ACS Medicinal Chemistry Letters
Cite This: ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX
Viewpoint
pubs.acs.org/acsmedchemlett

Focusing on Relevance: CETSA-Guided Medicinal Chemistry and Lead Generation

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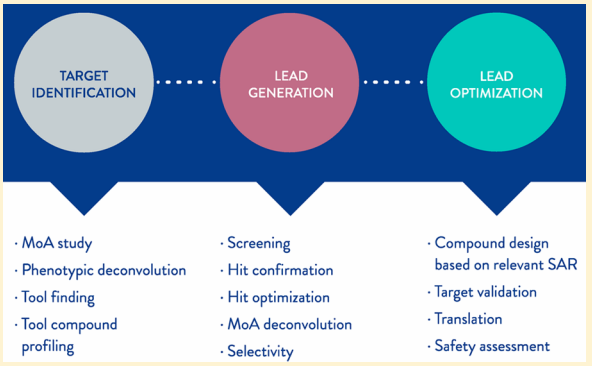
ABSTRACT: Confirmation of target engagement in relevant physiological environment ensures successful drug discovery and the right project prioritization. The Cellular Thermal Shift Assay (CETSA) offers a robust label-free method for studying protein−compound interactions in a cellular environment. This Viewpoint covers the broad applicability of CETSA in lead generation. The method can be used for deconvolution studies, target validation, screening of compound libraries, and hit confirmation. Moreover, the method is well suited for generation of relevant structure−activity relationship (SAR) data, enabling optimal compound design.



KEYWORDS: *CETSA, lead generation, medicinal chemistry, hit confirmation, screening*

In drug discovery, selective binding of the compound to the right target is important in order for the drug to be both effective and safe. To achieve this, the drug must be present at the site of action and occupy the intended target with high specificity. The high attrition rate in drug development is often attributed to lack of efficacy in the proof of concept studies or off-target induced toxicities.[1] A main reason for the low efficacy is insufficient target engagement at the intended site of action and incomplete understanding of the mode of action of the compound. The patented Cellular Thermal Shift Assay (CETSA) was developed to enable target engagement determination of a compound with its protein target in a physiologically relevant setting.[2] CETSA is a label-free method that assesses the thermal stability of proteins in living cells and tissue based on denaturation and aggregation as a result of heating. The remaining soluble proteins present in the supernatant after heating can be quantified, and the thermal melting curve of a protein can be generated. Compound binding often affects the thermal stability of proteins, and the shift in the melt curve is indicative of cellular target engagement (Figure 1). The method is applicable to all different types of modalities, for example, agonists, antagonists, allosteric binders, active site binders, and protein−protein interaction disruptors.

To date, there are three main formats in the CETSA technology platform. They all share the same principle assay protocol but differ in the method used for the protein quantification after the heat shock (Figure 2). Two of the formats, CETSA Classics and CETSA High Throughput (HT) are both targeted CETSA methods for confirming target engagement of a single known protein target using antibodies for the quantification. The third format, CETSA MS, is proteome-wide measurement of cellular target engagement using mass spectrometry for generating thermal profiles for 6−7000 proteins in a single experiment.

CETSA Classics is a Western blot-based method, normally used for confirming cellular target engagement for a limited number of compounds because of the low throughput. The format has mostly been used in the later stages of preclinical drug discovery for confirmation of cellular- or tissue target engagement prior to candidate drug (CD) selection. This is reflected in the majority of publications of projects in the late lead optimization, reporting on the use of CETSA Classics for target engagement determinations on a small selection of compounds. However, the impact on earlier projects could be of importance to ensure the right prioritization of projects and resources. Hence, target engagement assays in relevant cellular systems, such as CETSA, should be used for confirmation of hit compounds and preferably be included as a criterion in the lead target profile.

CETSA HT is based on dual-antibody detection of the folded target protein. The use of antibody proximity detection systems based on, for example, chemiluminescence or fluorescence makes the format suitable for miniaturization into 384 and 1536 well plates and subsequently enables rapid screening of medium sized compound libraries and multiple conditions with a short turnover. Almqvist et al. reported on the use of CETSA HT for screening of a compound library of more than 10,000 structurally diverse compounds, nucleosides, and known drugs against thymidylate synthase (TS).[3] The primary screen was conducted in single point testing (50 μM) resulting in a hit rate of 0.59%. The primary screen was followed by EC_{50} confirmation yielding novel TS inhibitors with cellular activity. The screened library included com-

DOI: 10.1021/acsmedchemlett.9b00112
ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

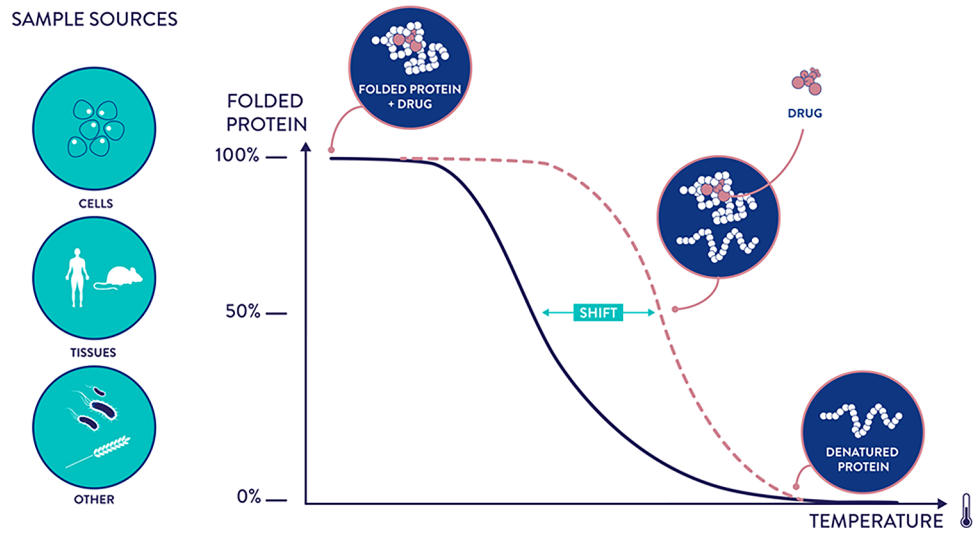


Figure 1. CETSA melt curves of the target protein in the presence or absence of compound.

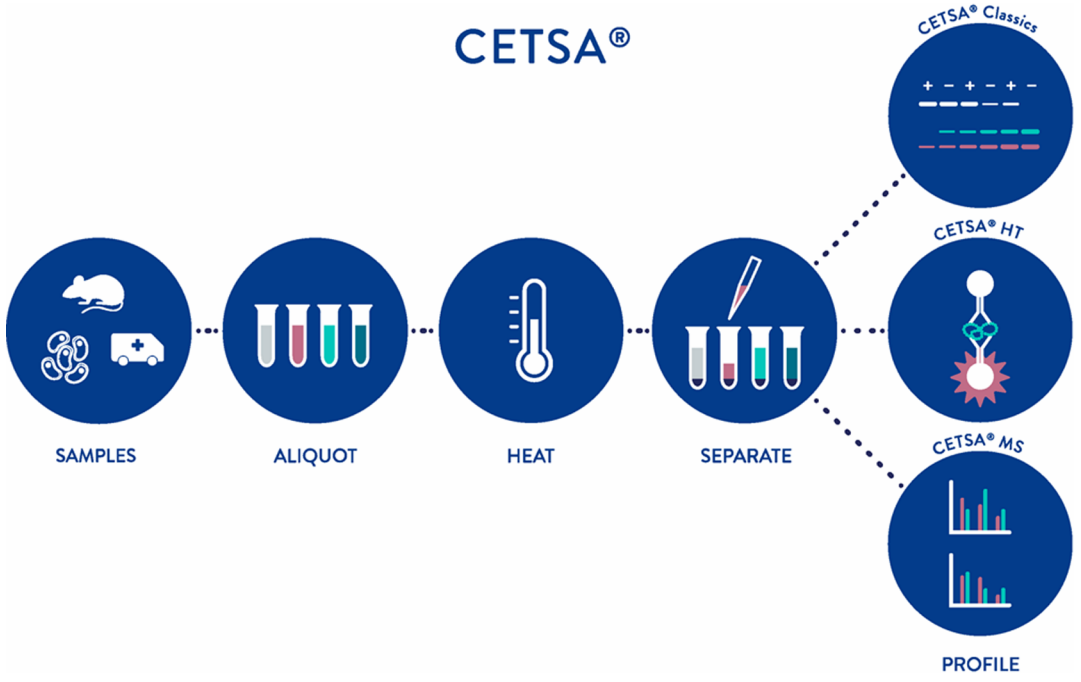


Figure 2. Overview of the principle assay protocol and the three main formats in the CETSA technology platform; CETSA Classic, CETSA HT, and CETSA MS.

pounds that are known to require metabolic activation before interaction with TS. The researchers performed time course studies by varying the compound incubation time prior to the heating step and observed change in several orders of magnitude in potency between the time points, highlighting the complexity in the CETSA EC_{50} data caused by cellular metabolism and permeability. Partly this can be resolved by comparing data generated in living cells with data from a lysate experiment. Although today there are no reports on CETSA-based structure−activity relationship (SAR)-driven ligand design, the data on cellular target engagement is nonetheless relevant for medicinal chemists to take into account in the generation of new ideas and prioritization of compound targets for synthesis.

Targeted CETSA has mostly been used for confirming cellular target engagement. However, Shaw et al. used CETSA to inform on binding mode and differentiating agonist and antagonist binders. While studying the androgen receptor (AR), they concluded that while binding of an agonist resulted in thermal stabilization, antagonist binders did not affect the thermal stability.[4] However, by competitive experiments they observed that the antagonists were able to reverse the thermal stability imposed by ligand binding, thereby allowing for quantification of apparent intracellular binding affinities and for calculating an apparent cellular K_i. AR is an example of a protein target that has proven difficult to produce sufficient quantities of the full-length recombinant protein. Therefore, target engagement measured by conventional biophysical methods has not been feasible, CETSA, however, not only enabled the confirmation of target binding, it did so in the physiologically relevant cellular environment.

DOI: 10.1021/acsmedchemlett.9b00112
ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

The potential and applicability of CETSA HT to be used in lead generation for SAR analysis and hit confirmation were clearly demonstrated in another study by Shaw et al. by the comparison of data from the CETSA HT assay with commonly used biochemical and cell-based assay data.[5] The publication evaluates the use of CETSA for screening, hit confirmation, and SAR generation for two protein targets: B-Raf and PARP1. To identify B-Raf inhibitors, the CETSA HT assay was utilized to screen a focused kinase library resulting in a reasonable hit rate (1.45%). Kinase inhibitors were identified including well-described B-Raf inhibitors and other structurally related compounds. For PARP1, CETSA HT was applied for hit confirmation following a biochemical screening campaign. In summary, the activity of the most potent PARP1 binders were confirmed in CETSA HT, with the advantage of instant confirmation of their permeability and target engagement in the cell. Interestingly, potencies were comparable between the CETSA HT and the cellular functional PARPylation assay, while higher potencies were observed in the biochemical assay. In addition, the level of correlation between CETSA HT and the biochemical assay was lower than with the cellular functional assay illustrating the differences between the biochemical and cellular assays.

This is a common phenomenon and not unique for PARP1. The lack of correlation could be explained by several factors, such as cell permeability, active transport, cell metabolism, and protein binding of the ligand in the cellular assay. In addition, the difference between an assay based on a purified recombinant protein and a full-length protein in a cellular context may have substantial impact. A cellular target engagement assay that determines the level of interaction between a ligand and the target protein in a physiologically relevant setting aids to understand the data and thereby the cellular context. Therefore, including CETSA in the screening cascade in lead generation and optimization is important to ensure a relevant compound design. Thus, CETSA-based SAR-driven ligand optimization and cellular target engagement data is highly relevant for medicinal chemists to take into consideration in the design of compounds and the prioritization of compound targets for synthesis.

Axelsson et al. described the first high-content imaging-based CETSA, suitable for in situ target engagement studies of P38α in adherent cells.[6] This imaging-based CETSA format was used in a screen of a kinase focused compound library in single point testing (50 μm) against p38α. The primary screen resulted in a 1% hit rate, including novel p38α binders, and was followed by a hit confirmation by CETSA EC_{50} determination. Although not included in the study, the imaging-based CETSA format enables the study of multiple targets simultaneously and studying drug and target localization.

The sensitivity and robustness of the CETSA HT assay allow high and low affinity binders to be identified, which enables screening of fragment compound libraries by using CETSA HT. There is also a potential to employ CETSA for iterative screening, which could be applied both to lead-like compound libraries and fragment libraries.

Preclinical projects are often unsuccessful in showing the intended effect in animal models despite access to well advanced lead compounds. This could be attributed to the lead optimization based on indirect functional data. The greatest strength of CETSA is the ability to confirm that the compound mediated cellular effects are a consequence of binding to the target in cells. A good correlation between functional readout and cellular or tissue target engagement to a target protein is an indication of its involvement in the biological pathway and strengthens the target validation. Developing and implementing the CETSA assay early in the discovery process provides valuable guidance to the research project. As a project in lead generation progress into lead optimization, the CETSA could be employed for generation of SAR for guidance of the medicinal chemistry program. Additionally, the CETSA assay could be employed for determination of target occupancy in tissue from animal studies and for translational studies from animal to human. The first report of using CETSA for verifying *in vivo* target engagement of novel RIPK1 inhibitors in various different samples matrices such as spleen and brain was published by Ishii et al.[7]

Equally important to verifying compound interaction with the intended target in the cell is the understanding of the selectivity profile of the compound. CETSA coupled to mass spectrometric readout measures not only the direct binding of compound to proteins in the cellular environment but also subsequent downstream consequences of the initial interactions. It is important to have access to well behaving and validated tool compounds in order to build relevant hypothesis around a specific target and biological function. Therefore, CETSA MS can be a powerful method for tool compound evaluation and identification of protein targets in lead generation. Moreover, assessment of the selectivity profile of the lead series by CETSA MS could highlight safety issues that needs to be addressed in the lead optimization phase.

As CETSA MS allows for unbiased proteome wide identification of direct interactions with individual proteins and impacts on cellular pathways, it can be used for deconvolution of hit compounds from a phenotypic screening. There are now several examples of this application in the public domain. Kitawaga et al. reported on the use of CETSA MS for understanding cellular targets and signaling pathways of hit compounds from a phenotypic screen.[8] The experiments provided strong evidence that PIP4Ks are the pharmacological targets of these novel compound hits. In another recent report, CETSA MS was used to investigate the targets of two antimalarial drugs.[9] The study effectively identified purine nucleoside phosphorylase as the target of quinine and mefloquine and provided critical knowledge for the understanding of the resistance mechanisms of these structurally related quinoline drugs.

In order for medicinal chemists to successfully design compounds and progress drug discovery projects toward CD selection, it is important that the compound design is based on a relevant SAR data true to the initial project hypothesis. CETSA offers a robust label-free method for studying protein compound interactions in a cellular environment regardless of whether it is a tool compound or lead molecule. Moreover, CETSA can enable binding assays for challenging targets with limited availability of substrates, for protein−protein interaction studies or in cases when recombinant protein cannot be produced. Understanding the mechanism of action of a compound, which targets it interacts with, which cellular pathways it affects, and which of these actions are important for the efficacy, provides very valuable guidance to the lead generation and ensures the right project prioritizations.

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DOI: 10.1021/acsmedchemlett.9b00112
ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX

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Notes

The author declares the following competing financial interest(s): S.L. is an employee of Pelago Bioscience AB.

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DOI: 10.1021/acsmedchemlett.9b00112
ACS Med. Chem. Lett. XXXX, XXX, XXX−XXX