CETSA® and its role in target engagement

Reduction the cost of drug development while increasing biological understanding

Despite significant advances in technology and increased public spending devoted to basic research, the duration and cost of drug discovery have both followed an upward trend for several decades. It currently takes at least 10 years to bring a new drug to market and the average cost is estimated at $1 billion. Furthermore, among the experimental drugs that enter Phase I clinical trials, only 9.6% are expected to reach the market. These high attrition rates in pharmaceutical development have led many companies to adopt a “fail early, fail fast” approach, meaning therapeutic candidates should fail before entering costly Phase II clinical trials. By failing early and failing fast, expenditures can be kept to a minimum.

In an effort to increase success rates, there has been a significant shift towards fine-tuning the pre-clinical stages of drug discovery. For example, a retrospective study of data from 44 Phase II decisions by Pfizer led to the evolution of three fundamental criteria to guide investment decisions for non-validated drug targets. Pfizer’s ‘three-Pillar’ paradigm enables the company to assess the quality of a molecule and the doses required to test the clinical hypothesis in future patient studies. This framework stipulates that a clinical candidate must demonstrate:

1. Sufficient exposure of ligands at the site of action
2. Proof of target engagement
3. Expression of pharmacological activity from the site of action.

In a similar vein, AstraZeneca developed the ‘5R Framework’ to improve the company’s R&D productivity. This involved the identification of five critical technical determinants of project success: the right target, the right patient, the right tissue, the right safety, and the right commercial potential.
Notably, the study points to target engagement as a key parameter for improving the probability of project success. Furthermore, the authors state that when assessing the ‘right tissue’, it is crucial to generate data that increase the understanding of a molecule’s pharmacokinetic (PK), pharmacodynamic (PD), and ADME (absorption, distribution, metabolism, and excretion) properties. This is to improve quality candidate selection, as well as help design the most appropriate studies to demonstrate evidence of target engagement.

**What is target engagement?**

Target engagement in drug discovery refers to the interaction of ligands with their intended target biomolecules. As discussed above, confirmation of target engagement is a fundamental component of modern drug discovery. Measuring target engagement can help research teams gain a better understanding of the relationship between a compound’s exposure and its pharmacological response, providing a quantitative understanding of drug efficacy and/or toxicity. Target engagement data can also be used for PK/PD modeling, whereby the relationship between the concentration and distribution of a small molecule administered to the study model (e.g., cultured cells, primary cells, organoids, or an animal model) and the observed effects on the target, downstream signaling, and desired phenotypic changes are determined over time.

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**Figure 1:** The drug discovery process. Clinical phases are the most time-consuming and also account for the majority of the overall cost. Duxin S. et al. (2022). Why 90% of clinical drug development fails and how to improve it? Acta Pharmaceutica Sinica B.

**Figure 2:** Simplified diagram of the relationship between exposure to a small molecule pharmaceutical agent and the resulting response. Sufficient engagement of the molecule with its intended target causes measurable changes downstream which can be measured through a biomarker or phenotypic changes.
Target engagement challenges

Establishing and confirming target engagement is a critical step in pharmacological research; however, it can prove quite challenging, especially for proteins with poorly understood function. There are a wide variety of methodologies that allow the measurement of target engagement. Currently, estimates are often based on downstream pharmacodynamic effects, which are often assumed to correlate with the binding of the drug to its target. While these measurements provide evidence of engagement with the target in some instances, many downstream processes are complex and so this approach becomes problematic in cases where there are numerous interconnecting pathways.

An alternative approach to measure target engagement is the establishment of affinity-based assays, either using a drug candidate as a direct affinity probe or a secondary affinity probe in a competitive assay. For example, emerging chemoproteomic-based approaches have been valuable for defining the selectivity of drugs within specific protein families, such as kinases, methyltransferases, and deacetylases. Affinity probes can also be applied to measure target engagement in cells, although these measurements are less direct when they depend on intermediate lysate steps or chemically altered ligands.

Ideally, target engagement should be monitored and assessed from initial hit generation through to preclinical and clinical development. The indirect nature of many target engagement assays, and the relatively high cost of establishing useful affinity probes, highlights the need for more direct and versatile assays which are applicable to a wider range of drug targets and cell systems.

Existing approaches to target engagement: disadvantages and limitations

Bioluminescence Resonance Energy Transfer (BRET)

BRET is the non-radiative transfer of energy between a bioluminescent donor and a fluorescent acceptor. BRET studies exploit the close proximity between a ligand and its target protein to study cellular target engagement. BRET can be used to monitor molecular interactions occurring in real time in living cells. The assay can be adapted to a microplate format and recent studies have successfully harnessed CRISPR/Cas9 to tag endogenous proteins, thereby circumventing the need for transfection with an exogenous copy of the gene.

- **Disadvantages**: expression of the luminescent donor requires fusion to the protein of interest, which is then exogenously expressed. Even with CRISPR/Cas9, it is limited to cell lines and modification can affect target protein properties. There is also a lack of physiological relevance.

- **Other limitations**: it is difficult to apply on primary cells and other more physiologically relevant disease models.

Fluorescence anisotropy

Another technique to examine cellular target engagement relies on fluorescence anisotropy. In a typical experiment, cells are dosed with fluorescently tagged molecules and after incubation, alterations in the isotropy of emission are measured by fluorescence polarization.

- **Disadvantages**: although this technique can be applied in biopsies, conjugation of the ligand of interest (candidate molecule) can affect its engagement profile.

- **Other limitations**: in general, the method is sensitive to quenching effects, difficult to optimize, and often results in high background.

Protein mass spectrometry

Numerous protein mass spectrometry (MS)-based approaches have emerged as tools for studying target engagement, including activity-based protein profiling (ABPP), photoaffinity labeling (PAL), and affinity chromatography. ABPP uses chemical probes that react with mechanistically related classes of enzymes. It can be combined with tandem MS to identify active enzymes from a single sample. Similarly, PAL in combination with MS, is a useful tool for identifying protein ligands. Affinity chromatography involves exposing cellular lysates to photo-affinity probes and analyzing the bound proteins by MS. While the principle of ABPP and PAL is identical to affinity chromatography, these techniques allow for the use of live cells instead of cell lysates.

- **Disadvantages**: false positives through non-specific protein binding to the scaffold or resin.

- **Other limitations**: difficult to apply in high throughput and complex data analysis.
Enter CETSA®

Scientific principle

The Cellular Thermal Shift Assay (CETSA®) is a patented method that allows the quantification of a compound’s target engagement within living cells or in disrupted cells. The CETSA® assay principle is based on the change in thermal denaturation profile of the target protein that occurs following the binding of a compound.

Upon heating, a protein will encounter a temperature at which it denatures (sometimes referred to as the melting point). This melting temperature is a physical property and a constant for any given set of conditions (pH, pressure, salts). Compounds that interact with a protein will change the melting temperature, i.e., induce a thermal shift. For a given temperature point where the thermal shift can be measured, compounds can be applied at different concentrations (dose-response), and the apparent effective concentration 50 (EC₅₀) values derived from such curves can be used to establish a rank order of potency of a series of compounds, which directly correlates to the rank order of affinity of the compounds.

The CETSA® method is patented and a license is required to run the technology in-house. Please contact Pelago Bioscience (https://www.pelagobio.com/) if you want to know more about CETSA® technology or hear more about Pelago Bioscience’s other services.


Figure 4: Simplified principle of the Cellular Thermal Shift Assay. Ligand binding to a protein target induces thermal stabilization which translates in an elevated melting temperature.
The CETSA® assay is performed by incubating the cells with the test compound, followed by heating of the compound-treated cells. The samples are then lysed and after a separation step the remaining soluble target protein is quantified.

**How CETSA® differs from other thermal shift assays**

There are several variations on the in vitro thermal shift assay, but the key technique was first described by Semisotnov et al. (1991)\(^8\). In this method, the melting and unfolding protein exposes hydrophobic surfaces that enable SYPRO orange to bind. The dyes’ fluorescence is quenched in water, so the binding to these hydrophobic surfaces reverses this, causing fluorescence to peak when the protein is fully unfolded. This type of thermal shift assay can only be applied to highly purified proteins, and so for low abundance species this may require an overexpression system to generate sufficient amounts.

While CETSA® is a method based on the same biophysical principle as standard thermal shift assays (i.e., that specific proteins will denature at a set temperature), it does not directly measure the specific temperature of unfolding. Instead, it relies on the fact that the presence of a compound on the protein will affect the amount of soluble protein present after heating to a set temperature. As such, CETSA® is in essence a total protein assay conducted after a specific heating event. Compounds with differing target engagement potencies will change the relative amounts of protein surviving the heating event. Because the assay measures this residual protein rather than the melting event, it can be applied to more complex in vivo systems such as primary cells and lysates (and in some CETSA® formats even solid tissue). In addition, CETSA® can identify compounds that destabilize a protein (i.e., reduce its melting temperature), which is less straightforward with other thermal shift methods.

**How CETSA® fits into the drug discovery lead development process**

CETSA® provides a unique measure of a compound’s on-target presence, or target occupancy. This occupancy will be affected by both the compound’s ability to engage the target and the presence at the right location (i.e., does it have the right solubility, permeability, metabolic stability, and availability at the right cellular compartment?).

Additionally, the potential and applicability of CETSA® to be used for structure activity relationship (SAR) analysis and hit confirmation was clearly demonstrated by Shaw et al. (2018)\(^9\) by comparing data from the CETSA® assay with commonly used biochemical and cell-based assay data. The publication demonstrates the added value of CETSA® for screening, hit confirmation, and SAR generation for two protein targets: B-Raf and PARP1.

**Advantages over non-cellular target engagement assays**

Non-cellular target engagement assays offer measures of affinity correlated only with the protein’s behavior in highly artificial environments, often using purified recombinantly expressed target proteins. Such proteins can prove challenging due to expression quantity and quality issues. By contrast, the CETSA® method has been validated for a range of different cellular model systems of various complexity and relevance to clinical situations.\(^10\) The experimental protocol is relatively simple, and there is no need for a target-specific probe. Furthermore, the critical experimental step of heat denaturation can be performed quickly after tissue isolation without homogenization, potentially minimizing in vivo changes to the target state and compound distribution.

**How CETSA® can be applied in high throughput**

As a label-free technology which is broadly applicable to a large number of protein targets, CETSA® may be the ideal strategy for high-throughput screening (HTS) as it can be performed directly in a physiologically relevant context. Implementing CETSA® in a high-throughput system constitutes a novel HTS method for identifying hits based on cellular target engagement. In addition, CETSA® can be used in secondary screens for orthogonal hit confirmation.

**Combining CETSA® and AlphaLISA®/HTRF® no-wash immunoassay platforms**

To achieve a high-throughput assay, CETSA® can be combined with a dual antibody proximity-based detection system, such as AlphaLISA or HTRF, which require no wash steps. In such an assay, a thermal melting curve is prepared using known positive and negative controls identifying a certain temperature set point where a compound-stabilized protein target stays in supernatant and non-compound binding leads to protein aggregation/precipitation. The compound-stabilized protein in solution is then detected by AlphaLISA or HTRF technologies.
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Both immunoassays are no-wash, highly sensitive technologies with a broad dynamic range, and fast time-to-results. These proximity assays enable the detection and screening of targets of interest in a reliable, high-throughput format.

Key differences to other cellular target engagement assays

Promega’s NanoBRET™ target engagement assay:
A luciferase fusion tag is combined with labeled tracer compounds which, when in close proximity to the luciferase, will lead to BRET. Compound binding in the same pocket will displace the tracer and BRET signal will decrease. Some compounds affecting the enzymatic reaction will ‘confuse’ data and produce false-positives.

Advantages:
- Cellular target engagement assay, but no heat shock required which means it is a simpler workflow.
- Some ‘ready-made’ targets are available (kinases, HDAC, and bromodomains). Other targets would need to be developed by the customer.

Disadvantages:
- Involves cloning/protein tagging: not truly endogenous, the cell line may be less physiologically disease relevant.
- Artificial expression can result in many issues (incorrect protein folding, incorrect trafficking, incorrect association with partner proteins.)
- Confirmation in alternate cell lines requires another cloning process and requires labeling of the tracer.

Eurofin/DiscoverX’s InCELL pulse™: This is based on Enzyme Fragment Complementation (EFC) technology. The target protein is fused with a small enzyme donor fragment of β-galactosidase (β-gal). An added reporter protein will bind to this fragment to reconstitute an active enzyme, which will then hydrolyze a substrate and generate a chemiluminescent signal allowing protein abundance quantification. There is a likelihood for false-positives if compounds affect the enzymatic reaction.

Advantages:
- Does not rely on unique target specific antibodies

Disadvantages:
- Involves cloning/protein tagging: not truly endogenous, the cell line may be less physiologically disease relevant.
- False data: artificial expression can result in many issues (incorrect protein folding, incorrect trafficking, incorrect association with partner proteins). All of these can affect a compound’s ability to bind its target.
- Reduced assay window: the assay measures aggregation event, which is a more ‘binary event’ than the denaturation that CETSA® measures.

The key difference between these recombinant target engagement systems and no-wash immunoassays like AlphaLISA or HTRF, is that they cannot be applied to native and unmodified cells. Generating a tagged protein will always have physiological consequences on the cell and the tagged protein may be: (i) overexpressed (wrong stoichiometry vs. partners), (ii) located in the wrong cellular compartment, (iii) not associated with key partner proteins, and (iv) have a different melting behavior than the native protein. Thus, the apparent effect of a compound may not reflect its real behavior in a patient’s tissue. These problems may be magnified in any temporary transfection system. In later stages of lead optimization, when more complex and physiologically relevant cellular models (primary, native cell lines, and tissue) are required, tagged protein approaches are simply not applicable.
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Case studies

**Vertex pharmaceuticals: using alpha CETSA® as a primary screen in drug discovery**

Julie Alexandre, Principal Research Scientist at Vertex Pharmaceuticals, looked as Alpha CETSA® for its potential to deliver hits for one of its particularly challenging targets in its relevant cellular context. The researchers anticipated the target of interest would present several challenges:

- The target was expected to be particularly challenging to drug owing to the high conformational flexibility of the protein
- There are no small molecule binders or inhibitors currently known for the target, meaning there are no positive controls for assay development
- The target is part of a multi-protein complex in vivo, making it difficult to reproduce with individual components in vitro
- Previous experience of working with this target highlighted the need to study it in the presence of its relevant interaction partners

Alpha CETSA® provided the team with an opportunity to study the target in its relevant cellular context. Of note, Alpha CETSA® formed part of a wider screening effort comprising biochemical assays such as ligand binding and enzyme activity, biophysical techniques in the form of a thermal shift assay with a recombinant protein and a focused compound library, as well as a dedicated fragment screening approach using focused fragment sets suitable for either X-ray crystallography or NMR. Thus, Alpha CETSA® data could be directly compared with those from the other screening approaches.

The researchers screened a library of ~50,000 chemically diverse small molecules with the overall goal of identifying small molecule binders of the target. In all, they identified 14 compounds that shifted the melting temperature by >1°C. The assay signal to noise ratio was maintained above four throughout the screening campaign, and the Z factor ranged between 0.4 to 0.75, with an average of 0.6.

Overall, Alpha CETSA® delivered new chemical scaffolds that were not identified in other screens. Vertex Pharmaceuticals are now considering the feasibility of extending the technique to larger libraries.

**Astrazeneca: lessons from using CETSA® to measure target engagement for three different protein targets and comparisons with functional assays**

At AstraZeneca, Joseph Shaw’s lab was an early adopter of Alpha CETSA®, using it to screen compound binding to Androgen Receptor (AR). The AR plays a key role in prostate development and homeostasis, and is implicated in most prostate cancers. Shaw et al. tested a library of compounds
which included AR binders as well as molecules expected to target AR co-regulators. The first surprising finding was that while the AR agonist α-Dihydrotestosterone (DHT) induced thermal stabilization of the protein, the same was not true for the well-established antagonist Enzalutamide, in whose presence no thermal stabilization was observed, similar to the negative controls.\(^\text{11}\) Co-treatment of the cells with both DHT and Enzalutamide gave rise to a concentration-dependent attenuating effect of the antagonist on the thermal stability conferred by the agonist.

The above observation was exploited in the design of a competition assay for measuring AR antagonist target engagement, whereby potency was not measured as a function of thermal stabilization but rather thermal destabilization of AR treated with DHT. The assay was applied to a set of 25 compounds comprising three known AR direct antagonists and 22 small molecules thought to exert their inhibitory effects by acting on AR co-regulators. Using CETSA\(^\text{®}\) in conjunction with Alpha reagents from Revvity, it was possible to distinguish between compounds that act directly on AR, such as Enzalutamide, Hydroxyflutamide and MK-2866 versus molecules such as Danusertib and Entrectinib, kinase inhibitors acting on tropomyosin receptor kinase A (TrkA), which is thought to act as a co-regulator of AR. It is worthwhile noting that all the above compounds exhibited inhibitory activity when using a downstream reporter assay measuring AR-driven transcription.\(^\text{11}\)

The success that Shaw’s lab experienced when assessing target engagement with AR spurred them to use CETSA\(^\text{®}\) with two more protein targets, B-Raf and PARP1. B-Raf is a kinase involved in the MAP kinase/ERK signaling pathway, which affects cell division, differentiation, and secretion. Its V600E mutation is the leading cause of melanomas and abnormal B-Raf function has been identified in a host of other cancers, including non-Hodgkin lymphomas, colorectal cancers, thyroid carcinomas, non-small cell lung carcinomas and more. Shaw and colleagues tested a library of 896 kinase inhibitors using Alpha CETSA\(^\text{®}\) in the A375 melanoma cell line, which contains the V600E mutation. Among the compounds tested, FDA-approved drugs vemurafenib and dabrafenib as well as the pan-kinase inhibitor ponatinib, showed clear thermal stabilization of B-Raf, whereas C-Raf or ERK specific compounds did not, demonstrating the selectivity of the technique. By testing different concentrations of B-Raf inhibitors it was also possible to construct dose-response curves and derive the 50% EC\(_{50}\) of different compounds based on the AlphaLISA signal intensity.\(^\text{1}\) This allowed the ranking of compounds according to potency.

PARP1 is yet another protein target of interest to the pharmaceutical industry. As one of the key enzymes involved in DNA repair, PARP1 is overexpressed in tumours. In the absence of functional BRCA-based repair pathways, tumours are reliant on its activity to ensure that cancer cells do not accumulate deleterious DNA damage. In this case, Shaw et al. used CETSA\(^\text{®}\) to assess the target engagement potential of 6288 compounds for PARP1, that had previously tested positive using a fluorescent polarization (FP) assay on purified recombinant PARP1. Although the results from the two methods generally correlated well, there were also some important differences:

- CETSA\(^\text{®}\), being a cellular assay, will invariably show no thermal stabilization for compounds that cannot permeate the cell membrane
- 127 compounds showed strong affinity for PARP1 in the FP assay but no or little thermal stabilization in CETSA\(^\text{®}\)
- Compounds identified as strong binders in both assays were on average nine-fold less potent in CETSA\(^\text{®}\) as opposed to the FP assay when comparing the former’s EC\(_{50}\) values versus the latter’s inhibitory concentration (IC\(_{50}\)) values\(^\text{5}\)

Further investigation of the compounds using a cellular assay measuring PARP1 activity as a downstream functional endpoint provided IC\(_{45}\) values that were more comparable with the CETSA\(^\text{®}\) EC\(_{50}\) values. More importantly, by comparing the three data sets (FP assay, CETSA\(^\text{®}\) and functional assay) the researchers concluded that Alpha CETSA\(^\text{®}\) was the only technique that did not produce any false negatives or false positives.

### Conclusion

Establishing and confirming target engagement in a physiologically relevant cellular environment is a critical step in pharmacological research and a key parameter for improving the probability of project success. CETSA\(^\text{®}\) allows such an assessment by quantifying the changes in the thermal stability of proteins upon ligand binding within living cells or in disrupted cells. The experimental protocol is relatively simple, and there is no need for a target-specific probe.
Furthermore, CETSA® can be applied in high throughput and combined with AlphaLISA and HTRF no-wash immunoassays. Using CETSA®, biologically relevant values can be obtained from any type of cellular matrix – i.e. cell lines, animal tissue, or patient samples – to help researchers explore, discover, and confirm drug candidates faster.

References


