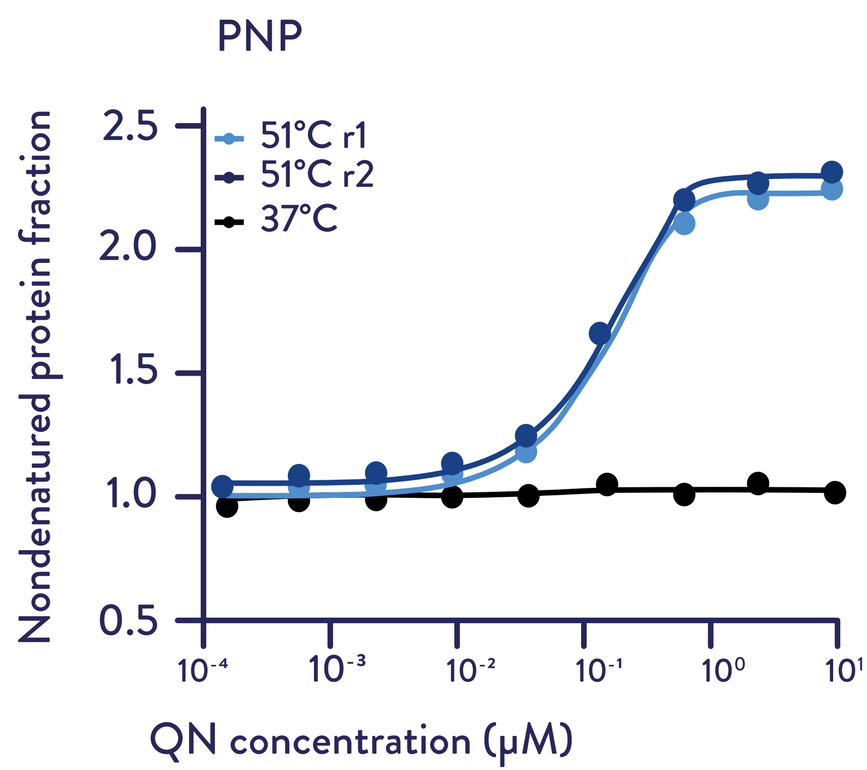

Figure 3: The protein stabilization profile of PNP using CETSA MS.

> "One of the best target ID technologies available."
>
> **DEREK LOWE, DRUG DISCOVERY EXPERT**
>
> **IN THE PIPELINE, SCIENCE, 2019**

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3. Off-target monitoring for safety assessments

Studies using CETSA have elucidated the molecular causes of the harmful side effects of certain drugs. For example, the kinase inhibitors vemurafenib and alectinib cause phototoxicity and skin rashes in patients due to increased tissue levels of protoporphyrins. Savitski et al. (2014) used unbiased CETSA profiling to study the thermal response of the proteome under increasing concentrations of these drugs. The study found that, alongside the primary drug target (BRAF), the two compounds also bind to the heme biosynthesis enzyme ferrochelatase (FECH), the inhibition of which is known to cause increased protoporphyrin levels. Savitski et al., (2014) also revealed that alectinib affected FECH more potently than vemurafenib, while another oncology drug with no such side effects (crizotinib) did not affect FECH stability. These findings further support that FECH inhibition underpins phototoxicity (Figure 4).

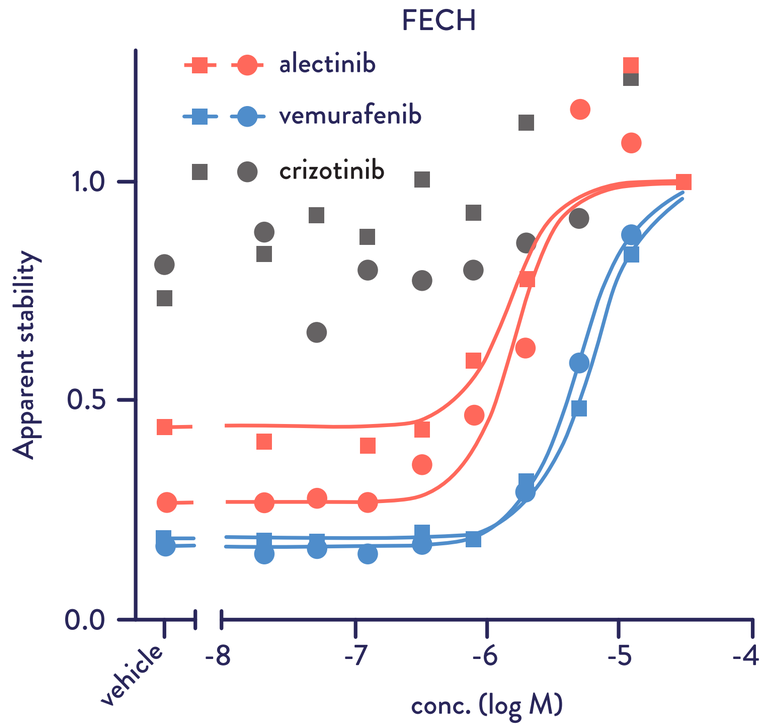


Figure 4: CETSA MS concentration-response profile demonstrating stability shifts of FECH induced by vemurafenib and alecitinib but not crizotinib over a range of concentrations.

> One of the best target identification technologies available is proteome-wide CETSA profiling, sometimes also referred to as TPP and PISA. This technique provides physiologically relevant data on compound-protein interactions.
>
> **MICHAEL DABROWSKI,**
>
> **CEO PELAGO BIOSCIENCE**

4. Protecting against unknown liabilities

Protecting patients from the potentially toxic effects of candidate drugs isn't always possible when the compound's effects on the entire disease pathway are unknown. In more serious cases, this can lead to retraction after a drug has been approved. For example, the drug thalidomide caused devastating limb malformations and severe birth defects in thousands of children in the late 1950s.

Only in the last ten years have studies identified the targets and determined the MoA of thalidomide and its more potent analogues, lenalidomide and pomalidomide (IMiDs). For example, Ito et al. (2010) used high-performance affinity bead purification to identify the protein CRBN (Cereblon) as a direct interaction partner of thalidomide. Other studies have found that IMiD-mediated ubiquitination and subsequent degradation of the transcriptional factor SALL4 was responsible for the birth defects seen in children whose pregnant mothers took thalidomide during a sensitive period of embryonic development (Donovan et al., 2018; Matyskiela et al., 2018).

Recently, the in-house research team at Pelago Bioscience used unbiased proteome-wide CETSA profiling to yield more insights into the functional consequences of IMiDs on the target and the entire signaling cascade (Chernobrovkin et al., 2021). In a single experiment, CETSA confirmed CRBN as a target of pomalidomide. It also revealed a time-dependent decrease in abundance or increased stability of several previously published and novel protein targets of the E3 ubiquitin ligase complex (Figure 5).

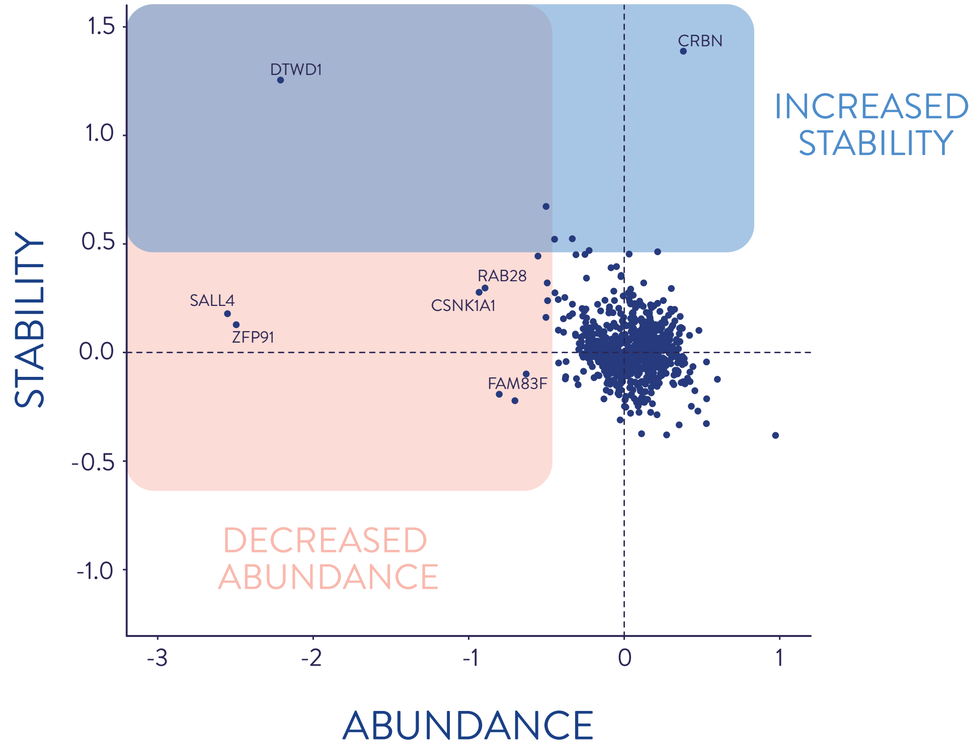


Figure 5: Compressed CETSA MS concentration response profile demonstrating time-dependent decreases in abundance or increases in the stability of known and novel protein targets of the E3 ubiquitin ligase complex. Highlighted in the figure are SALL4, ZFP91, RAB28, CSNK1A1, FAM83F and DTWD1, which had been discovered previously as substrates of the IMiD-activated E3 ubiquitin ligase.

How CETSA MS can help repurpose marketed drugs and evaluate protein degraders

Although Thalidomide was retracted as a treatment for morning sickness in pregnant mothers, the drug has since proved to be a useful treatment for other diseases, including leprosy, inflammation and different types of cancers, such as multiple myeloma (Zhou et al., 2013). Repurposing such 'old' drugs can potentially offer lower overall costs and quicker timelines to approval. Using CETSA MS could be highly beneficial in this regard, by quickly and reliably detecting promising new targets, off-target effects and the MoA of your compound to support its value as a repurposed drug.

Additionally, CETSA MS could be used to evaluate targeted protein degraders, such as PROTACs (proteolysis-targeting chimaeras) and molecular glues, which are emerging drug strategies that degrade target proteins in order to produce the intended therapeutic effect. As the Pelago team has successfully used CETSA MS to detect the targets of protein-degrading IMiDs, this shows great promise for using it to evaluate the targets and MoA of other degraders like PROTACs. This includes generating degradation profiles and information on how the PROTAC interacts with other targets and the effect on associated proteins.

5. Improving translational models

A research collaboration between Cellzome GmbH (a GlaxoSmithKline company) and the European Molecular Biology Laboratory in Heidelberg, Germany, has recently demonstrated how CETSA can be used to enhance translational models (Perrin et al., 2020, Nature Biotechnology). The team developed and tested a new approach for measuring proteome-wide thermal stability and target profiling in vivo in the study.

The new method, termed 'tissue-thermal proteome profiling' or 'tissue-TPP', applies CETSA to measure thermal stability in tissue proteomes. Using tissue-TPP, the researchers quantified the thermal stability of rat-derived tissue proteomes (liver, lung, kidney, and spleen) and found that the method recapitulates the physiological processes of these organs, as well as differences related to energy metabolism, signaling, and protein homeostasis. The team also used tissue-TPP to identify targets and off-targets in tissues derived from animals dosed with panobinostat (a histone deacetylase inhibitor).

These findings demonstrate how unbiased CETSA profiling can help improve translational models by enabling in vivo target engagement measurements in physiologically relevant tissue proteomes. The efficient generation of such relevant data can help to quickly and reliably predict the efficacy and adverse side effects of candidate drugs, even in early preclinical phases.

Chapter 5: Why it's crucial to use the right tools, at the right time

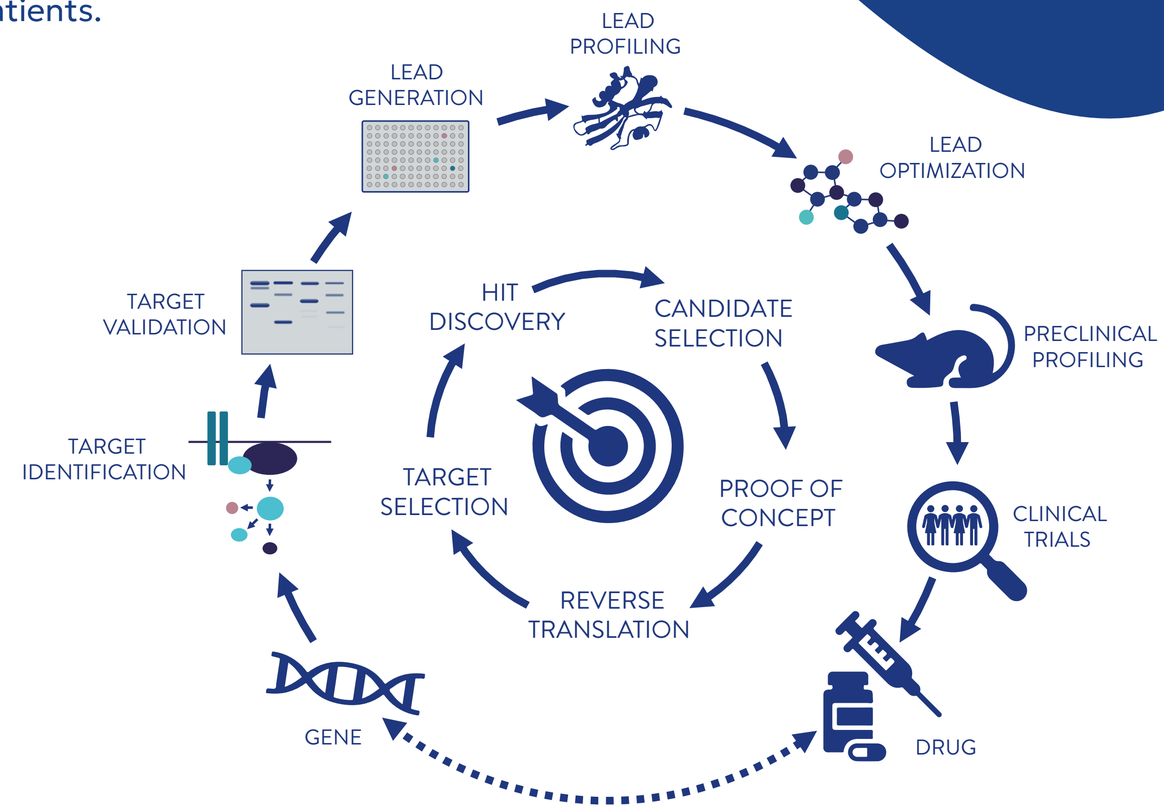
As late-stage attrition rates continue to rise, drug discoverers face increasing pressure to pass efficacious and safe compounds down the pipeline faster. Mitigating risks to the pipeline, such as delayed timelines and increasing costs, makes this even more challenging.

In this high-stakes environment, the early adoption of proteome-wide, MS-based tools like unbiased proteome-wide CETSA profiling is one solution you could consider. As in our exploration through uncharted territory, using an alternative tool instead of a more tried-and-tested method can be a daunting prospect. However, together, we must act when we know that another technology can help us collect the information we need more quickly and with far fewer risks.

As we have seen throughout this eBook, early adoption of proteome-wide CETSA profiling can not only save you time, but can also ensure you will get the data you need within budget. It can also help generate unbiased data relating to the effects of your compound on the whole pathway, not just on the primary target. Moreover, the technique allows you to simultaneously interrogate the entire human proteome without probe-induced bias to uncover novel targets—which more traditional methods might never discover. Overall, this enables you to more efficiently validate disease-modifying targets, quantify the selectivity of your compound, and fully elucidate the complexity of its MoA. The richer insights you gain can boost the success of downstream preclinical studies and clinical development. Indeed, since it was introduced, unbiased CETSA profiling has had a remarkable impact, such as improving translational models, minimizing the risk of late-stage failure, and elucidating previously unknown drug MoA. Adopting such powerful tools clearly has the potential to advance drug discovery and enable the development of new and improved medicines to help patients.

Book a complimentary consultation with us to learn how CETSA can help boost the success of your target identification studies.

BOOK YOUR CONSULTATION



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About Pelago Bioscience

Pelago Bioscience AB was founded to provide and develop the patented Cellular Thermal Shift Assay (CETSA®) in 2013. CETSA was invented at Karolinska Institute, a prestigious medical university in Stockholm, Sweden, and developed by Pelago Bioscience to deliver in situ target engagement studies to expedite clinical drug discovery and diagnostics development.

Pelago Bioscience has vast experience and expertise in drug discovery, including the areas of screening and assay technology development as well as lead generation and lead optimization research. By tapping into this wealth of knowledge, you can ensure you effectively prioritize your projects to make an impact and get the most value out of your drug discovery projects.

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Stina Lundgren is the Head of Business Development at Pelago Bioscience. She has long experience as a medicinal chemist working on multiple small molecule programs across all phases of drug discovery. Stina received her PhD in Chemistry at the Royal Institute of Technology in Stockholm in 2007 in the research group of Professor Christina Moberg. Prior to joining Pelago Bioscience, she was a Principal Scientist at Medivir responsible for establishing a lead generation platform and managing projects in the drug discovery pipeline.

Alexey Chernobrovkin is a Principal Scientist at Pelago Bioscience with experience in mass-spectrometry-based proteomics and data analysis. He obtained his Master of Science in Applied Math and Physics at the Moscow Institute of Physics and Technology and his PhD in Bioinformatics at the Russian Academy of Medical Sciences. Before joining Pelago Bioscience in 2018, Alexey worked at Karolinska Institute in Roman Zubarev's laboratory, developing mass-spectrometry-based methods for the characterization of protein targets and mechanisms of action of small-molecule drugs.

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