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Review article

Mass spectrometry-based Cellular Thermal Shift Assay (CETSA®) for target deconvolution in phenotypic drug discovery

Tomas Friman*

Pelago Bioscience AB, Solna, Sweden

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ABSTRACT

The recent renewed interest in phenotypic drug discovery has concomitantly put a focus on target deconvolution in order to achieve drug-target identification. Even though there are prescribed therapies whose mode of action is not fully understood, knowledge of the primary target will inevitably facilitate the discovery and translation of efficacy from bench to bedside. Elucidating targets and subsequent pathways engaged will also facilitate safety studies and overall development of novel drug candidates. Today, there are several techniques available for identifying the primary target, many of which rely on mass spectrometry (MS) to identify compound – target protein interactions. The Cellular Thermal Shift Assay (CETSA®) is well suited for identifying target engagement between ligands and their protein targets. Several studies have shown that CETSA combined with MS is a powerful technique that allows unlabeled target deconvolution in complex samples such as intact cells and tissues in addition to cell lysates and other protein suspensions. The applicability of CETSA MS for target deconvolution purposes will be discussed and exemplified in this mini review.

1. Introduction

In the last decade many drug discovery programs on novel modes of action and even first in class projects have made good progress using phenotypic approaches rather than target centered strategies.[1,2] As a consequence of the increased interest in phenotypic drug discovery (PDD) the demand for follow-up target deconvolution has also increased. Although, knowing the target is not always a prerequisite for further development of a drug candidate, it is well accepted that most aspects of the future development would be easier and the risk associated with a project would be more comprehensible if the target is known.[3] Researchers having obtained hit compounds from PDD screening have several choices regarding the method they should use for deconvolution; each method comes with a range of pros and cons. Firstly, a decision on a direct or indirect method must be made. Indirect methods are more suitable for deciphering the pathways that are associated with the compound's mode of action than finding the direct target(s). Indirect methods can assess the trace that a compound leaves in a biological system, for example by cell painting,[4] changes in gene expression, or how the phenotype (as observed in the PDD screen) is altered as a consequence of specific gene expression alterations (typically RNAi treatment or CRISPR/Cas9 engineering).[5] On the other hand, direct methods are able to identify the target by occupancy or target engagement directly, *i.e.* the compound – target interaction is directly assessed. Mass spectrometry (MS) coupled with liquid chromatography (LC-MS) is an integral part of target deconvolution by direct methods as it allows for an unbiased investigation of isolated proteins. If a direct method is preferred, the researchers now have to decide if they want to pursue a technique that requires modification of their compounds and/or targets, or if they would like to pursue a label-free method.

1.1. Labeled methods

Labeled methods are typically comprised of a pull-down assay where the hit compound is covalently conjugated to a matrix[6,7] or chemically modified such that the compound can be attached to the matrix, *i.e.* a chemical tag such as biotin.[8] The conjugated probe is then used to fish out proteins that bind and subject them to MS analysis.[9] The success of this strategy invariably depends that the compound's activity and affinity is not affected by the modifications of the hit compound as well as the sample matrix. This is a major drawback since the derivatization requires significant efforts to achieve.[10] On the other hand, enrichment of target proteins is an inherent feature of the method,

Abbreviations: LC-MS, liquid chromatography mass spectrometry; PAL, photo affinity labeling; ABP, activity-based probes; DARTS, drug affinity-responsive target stability; SPROX, stability of proteins from rates of oxidation; TSA, thermal shift assay

* Address: Pelago Bioscience AB, 171 48 Solna, Sweden.
E-mail address: tomas@pelagobio.com.

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which can be beneficial for low abundance targets. Photo affinity labeling (PAL) and activity-based probes (ABP) are two other types of labeled methods where hit compounds are equipped with chemical groups that allow covalent binding to bound target proteins upon exposure to light of certain wavelengths (in the case of PAL)[11] or exploitation of the target protein's own enzymatic activity (in the case of ABP).[12]

1.2. Non-labeled methods

Non-labeled methods do not require derivatization of the hit compound, which affords faster transition from hit generation to target deconvolution. Non-labeled methods are reliant on compounds altering the biophysical characteristics of the target protein(s); such as thermal stability, susceptibility to proteolytic degradation or oxidation. All such methods can be combined with either a targeted protein quantification step or LC-MS for multiple protein quantification. The proteolytic methods exploit the fact that protein targets can display an altered sensitivity to proteolytic degradation upon binding of a small molecule ligand. There are several variants of this concept with DARTS (Drug affinity-responsive target stability)[13] being the original protocol that has been developed further into limited proteolysis[14] and pulse proteolysis.[15] The latter differs from the former two in the way that proteolysis is assessed as a function of a gradient of denaturant, where higher denaturant concentration will result in more extensive proteolysis. The protein degradation curve (as a function of the denaturant gradient) will be shifted if a compound has engaged a protein target.[15] In SPROX (Stability of proteins from rates of oxidation) denaturants are used in a similar fashion as in pulse proteolysis, but susceptibility to oxidation in presence of ligand is measured instead of proteolysis.[16] The Cellular thermal shift assay followed by MS (CETSA MS) or thermal proteome profiling (TPP - as the method is also referred to) is the latest addition to the tool-box for label free target deconvolution. Instead of applying proteases, oxidizing agents and denaturants, applied heat is used to stress the system.[17] In contrast to the other non-labeled methods, CETSA can be applied to assess target engagement in live cells and tissues in addition to cell lysates and other types of protein suspensions, e.g. serum or plasma.[18] This mini-review will describe the qualities of CETSA MS for target deconvolution in conjunction with PDD.

2. Cellular thermal shift assay (CETSA)

CETSA is based on the same principle as conventional thermal shift assays (TSA), in that proteins that are engaged by a small molecule compound display an altered melting or aggregation behavior upon exposure to increased temperature. However, in contrast to traditional TSAs, that are carried out in highly purified and isolated systems monitoring a single protein species, CETSA can be performed in complex protein samples and in live cells. This realization was first published in 2013 by researchers from Karolinska Institutet, Sweden and Nanyang Technological University, Singapore.[17] The format offers a physiologically relevant environment and due to the generally applicable principle of protein melting it also enables investigation of a large part of the proteome.[19] Protein melt curves (amount of soluble protein as a function of temperature) can show a shift in either direction (stabilization or destabilization) upon compound binding (Fig. 1A). A melt curve shift can only tell if there has been a compound target interaction at the assayed concentration, but does not report on the potency of the compound. However, relative potencies can be determined by lead of a melt curve experiment, as it can be used to determine a temperature at which Concentration – Response (CR; also referred to as isothermal dose response (ITDR)) experiments can be performed. A temperature that gives a large amplitude shift in the melt curve is used to assay samples treated with a dilution series of the compound. The resulting CR curve enables determination of relative compound potencies between several compounds that engage the target protein[17] (Fig. 1B).

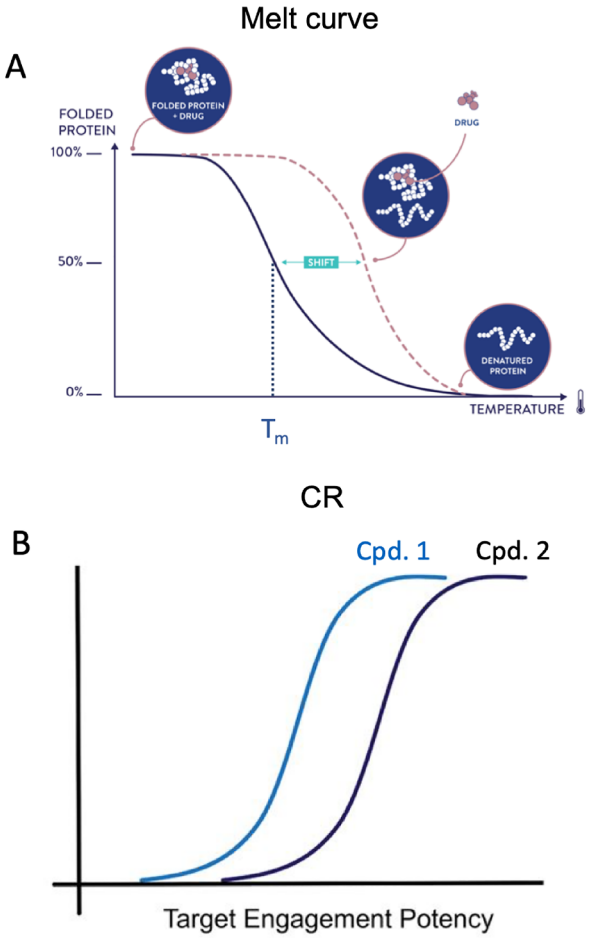

Fig. 1. The CETSA method. (A) A protein melt curve. T_m is the temperature where half the amount of protein remains. There is a shift in the melt curve if a compound that binds the protein is present at high enough concentration. (B) Concentration – Response (CR) curves. Different compounds (Cpd.) that bind the same target protein can be ranked relative to each other regarding their potency in a CR experiment. A single screening temperature (usually close to the T_m) is used to heat cells exposed to a dilution series of compound.

3. CETSA MS methods

There are both targeted and non-targeted formats of CETSA, where the targeted approaches are typically based on antibody detection for quantification of protein amounts and the non-targeted format uses LC-MS readout.[19,20] CETSA in the MS format (CETSA MS) can be performed according to different project scopes and demands. Protein identification after the heat shock can be studied as a function of temperature, drug concentration and incubation time or combinations thereof as the variables. In a melt curve experiment, samples are incubated with a saturating concentration of compound before being subjected to a temperature gradient. The samples are then centrifuged and the supernatant (soluble fraction) is subjected to tryptic digestion and typically Tandem Mass Tag (TMT) labeling to enable multiplexing of the conditions (temperature, concentration and time).[21] This approach enables detection of proteins that are more or less heat stable in the presence of compound (Fig. 2A). Again, the observed thermal shift is qualitative and does not reflect affinity; it demonstrates whether the compound affects the protein or not. In contrast, a concentration – response experiment, for example a 2D CETSA MS, requires that samples are treated with a dilution series of compound and that each series is exposed to all temperatures in the temperature gradient.[19] This is an

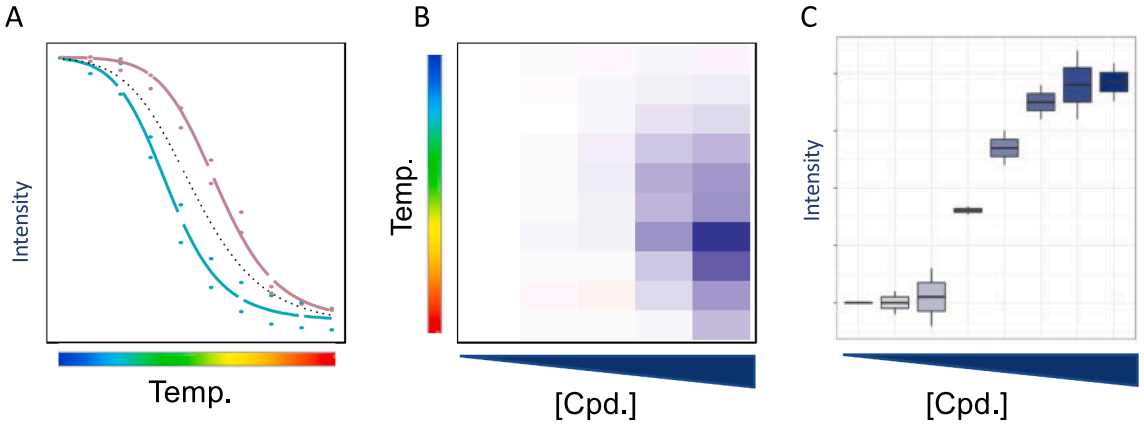

Fig. 2. Different formats of CETSA MS. (A) Melt curve of each protein in the assessed proteome. If a compound has affected a target protein, a melt curve shift will be observed. (B) 2D CETSA MS results presented as a heat map for each protein. Each row is a CR (5 concentration points) at a single temperature. Darker blue color indicates a stabilization of the protein relative the vehicle control (leftmost column). (C) CETSA compressed. Each concentration is comprised of a range of pooled temperatures and can be presented as a concentration response curve for each protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effective way of exploiting the CETSA method since what is practically achieved is a melt curve at each concentration or alternatively it can be viewed as an CR at each temperature (Fig. 2B). Consequently, determining target engagement is not just limited to observed shifts in a melt curve, but also a change in protein amount (fold change) at a select temperature as a function of increasing concentration of compound. These concentration – response data are typically easier to interpret than a potentially small shift in a protein T_m.[19] This format also allows for clustering of heat maps, which also can inform which proteins are part of protein complexes, similar to what was shown by Tan et al.[22] Recently, the CETSA MS format has been developed further into a compressed format in which all temperature points stemming from a certain condition have been pooled after the heat treatment step, *i.e.* the heatmap (Fig. 2B) has been *compressed* into a concentration – response curve (Fig. 2C). This approach allows for use of less sample material and shorter running times on the MS instrument, whereas most of the information from a 2D experiment is maintained.[23] However, the pooled temperature samples in the compressed CETSA MS format will not detect if there have been changes in expression or degradation that affect protein abundance, *i.e.* non CETSA effects. This can in turn easily be controlled for by including non-heated control samples with and without the compound into the experimental design. Another important issue to address with regards to experimental design is what sample matrix that is going to be used; intact cells/tissues or lysates thereof. The biology is *on* in the intact format since proteins reside in their native cellular compartments and more elaborate protein complexes are maintained. On the other hand, biology is *off* in the lysate formats since proteins are displaced from their native compartments and protein complexes are to a large extent disrupted. However, direct ligand – protein interactions are still present in lysate, which makes this format more suitable for identifying direct binders, whereas the intact cell format can inform on both direct ligand – protein interactions as well as pathway effects.[22] Preferably, both sample matrices should be used in parallel in order to distinguish between direct and indirect effects. Also, some ligand – target interactions are only observed in either sample matrix,[24,25] which can be observed for proteins that reside in large protein complexes.[22] The aforementioned pathway effects that can be picked up by CETSA MS protocols are due to compounds that indirectly affect thermal stability of proteins. These alterations, which often reflect changes in the functional state of the protein can be due to changes in protein – protein/DNA/RNA/lipid[26] interactions, posttranslational modifications,[27] and levels of small metabolites.[28]

4. Examples of target deconvolution with CETSA MS

To date CETSA MS and variants thereof[18,29–31] have been used in several studies for target deconvolution, which have both confirmed previously known compound – target interactions and discovered new ones (comprehensively reviewed in Dai et al., 2019).[26] Prominent examples of studies where CETSA MS has been used to deconvolute target engagement include profiling of the HDAC inhibitor panobinostat,[19] the identification of ECM29 as a biomarker for the CDK4/6 inhibitor palbociclib,[32] and the identification of P. *falciparum* purine nucleoside phosphorylase as the target for the antimalarial drugs quinine and mefloquine.[25]

In the case of panobinostat, both intact cells and cell lysates were treated and thereafter subjected to 2D CETSA MS analysis. The intact cell sample matrix yielded more hits (23 proteins showed dose-dependent thermal stability shifts) compared to the lysate (5 proteins had changed thermal stability), which is consistent with that more downstream/indirect effects are observed in intact cells; *biology is on*. However, the hits obtained in lysates are more likely to be direct binders. In this study, Mikhail Savitski and co-workers found that phenylalanine hydroxylase (PAH) was thermally stabilized in addition to panobinostat's known targets HDAC1/2/6. Binding and inhibition of PAH by panobinostat was confirmed with a labeled method (sepharose conjugated panobinostat) and an enzymatic assay, respectively. This pivotal work is a good example of how CETSA MS can aid researchers explore liabilities of their candidate compounds, as inhibition of PAH correlates well with some of the side effects associated with panobinostat, but not with other structurally unrelated HDAC inhibitors.[19] As mentioned, CETSA MS applied to intact cells gives the possibility to investigate a compound's effect on cell biology processes. Miettinen et al showed in a CETSA MS study employing melt curves that the CDK4/6 inhibitor palbociclib induced thermal stabilization of the 20S subunit of the proteasome in addition to several kinases, including CDK4 and 6.[32] By lead of the effect on the proteasome, Miettinen and co-workers identified ECM29 as a protein regulated by palbociclib that in turn regulated proteasome activity. De-regulation of ECM29 by palbociclib resulted in increased proteasomal activity, which in turn explained anti-neoplastic effects independent of CDK4/6 and other kinase targets of palbociclib. This study shows that MS CETSA is a powerful tool not just to prove direct target engagement, but also to identify changes in cellular processes, and thereby gaining more insight into the characteristics of a phenotype. Moreover, in this case mRNA levels of ECM29 was shown to have a negative correlation with survival in HER2 positive breast cancer patients, inferring that ECM29 can be

used as a patient stratification biomarker.

Intact cell CETSA MS is not just limited to cells of the animal kingdom, target deconvolution has also been studied in bacteria[24] and protozoans. The protozoa in question was the malaria causing parasite *P. falciparum* and 2D CETSA MS was used to identify the target for two conventional drugs used to treat malaria; quinine and mefloquine. In this study led by the inventor of the CETSA method Pär Nordlund, *P. falciparum* parasites were isolated from infected erythrocytes and both intact parasites and parasite lysates were treated with compounds.[25] Both quinine and mefloquine were found to bind *P. falciparum* purine nucleoside phosphorylase (PfPNP) in lysates, whereas only quinine bound PfPNP in the intact cell setting. Once again showing that the intact cell setting includes more complex biology. However, both compounds were validated as direct PfPNP binders in subsequent differential scanning fluorimetry and surface plasmon resonance[25] experiments. This study is an example of where CETSA MS has been successfully applied for target deconvolution in a completely unbiased way.

5. Considerations with CETSA MS

Although CETSA MS has proved successful in many instances[26] and with no apparent false positives, there are caveats to the method. A small fraction of proteins display no shift upon compound binding, which can be attributed to the particular properties of the ligand – protein interaction. For instance, a melt curve shift is less likely when protein unfolding is determined by a protein domain that is not affected by the compound binding, or when the protein target is stabilized by interactions with another protein and this interaction in turn is unaffected by the binding of a small organic molecule. Some types of protein are inherently hard to shift, such as integral transmembrane proteins, yielding only small changes in thermal stability upon compound binding. In the targeted formats this can in general be overcome by appropriate optimization of the assay conditions (mainly the detergent of choice) for such individual targets.[33] Despite this, CETSA MS quantification of target engagement has been reported for several membrane proteins.[34] The majority of proteins melt within the standard assay temperature range (40–75 °C). However, there are a few examples of proteins that require an extended temperature range, e.g. albumin and superoxide dismutase 1 (SOD1). On the other hand, such proteins are valuable as loading controls when assessing other proteins that do melt within the standard range.[32]

6. Future perspectives

The CETSA technology platform is an evolving method format that has gone through fast development; from being based primarily on immunological methods focusing on individual target engagement to having a large range of un-biased whole proteome profiling applications with LC-MS readout. CETSA MS has been adopted in several published studies for target deconvolution in both intact cells and lysates. Furthermore, the technique is increasingly implemented in the pharmaceutical industry for both compound profiling and target deconvolution. Companies with a license to use CETSA such as AstraZeneca, Merck, MSD, Pfizer, Sanofi and Vertex are prominent adopters of the methods described. Among these, many proof of concept publications on CETSA MS have been authored by researchers from GlaxoSmithKline, which is one of the leading innovators in the field of target engagement studies and in particular with CETSA MS.[19–21,35] CETSA MS has several benefits compared to other available methods for target deconvolution: no requirement for modification of the test compound, compatibility with intact cells and tissues, and that a large portion of the proteome can be assessed with this method.[35] Moreover, a CETSA assay can be used along the whole drug development chain: from target deconvolution to monitoring target engagement in patients.

The future development of CETSA MS is in striving to incorporate more complex samples, but at the same time use less material. Thus, enabling assays in precious, but more relevant material, such as patient biopsies. Also, the future advancement of CETSA MS is closely related to the general advancement of MS methods, which would likely allow for faster and more cost-efficient processing due to the introduction of more advanced multiplexing.

As is abundantly clear, one method is rarely enough information to confidently deconvolute a hit compound's target, researchers must also be ready to use a multi-disciplined approach for follow up target validation. The investment in deconvoluting the primary efficacy targets and liability targets of phenotypically identified compounds, may seem significant in the early phases of a PDD program. However, the return of this investment is certain to far outweigh the initial expenditures as the results will allow better prioritization and de-risking of hits for further development into novel medicines.

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Declaration of Competing Interest

Tomas Friman is a full-time employee of Pelago Bioscience AB, which holds exclusive patent rights for the commercial use of the CETSA method

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Tomas Friman was born in Stockholm 1980, and performed his undergraduate and graduate studies at Uppsala University (Uppsala, Sweden) where he obtained his PhD in medical sciences 2010. Dr. Friman's research interests has mainly been focused on tumor biology and the drug distribution problem in solid tumors. Since 2017, Dr. Friman is working for the R&D department at Pelago Bioscience AB in Solna, Sweden.