The Cellular Thermal Shift Assay: A Novel Biophysical Assay for In Situ Drug Target Engagement and Mechanistic Biomarker Studies

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Abstract

A drug must engage its intended target to achieve its therapeutic effect. However, conclusively measuring target engagement (TE) in situ is challenging. This complicates preclinical development and is considered a key factor in the high rate of attrition in clinical trials. Here, we discuss a recently developed, label-free, biophysical assay, the cellular thermal shift assay (CETSA), which facilitates the direct assessment of TE in cells and tissues at various stages of drug development. CETSA also reveals biochemical events downstream of drug binding and therefore provides a promising means of establishing mechanistic biomarkers. The implementation of proteome-wide CETSA using quantitative mass spectrometry represents a novel strategy for defining off-target toxicity and polypharmacology and for identifying downstream mechanistic biomarkers. The first year of CETSA applications in the literature has focused on TE studies in cell culture systems and has confirmed the broad applicability of CETSA to many different target families. The next phase of CETSA applications will likely encompass comprehensive animal and patient studies, and CETSA will likely serve as a very valuable tool in many stages of preclinical and clinical drug development.

INTRODUCTION

TE: target engagement

CETSA: cellular thermal shift assay

TSA: thermal shift assay

A key concept in targeted therapies is target engagement (TE): the extent to which a drug binds to its target protein in situ. TE in targeted therapies should be optimal in the target cells and tissues but should be minimal in other cells and tissues that are prone to adverse effects. However, conclusive measurements of TE in situ are difficult to obtain (1–3), and the limitations in accessing TE are important factors contributing to the relatively poor success rates of clinical drug development (4, 5). As an illustration of this problem, several drugs have failed in advanced clinical trials (6–8) and have subsequently been shown to not act on the predicted drug target within cells (9).

Ideally, TE should be monitored and controlled from initial hit generation through preclinical and clinical development. Currently, estimates of TE are often based on indirect close-to-target downstream pharmacodynamic effects, which are shown, or are assumed, to correlate with the binding of the drug to its target (2, 10). Although the measurement of such effects can be a good correlate to TE, many downstream processes are complex, and the interpretation of these data is complicated by interconnecting pathways. An alternative to measuring TE is the establishment of affinity-based assays either using a drug candidate as a direct affinity probe or using a secondary affinity probe in a competitive assay (3). Chemoproteomics-based affinity assays of lysates have been valuable for defining the selectivity of drugs within specific protein families, such as kinases, methyltransferases, and deacetylases (11). Affinity probes can also be applied to measure TE in cells, although these measurements are less direct when they depend on intermediate lysate steps or chemically altered ligands (3). The indirect nature of many TE assays, and the relatively high cost of establishing useful affinity probes (3), stresses the need for more direct and versatile TE assays applicable to a wider range of drug targets and cell systems.

THE CELLULAR THERMAL SHIFT ASSAY

In July 2013, we published the first report on the cellular thermal shift assay (CETSA) (12), a broadly applicable strategy for studies of TE in cells and tissues. The assay is based on the concept that ligand binding affects protein stability (13–15). Thermally induced unfolding experiments provide distinct melting curves for a protein, and these melting curves typically shift to a higher temperature when a ligand binds to and stabilizes the protein (**Figure 1**).

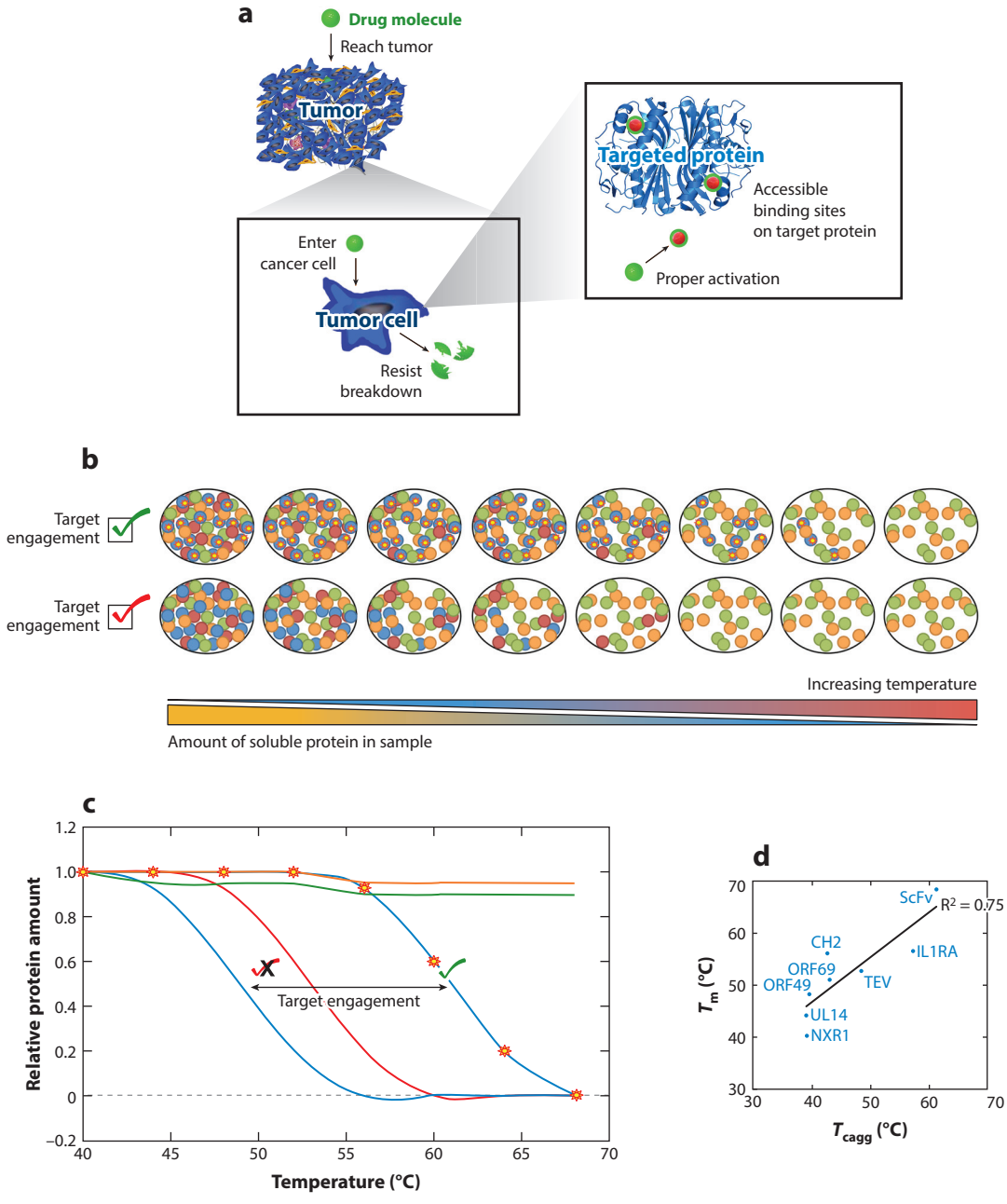
Thermal Shift Assays of Purified Proteins

Thermal shift assays (TSAs) have been explored extensively in studies of ligand binding to purified proteins. The thermally induced unfolding of a protein often shows a sigmoidal melting curve,

Figure 1

(*a*) Processes affecting target engagement of a cancer drug. Once the drug has been absorbed and distributed in the body, it needs to be present in a sufficient concentration at the tumor site, enter the tumor cells, and, once inside the cell, find and bind the intended target in a responsive form. (*b*) Illustration of the principle of CETSA, in which protein unfolding is coupled to concomitant precipitation in cells. Here, we follow four proteins (*blue*, *orange*, *green*, and *red*), where the blue protein is the protein target of the added compound; thus, its stability shifts upon compound binding, as observed both in the schematic figure of the cells and in the hypothetical melting curves in panel *c*. The green and orange proteins are highly stable and do not precipitate in this temperature range. (*c*) CETSA is based on the same physical principles as thermal melting curve–based assays used to study ligand binding to purified proteins. (*d*) Comparing the T_{cagg} of eight different proteins overexpressed in *Escherichia coli* cells with the T_m of the corresponding purified proteins showed a strong correlation, supporting the hypothesis that CETSA indeed measures protein unfolding in cells. Figure adapted with permission from Reference 27. Abbreviations: CETSA, cellular thermal shift assay; T_{cagg}, CETSA-based melting temperature; T_m, melting temperature.

from which a characteristic melting temperature (T_m) can be derived. When a protein binds to a ligand, the T_m is typically shifted to a higher temperature, producing a thermal shift (ΔT_m). This is the principle of TSAs, and this well-established method has demonstrated substantial impact and utility for the investigation of purified proteins in drug discovery, mechanistic biochemistry, and structural biology (16–18). The most popular TSA technique for analyzing purified proteins is differential scanning fluorimetry (DSF), or ThermoFluor, in which a fluoroprobe recognizes the



PPI: protein-protein interaction

exposed hydrophobic patches of unfolded proteins (18). An alternative TSA method is differential static light scattering, or Stargazer, which exploits the fact that many proteins rapidly aggregate after they unfold (17). Light scattering is used to quantify the amount of precipitated protein, which correlates with the extent of protein unfolding. TSAs implemented in multiwell formats are now used extensively in both academia and industry to study protein stability and ligand binding. TSAs enable the examination of a wide range of interactions in addition to protein-drug interactions, such as protein-protein interactions (PPIs) (19) and protein–nucleic acid (20) and protein-metabolite interactions (17). Furthermore, TSAs have been shown to be useful for assessing ligand binding to detergent-solubilized membrane proteins (21, 22). In many cases, TSA measurements correlate well with binding constants measured using other methods (23, 24), and it is likely that the more similar the binding mode and chemical properties of the examined compounds, the stronger this correlation becomes (25). However, different proteins display different slopes in this correlation; that is, a 5° shift in T_m represents a different ligand affinity in different proteins. Generally, ligand-binding studies using TSAs, as for other biophysical methods, are considered to produce false positive signals rarely (see below). However, some proteins do not respond well in TSAs, showing no or only small shifts in T_m upon ligand binding. Researchers anticipate that in general, larger and more complex proteins exhibit smaller T_m shifts upon ligand binding. For most applications, however, the thermal shift information is used as a correlative measurement. For cases in which the protein follows a thermodynamic two-state equilibrium unfolding model, binding constants can be determined directly from TSA measurements using dose-response studies (26). However, many proteins do not follow this model—for example, when the protein rapidly precipitates after unfolding.

The CETSA Experiment

CETSA (12) is based on our discovery that when heated in cells, many proteins apparently unfold and precipitate in a similar manner as do purified proteins. Thus, heat-induced unfolding leads to rapid precipitation, and the amount of remaining soluble protein in the cell correlates with the amount of protein that remains folded. After the heating step, the remaining soluble protein is isolated and quantified, and plotting the amount of soluble protein against the temperature provides the CETSA melting curve (**Figure 1*b***). The subsequent addition of a ligand results in TSA-like shifts in the melting curve. Comparison of the CETSA melting points for eight human and herpes proteins overexpressed in *Escherichia coli* cells showed good correlation with the corresponding melting points of the purified proteins determined via DSF (**Figure 1*c***) (27). This result supports the notion that melting curves generated using CETSA reflect a physical process similar to that of melting curves generated for purified proteins, i.e., that CETSA is a biophysical measurement in the cell. This result also supports the notion that unfolding and subsequent precipitation in the cellular environment are relatively independent of other molecular processes in the cell, except, of course, direct physical interactions between ligands and the protein (**Figure 1*b***). This phenomenon may have been difficult to predict before these experiments owing to the complexity of the cellular environment, the very high level of molecular crowding in cells (28), and the presence of several systems that function to chaperone unfolded proteins (29). One could speculate that evolutionary reasons may even explain why proteins rapidly precipitate after unfolding, as this minimizes potential deleterious interferences with other processes in the cell.

As indicated above, the experimental procedure used to generate CETSA data involves three principal steps after the sample (typically treated or control) has been collected (**Figure 2*a***). The CETSA method is applicable to any complex mixture containing proteins, including lysates, cells, and tissue samples.

The first is a temperature exposure step, a short heating step of lysates, cells or tissue samples at various temperatures. Cell membranes and overall cell structure remain intact under typical conditions at temperatures of up to 60–65°C. Temperature exposure steps of 3 min have been used in most of the initial CETSA studies, but for many proteins, this step can be shortened markedly to less than 30 sec and still retain prominent signals.

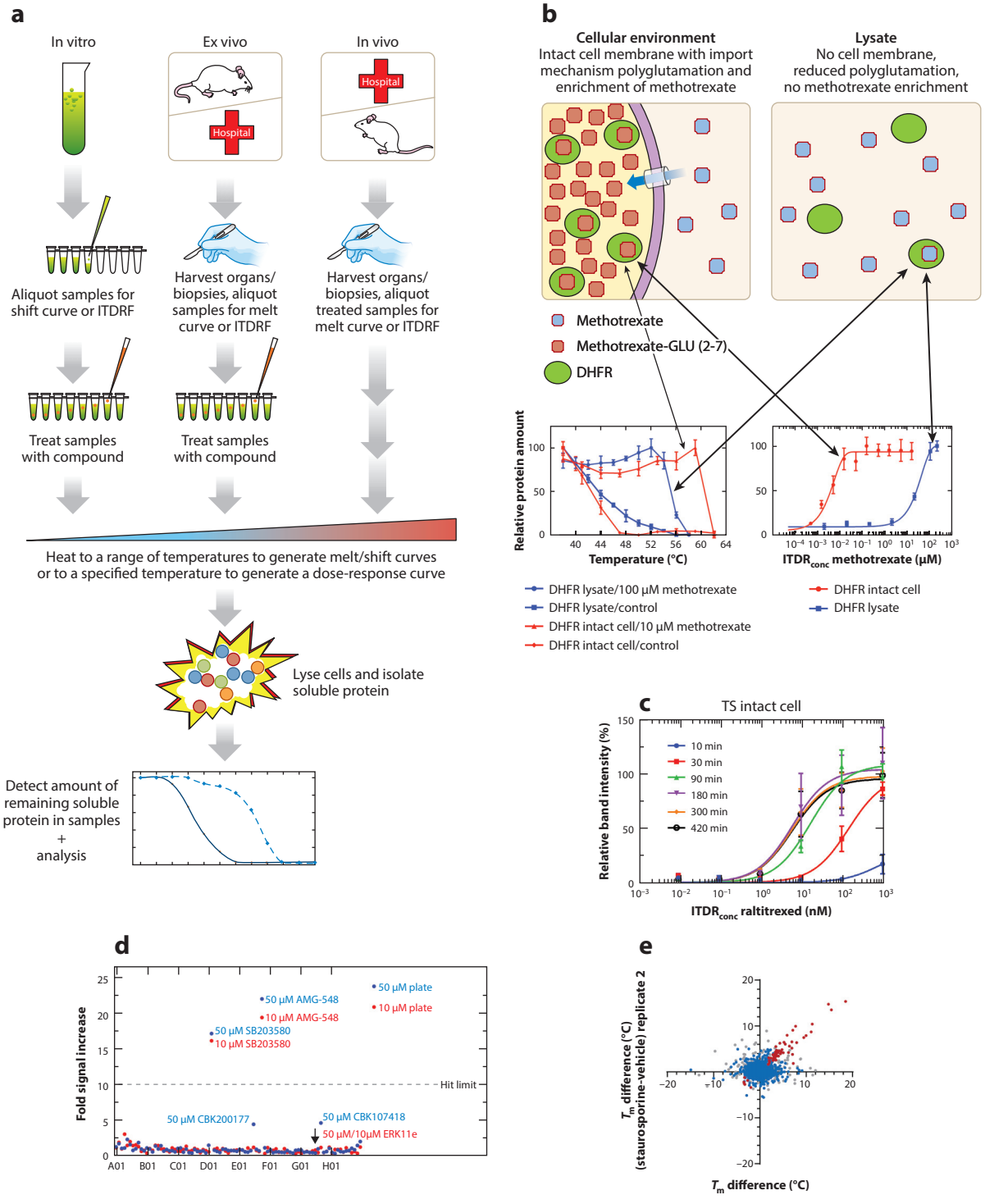
MS: mass spectrometry

In the next step, the soluble fraction of proteins is isolated. The isolated proteins are predominantly those that remained folded after the heating step. Cells and tissues require lysis; subsequently, the isolation of the soluble fraction from the lysates is typically accomplished by centrifugation or filtration.

Finally, the protein quantification step involves the quantification of the remaining soluble proteins. This can be achieved via immunoassays such as Western blots, ELISAs, or proximity ligation assays (30) using specific target antibodies, or alternatively via mass spectrometry (MS), as further discussed below.

In the first proof-of-principle study of CETSA (12), we used human cell lines and mouse models to demonstrate that CETSA is a distinct and potentially broadly applicable assay for measurements of drug TE in lysates, cells, and tissues. We established CETSA based on the Western blot–mediated detection of 10 different drug targets from several different protein families and demonstrated that significant and informative thermal shifts induced by drug binding can be measured. Analogous to TSA for purified proteins, CETSA gives melting curve shifts that confirm drug binding, and, at saturating drug concentrations, these shifts can be used to directly rank the relative affinity of compounds to the physiologically relevant form of the protein in the cell. To relate the CETSA measurements to target occupancy, we introduced a dose-response measurement performed at a constant temperature: the isothermal dose-response fingerprint ($ITDRF_{CETSA}$). We used $ITDRF_{CETSA}$ as a relative measurement of concentration-dependent TE. $ITDRF_{CETSA}$ often correlates well with the IC_{50} values measured via other methods (see below). However, in some cases, the absolute value of $ITDRF_{CETSA}$ depends on the temperature used for the isotherm; therefore, we used $ITDRF_{CETSA}$ primarily as a relative measurement for $ITDRF_{CETSA}$ performed at the same temperature in this study (12). A better understanding of the circumstances in which the absolute value of $ITDRF_{CETSA}$ shows a temperature dependence will clearly be valuable, and appropriate mathematical models may allow the direct derivation of absolute target occupancy at specific drug concentrations based on $ITDRF_{CETSA}$ measurements.

One key aspect of the CETSA assays is that they are easily translated between different cell and tissue types. Because of the intrinsic protein-centered nature of the assay, i.e., it is relatively independent of other events in the cell, comparison of CETSA data between different cell and tissue types can be valuable and allow the rapid estimation of relative TE in cells. Additionally, comparison of CETSA experiments between lysates and cells could be very informative and could, for example, shed light on whether the protein is in the same functional form in the two examined systems. We have observed cases in which the protein displays rather different melting profiles and drug responses in cells compared with their lysates. In specific cases, this discrepancy can be corrected for by adding a cofactor that has been diluted in the lysates. For cases in which the protein is likely to be in a similar form in cells and lysates, the comparison of $ITDRF_{CETSA}$ between lysates and cells provides a valuable strategy to rapidly dissect aspects of cell transport and metabolism. This concept is illustrated in **Figure 2*b*** for dihydrofolate reductase, in which the TE for the antifolate methotrexate is shown both as a CETSA curve shift at a high drug concentration and as $ITDRF_{CETSA}$ (12). The drug effects on the melting curves are similar between the two methods, supporting the hypothesis that the proteins are in a similar functional form. Comparisons of $ITDRF_{CETSA}$ between lysates and cells make it clear that the drug is dramatically concentrated in cells owing to its efficient transport and activation by polyglutamation; the latter also blocks the


a
In vitro
Ex vivo
In vivo
Hospital
Hospital
Hospital
Aliquot samples for shift curve or ITDRF
Harvest organs/ biopsies, aliquot samples for melt curve or ITDRF
Harvest organs/ biopsies, aliquot treated samples for melt curve or ITDRF
Treat samples with compound
Treat samples with compound
Heat to a range of temperatures to generate melt/shift curves or to a specified temperature to generate a dose-response curve
Lyse cells and isolate soluble protein
Detect amount of remaining soluble protein in samples + analysis
b
Cellular environment
Intact cell membrane with import mechanism polyglutamation and enrichment of methotrexate
Lysate
No cell membrane, reduced polyglutamation, no methotrexate enrichment
Methotrexate
Methotrexate-GLU (2-7)
DHFR
Relative protein amount
Temperature (°C)
ITDRconc methotrexate (μM)
DHFR lysate/100 μM methotrexate
DHFR lysate/control
DHFR intact cell/10 μM methotrexate
DHFR intact cell/control
DHFR intact cell
DHFR lysate
c
TS intact cell
Relative band intensity (%)
10 min
30 min
90 min
180 min
300 min
420 min
ITDRconc raltitrexed (nM)
d
Fold signal increase
50 μM plate
10 μM plate
50 μM AMG-548
10 μM AMG-548
50 μM SB203580
10 μM SB203580
Hit limit
50 μM CBK200177
50 μM CBK107418
50 μM/10μM ERK11e
A01
B01
C01
D01
E01
F01
G01
H01
e
Tm difference (°C) (staurosporine-vehicle) replicate 2
Tm difference (°C)

export of the drug from the cell. Additionally, the time frames of drug transport and metabolism can now be accessed directly in time-dependent CETSA TE experiments (**Figure 2*c***). The active import and polyglutamation-mediated activation of antimetabolites such as methotrexate not only are important for their activity but also constitute processes that are typically modified in drug-resistant cancer cells (31). This suggests that CETSA will provide a novel tool to identify and characterize such resistance mechanisms.

For some experiments, compact high-throughput CETSA formats are likely to be very valuable. In the development of a targeted therapy, CETSA should provide an ideal readout in a high-throughput assay, as it can access TE in a physiologically relevant cellular environment. As an alternative method to the three-step CETSA experiment procedure discussed above, the specific immunodetection of the folded form of proteins can be established, thereby abolishing the soluble protein isolation step (32). For example, using an AlphaScreen-based assay (33) that includes two different antibodies that recognize distinct epitopes on the same folded protein, we showed that after the heating step, CETSA data can be generated directly using this homogenous assay (32). Using isothermal conditions provides an efficient method for performing high-throughput screening for direct cellular TE in a physiologically relevant context (**Figure 2*d***) (32). The direct detection of the folded form of a protein may also be a means of establishing CETSA assays for membrane proteins, which contain extramembrane domains that are likely to unfold similarly to soluble proteins (34). However, the extent to which membrane proteins can be examined via CETSA remains to be determined.

Proteome-Wide Mass Spectrometry–CETSA

We (32, 35, 36) and several other groups realized early on that the CETSA experiment could potentially be implemented using quantitative MS as a detection method in a proteome-wide experiment. In principle, such MS-CETSA experiments should enable parallel binding studies of drugs to many proteins in the lysate, cell, or tissue context. In the first published report, MS-CETSA data generated by a team at Cellzome GmbH/GlaxoSmithKline, complemented by some

Figure 2

(*a*) CETSA melting curve and ITDRF CETSA procedures using samples of various origins: cultured cells or animal or patient material both in vivo and ex vivo. The different steps are the same for all sample types. First, treat (aliquoted) cells with a compound or vehicle, and heat the cells to a range of temperatures or a specified temperature (for dose-response curve generation). Lyse the cells and isolate soluble protein. Finally, detect the amounts of soluble protein via one of several means, such as Western blot or, for large numbers of proteins, proteome-wide mass spectrometry. (*b*) Illustrative examples of CETSA melting curves and ITDRFs. When intact cells are assessed via CETSA, factors contributing to target engagement, such as membrane transport of the compound, accumulation, and specific activation events, are detectable and distinguishable from the lysate results. DHFR and the drug methotrexate serve as examples. Methotrexate is imported by cells via specific transport mechanisms and is subsequently polyglutamated in the cell; this modification hinders it from exiting the cell, resulting in its accumulation and apparently increased concentration. In contrast, in a cellular extract (*right*), no accumulation occurs, and methotrexate and DHFR exist in a more dilute environment and are less likely to engage with each other. (*c*) A similar example as in panel *b*, here using dose-response curves for TS and raltitrexed to study drug uptake and the time dependence of the productive binding between the two compounds. An exposure of more than 90 min is required to obtain a stable dose-response curve. Panel *c* adapted with permission from Reference 12. (*d*) A small screening campaign using AlphaScreen technology to select binders of p38α was performed in a high-throughput manner. In this example, two known binders of p38α were included in the compound plate and yielded significant increases in AlphaScreen signal. Panel *d* adapted with permission from Reference 32. (*e*) CETSA with mass-spectrometry readout on staurosporine-treated samples identifies induced T_m shifts for more than 50 kinases. Shown here is a scatter plot of the T_m shifts from replicate experiments; red dots indicate shifts that passed the significance criteria. Panel *e* adapted with permission from Reference 37. Abbreviations: CETSA, cellular thermal shift assay; DHFR, dihydrofolate reductase; $ITDRF_{CETSA}$, CETSA isothermal dose-response fingerprint; T_m, melting temperature; TS, thymidylate synthase.

of our own mechanistic biomarker studies discussed below (37), showed the feasibility of this approach and allowed several interesting conclusions to be drawn. The MS-CETSA work in this study used the tandem mass tag (TMT)-based isobaric labeling strategy (38), in which the relative quantification of proteins from typically 8–10 different temperatures or drug concentrations are made in parallel. Overall, the data from this approach are of high quality, and some 80% of the proteins showed highly sigmoidal melting curves. In our own laboratory, we routinely apply a slightly modified TMT-based strategy and detect 6,000–7,500 proteins in the full temperature or dosage range in an MS-CETSA data set. We have also explored a SILAC-based strategy (39) that further improves data quality but requires a longer data collection time because a maximum of only three related data points can be collected in parallel.

The first published MS-CETSA study (37) focused on kinase inhibitors using affinity proteomics data obtained via the kinobead strategy as a reference (40). MS-CETSA thermal shift data on the pan-kinase inhibitor staurosporine in lysates identified more than 50 protein kinases that showed significant thermal shifts (**Figure 2*e***). Importantly, for proteins displaying highly sigmoidal melting curves, these results support the notion that the rate of false positives is very low. This finding is consistent with the results from TSAs on purified proteins, as discussed above. However, approximately 30% of the overlapping kinases observed in the kinobead experiment did not show significant shifts in the CETSA experiment. This suggests that some protein kinases are not responsive in TSAs. Another contributing factor may be that the kinobead experiment enriches the actively bound form of a kinase, whereas this form of the kinase is not enriched in the CETSA experiment and may be masked by other forms. However, there is potential to improve the CETSA experiment in this respect by monitoring, for example, specific phosphorylated forms of the kinase that are more likely to bind to the drug. Research suggests the drug binding profiles of kinase inhibitors in cells and lysates are quite different (41), and because MS-CETSA constitutes a more direct means of profiling drug interactions in cells and tissues, it is likely to complement the current affinity proteomics strategies nicely for studies of kinase inhibitors in cells.

The Cellzome MS-CETSA data also provide insight into the quantitative aspects of CETSA (37). Consistent with the results for purified proteins, these data support the notion that the ΔT_m measurements for each protein correlate well with its apparent affinities. In an isothermal dose-response MS-CETSA experiment performed at 53°C, $ITDRF_{CETSA}$ of staurosporine was measured in parallel for approximately 30 kinases, and the results showed good correlation with the EC_{50} values obtained from a kinobead assay. Therefore, an MS-CETSA $ITDRF_{CETSA}$ experiment provides a novel means of estimating ligand affinities at the proteome level, and this method should also be applicable to cell and tissue studies. In the kinase $ITDRF_{CETSA}$ measurements, the $ITDRF_{CETSA}$ values were shifted overall to slightly higher concentrations than the results obtained from the kinobead experiment. This can be explained by the softness of the $ITDRF_{CETSA}$ measurement for subsets of proteins, as discussed above.

The Cellzome MS-CETSA data (37) also revealed a novel off-target interaction of the BRAF inhibitor vemurafenib (42) and the Alk inhibitor alectinib (43) with ferrochelatase, the terminal enzyme in the biosynthesis of heme. Inactivating mutations in this enzyme are known to yield conditions such as erythropoietic protoporphyria, resulting in severe photosensitivity and liver damage (44). These are also typical adverse effects of vemurafenib (45). Taken together, the results of this study show that MS-CETSA provides a novel means to rapidly access proteome-ligand interactions, and this method is likely to be valuable not only in understanding off-target toxicity and polypharmacology but also in providing an alternative strategy for deorphanization of hits obtained from phenotypic screening.

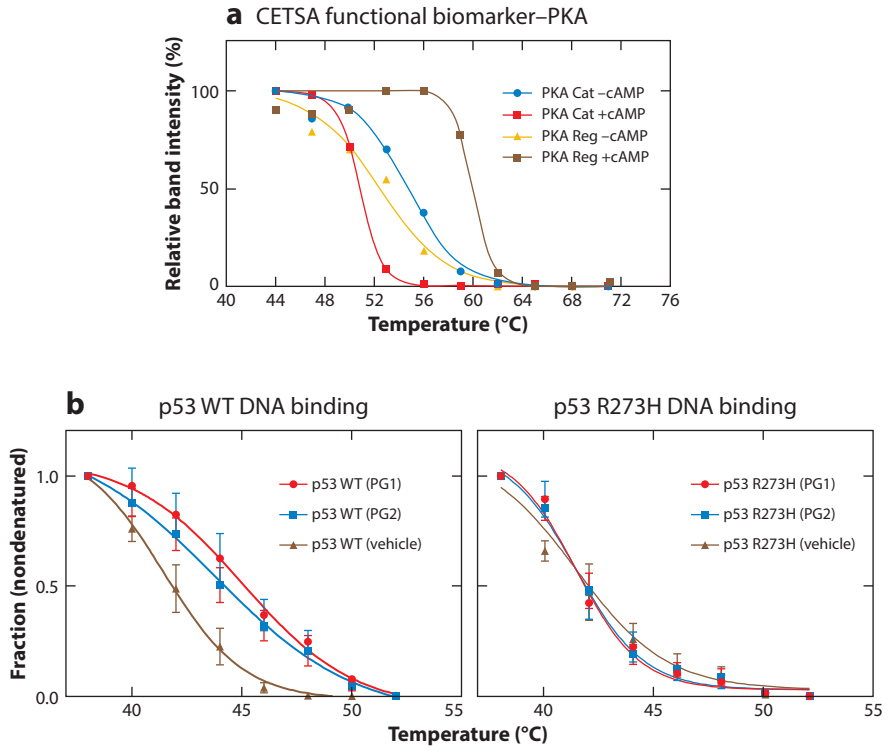


Figure 3

Examples of mechanistic CETSA biomarkers. (*a*) CETSA shifts resulting from cAMP binding to PKA. The binding of cAMP to the catalytic subunits (Cat) of PKA triggers the dissociation of the catalytic subunits from the regulatory subunits (Reg). As a consequence, the catalytic subunits become stabilized, whereas the regulatory subunits become destabilized, probably as a consequence of the lost interaction with the regulatory subunits. (*b*) CETSA analysis of DNA binding to WT and mutant (R273H) p53 in cellular extracts. The two cognate DNA oligonucleotides bind to and stabilize WT p53 but not the p53 mutant R273H, which is known to not bind to these DNA sequences. Figure adapted with permission from Reference 37. Abbreviations: cAMP, cyclic adenosine monophosphate; CETSA, cellular thermal shift assay; PKA, protein kinase A; WT, wild-type.

CETSA-BASED MECHANISTIC BIOMARKERS

CETSA-Mediated Measurements of Cell Biochemistry

Because CETSA is a general biophysical technique for detecting changes in the extent of ligand binding in cells, it is not surprising that this method also permits the detection of the binding of physiological ligands to proteins in cells and lysates. As an example, we have shown that CETSA can be used to monitor the direct binding of the second messenger cyclic AMP (cAMP) to protein kinase A (PKA) (**Figure 3*a***) (37). This is the pathway through which G protein–coupled receptors (GPCRs) signal via adenylate cyclase. When cAMP binds to the regulatory subunits of the inactive PKA complex, the catalytic subunits of the complex are released from the regulatory subunits, leading to their activation. In the CETSA experiment, the regulatory subunits of PKA are stabilized strongly by cAMP binding, whereas the catalytic subunits are destabilized when it is released from the regulatory subunits (**Figure 3*a***). Therefore, the CETSA shifts for PKA may serve as a

PKA: protein kinase A

mechanistic biomarker of adenylate cyclase–mediated GPCR signaling. Another example of a class of functional interactions that can be detected by CETSA is the binding of DNA to proteins. We showed that the binding of cognate oligonucleotide duplexes to the tumor suppressor p53 in lysates could be detected via CETSA (37, 46). Binding to cognate DNA significantly stabilized wild-type p53, whereas binding to noncognate DNA did not stabilize p53 (**Figure 3*b***). Furthermore, the R273H mutant of p53, which is known to lack binding affinity to cognate DNA, was not stabilized by DNA. p53 is the most frequently mutated protein in cancer, and hundreds of different mutations have been detected in this tumor suppressor, although the functional roles of many of these mutations remain uncharacterized (47, 48).

The proteome-wide MS-CETSA experiment provides a particularly exciting strategy for the discovery of mechanistic CETSA biomarkers. In the Cellzome study, two of the four most prominent CETSA thermal shifts caused by the inhibition of Bcr-Abl by dasatinib in K562 cells are two proteins involved in the downstream signaling of Bcr-Abl: CRKL and SHIP2 (49). These proteins act downstream of the kinase Bcr-Abl as signaling factors in the endocytosis and actin remodeling pathways, respectively (37). Both proteins show negative thermal shifts, which could be explained by the loss of their interaction with the Bcr-Abl complex (49). Interestingly, the T_m of CRKL in dasatinib-treated K562 cells is very similar to its T_m in normal Jurkat cells. This may be explained by the fact that Jurkat cells express a wild-type Abl tyrosine kinase that is in the same inactive signaling state as the constitutively activated Bcr-Abl in K562 cells when inhibited by dasatinib (37). The melting curve of CRKL therefore likely reflects the activation state of the protein (and the pathway) directly, and its melting point has an absolute meaning that may be translatable between different cell and tissue types.

Extrapolating from the results of these cell biochemistry–based CETSA experiments, changes in many different types of physiological interactions in the cell, including PPIs and protein-metabolite, protein-cofactor, protein-lipid, and protein–nucleic acid interactions, will plausibly produce informative CETSA signals in a physiologically relevant context. Importantly, the CETSA signals produced by key cellular events may be translated directly as mechanistic biomarkers for applications in animals and patients. The extent to which different protein types and families of the proteome will be accessible as mechanistic CETSA biomarkers is difficult to predict at this point.

CETSA IN MAMMALIAN TISSUES

In our initial report on CETSA (12), we performed a series of mouse experiments, demonstrating the feasibility of CETSA TE measurements in mammalian tissues. TNP-470 is an antiangiogenic drug currently in clinical development for the treatment of solid tumors (50). TNP-470 targets the protease Methionine aminopeptidase 2 (METAP2) (50), and in liver tissues from mice treated with TNP-470, strong drug binding–induced thermal shifts in METAP2 are observed. Relative $ITDRF_{CETSA}$ measurements in mouse liver and kidney also demonstrated that relevant TE and drug distribution in mice could be measured directly via CETSA (**Figure 4**). Notably, the mouse studies on TNP-470 TE to METAP2 may be considered special cases because METAP2 melts at temperatures above that at which cells typically lyse and because TNP-470 covalently binds to METAP2. Therefore, these TE measurements were performed in tissue lysates rather than in cells. However, subsequent evidence from our lab supports the notion that very good mouse CETSA data can be generated for reversibly binding drugs at temperatures at which cells are intact. Therefore, CETSA on animal tissues will likely provide a novel means of performing more highly resolved pharmacokinetic and absorption-distribution-metabolism-excretion (ADME) studies in animals, as both tissue distribution and effective TE are reflected in

ADME: absorption-distribution-metabolism-excretion

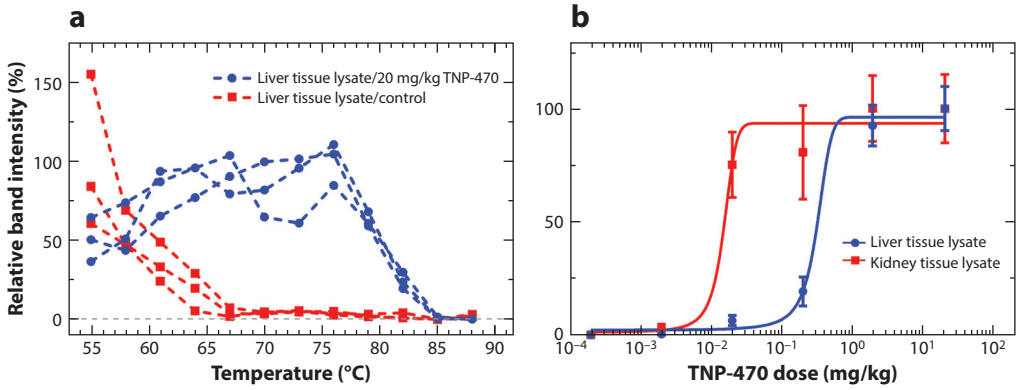


Figure 4

Monitoring of TNP-470 target engagement in tissue samples from in vivo treated mice. (*a*) CETSA melt and shift curves of METAP2 in mouse liver lysates displays a large stabilization of METAP2. (*b*) $ITDRF_{CETSA}$ of METAP2 in liver and kidney lysates at six different doses. Figure adapted with permission from Reference 12. Abbreviations: CETSA, cellular thermal shift assay; $ITDRF_{CETSA}$, CETSA isothermal dose-response fingerprint.

CETSA measurements. It should also be possible to monitor mechanistic CETSA biomarkers for downstream pharmacodynamic effects in parallel with TE in both target tissues and toxicity-prone tissues. This may be of interest because for many targets, relevant toxicity assays are difficult to establish in multiple tissues (51).

Similar to CETSA experiments on mice, CETSA experiments on human tissues are feasible, and CETSA may become a valuable future tool for monitoring TE during clinical development. The critical experimental step (heating and cooling) is well suited for the processing of clinical samples when the method is robust and readily standardized. However, the melting properties of some drug targets may not be appropriate for such assays, e.g., in cases in which the T_m is in the range in which the cell membrane ruptures. Still, many clinical drugs have relatively long off-rates. In our experience, this problem can be partly overcome by applying a temperature exposure step that is much shorter than the drug off-rate. As an alternative to the study of drug binding in tissues from treated patients, ex vivo experiments on fresh tissue samples exposed to the drug can be used to rapidly measure the local effects of drugs. Preliminary ex vivo experiments in our lab on tumor samples have demonstrated the feasibility of this approach. Such experiments may represent a useful strategy for rapidly examining several anticancer drugs in parallel, such as for the determination of innate and acquired drug resistance, as reflected by a decrease in TE.

THE FIRST YEAR OF CETSA

We published the first report on the CETSA method in *Science* in July 2013 (12), and the first original publications by other groups came in April 2014, with two back-to-back studies in *Nature* exploring CETSA to confirm TE to MTH1, an oncologic target that displays a synthetic lethality mechanism (52, 53). In the year since then, as of this writing, researchers have published more than 15 original papers in which CETSA has been used in different systems and applications (see **Table 1** for a summary of the most relevant studies). These studies examined more than 20 different protein targets, and most studies aimed to demonstrate TE by novel compounds in cells or lysates. Interestingly, none of these latter studies included protein kinases as targets, although, as discussed above, they are highly feasible for CETSA measurements. This could be explained

Table 1 Selected publications from the first year of CETSA adoption

Protein(s)	Sample type and method	Results	Comments	Reference
CDC20: activator of anaphase-promoting complex; WD40 domains	Lysates; ITDRF	TE in cells confirmed for apcin, a novel inhibitor of CDC20 that blocks the PPI of CDC20 with the anaphase-promoting complex	Performed in *Xenopus* egg extracts; monitored total protein levels to rule out general, nonspecific stabilization	70
Bcl-xL and Bcl-2: inhibitors of apoptosis	Cells; CETSA shifts and ITDRF	TE in cells confirmed for BM-1197, a novel inhibitor of PPI in Bcl-xL and Bcl-2, and comparison of TE between BM-1197 and the clinical candidate ABT-263	Actin used as a negative control	54
PARP-1: poly-ADP-ribose polymerase 1; BRCT domain	Cells; CETSA shifts	TE confirmed in cells for the natural product gossypol, a potential atypical inhibitor of the PPI between PARP-1 and the BRCT domain	Olaparib, a classical PARP-1 active-site inhibitor, used as a reference	67
MDM2: E3 ubiquitin protein ligase acting on, for example, p53	Cells; CETSA shifts	TE addressed in two cell lines of JapA, a tentative inhibitor of MDM2 PPI, although the data remain ambiguous	Stabilization surprisingly large for this nonoptimized ligand; lysate studies could distinguish direct from indirect MDM2 effects	59
MDM2 and MDM4: E3 protein ligases	Lysates and cells; CETSA shifts	Differential TE confirmed in cells and/or lysates for several stapled peptides and Nutlin-3 to MDM2 and MDM4	TE also quantified for fluorophore-labeled peptides, indicating changes in affinity compared to nonlabeled peptides	57
Menin: component of coactivator of androgen receptors	Cells; CETSA shifts	TE confirmed in two cell lines of MI-503, a Menin PPI inhibitor	Three proteins (ASH2L, WDR5, and GAPDH) used as negative controls	71
NQO2: quinone reductase; flavodoxin-like domains	Cells; CETSA shifts and ITDRF	TE confirmed in cells of several drugs, including acetaminophen (paracetamol), to NQO2; these interactions may explain the adverse effects of these drugs	SOD1 used for sample normalization; cell integrity confirmed at 72°C, the temperature at which ITDRF was measured	54
eIF2B: translation initiation factor subunit(s)	Lysates; CETSA shifts	TE in lysates of subunits of the potential eIF2B4 complex inhibitor ISRIB; a small shift in eIF2B4 was observed, but no shifts were observed for eIF2B1/5, eIF2alpha, or eIF2a subunits	Error estimates would have been helpful to judge the relevance of the shift in eIF2B4	4, 72
EZH2: histone lysine methyltransferase	Cells; CETSA shifts and ITDRF	TE confirmed in two cell lines of CPI- 360, a novel EZH2 inhibitor; time-dependence of TE at 4 and 24 h was shown	At 24 h, isotherm at 37°C was also measured to show constant protein levels	65

(*Continued*)

Table 1 (*Continued*)

Protein(s)	Sample type and method	Results	Comments	Reference
PRMT5: protein arginine methyltransferase	Lysates; CETSA shifts	TE confirmed in lysates of EPZ015666, a PRMT5 inhibitor	Inactive compound 3 used as a reference; actin used as a negative control	64
MTH1: oxo-purine triphosphatase	Cells; CETSA shifts	TE confirmed in cells for TH287 and TH588, two novel MTH1 inhibitors	Cell ITDRF would have enabled interesting correlations with other cell effects	53
		TE confirmed in cells of the *S* form of crizotinib to MTH1, although no TE of *R*-crizotinib was observed	Cell ITDRF would have enabled interesting correlations with other cell effects	52
AMPD3/VDR/CBS/PAFAH1B1: diverse protein families	Cells; CETSA shifts	Destabilization of mutated proteins in association with increased chaperone binding	No small molecules; stabilization due to PPIs	70

Abbreviations: CETSA, cellular thermal shift assay; ITDRF, isothermal dose-response fingerprint; PPI, protein-protein interaction; TE, target engagement.

by the notion that other, albeit less direct, assays exist for TE studies of protein kinases in cell systems, such as the monitoring of auto- or downstream phosphorylation sites or affinity probe–based approaches (3). Nevertheless, the wide range of targets assessed via CETSA in its first year of use clearly illustrates the broad applicability and robustness of this method and supports the notion that CETSA will indeed be applicable to many target families aside from protein kinases.

The drugs and targets studied in the first year of CETSA adoption include both drugs that bind to protein-protein interaction (PPI) surfaces and drugs that act by binding to enzyme sites. The first example of the use of CETSA of a PPI inhibitor was in a study by Bai et al. (54) on a novel inhibitor of the antiapoptotic Bcl-2 family proteins Bcl-xL and Bcl-2. The novel inhibitor BM-1197, which was generated via a structure-based design approach, was shown to induce strong stabilization of Bcl-xL and, to a lesser extent, stabilization of Bcl-2 based on CETSA. $ITDRF_{CETSA}$ demonstrated significant stabilization at the lowest dose tested, 16 nM, whereas ABT-263, an inhibitor of multiple Bcl-2 family members tested in clinical trials, required a concentration of at least 32 nM to yield a similar stabilization of Bcl-xL. The low nanomolar $ITDRF_{CETSA}$ of BM-1197 on Bcl-xL can be related to its subnanomolar affinity to purified Bcl-xL and to its inhibition of cell growth at a concentration of approximately 2 nM (55). These data were presented only as Western blots (as for several other CETSA studies in its first year of adoption), and it would have been helpful for interpretation if the data had been quantified to enable T_m and error estimates. Actin was used as a control in the $ITDRF_{CETSA}$ experiment, and the compound did not stabilize actin. In our experience, the use of one or more negative controls can be highly valuable. For example, using poorly soluble compounds at very high concentrations could lead to global protein aggregation in lysates and produce false shifts in a CETSA experiment.

Investigators have also applied CETSA to study TE to the E3 ligase MDM2. MDM2 targets the tumor suppressor p53 for proteasomal degradation; therefore, MDM2 represents a potential target for cancers that are deficient in p53 activity (56). A recent study by Tan et al. (57) from David Lane's lab used CETSA to confirm TE to MDM2 and its close homolog MDM4 using different stapled peptides or the MDM2 inhibitor Nutlin-3. Stapled peptides, which provide exciting new

chemistry for drug development, might be broadly applicable to the inhibition of PPIs (58). In lysates, the peptide Staplin-2 induces a ΔT_m of 6.7°C in MDM2 and of 5.4°C in MDM4. In cells, these shifts are smaller but significant—at 2.5°C and 1.8°C, respectively—supporting the notion that Staplin-2 indeed establishes TE in cells. This study suggests that CETSA may become a generally applicable strategy to confirm and optimize the TE of stapled peptides in cells and animal tissues.

NQO2: NAD(P)H dehydrogenase quinone 2

In another study of MDM2 using CETSA, Qin et al. (59) investigated the binding of JapA to MDM2. JapA, originating from a computational structure-based screen of a natural compound-based library, induced shifts of >15°C in MDM2 in cells, and similarly large shifts were observed in two different cell lines (MCF-7 and MDA-MB-231). The in vitro affinity of JapA for purified MDM2 remains to be determined, but the very large CETSA shift observed for this nonoptimized compound in cells is surprising. In the studies by Tan et al. (57) discussed above, the low-molecular-weight compound Nutlin-3, which binds to purified MDM2 in the 10-nM range, induces only an approximately 2°C shift in MDM2 in cells and lysates (58). An assessment of JapA in lysates would be valuable to shed light on whether this compound truly displays direct TE to MDM2 or whether the observed stabilization effect might be due to other processes affecting MDM2 in these experiments. Researchers have addressed several other interesting PPIs using CETSA during its first year of adoption (see **Table 1**), but space limitations make it impossible to discuss all these studies in detail in this review.

CETSA has also been applied to several enzyme targets that demonstrated plausible TE in cells or lysates (**Table 1**). In particular, metabolic enzymes should have high feasibility for CETSA experiments because they are often low in complexity. Moreover, robust downstream measurements of the inhibition of such enzymes in cells can be difficult owing to the rapid metabolic transformation of enzyme products. MTH1 plays a role in degrading potentially toxic oxidized nucleotides, and CETSA melting curves confirmed TE of several newly discovered MTH1 inhibitors in cells (52, 53). Differences between the affinities of these compounds to the purified protein and their IC_{50} values for cell death ranged from one to three orders of magnitude. Cellular $ITDRF_{CETSA}$ was not determined in these studies, although such data would have been helpful to shed light on the cause of the large deviations in the in vitro and cellular effects of these compounds.

In another study, NAD(P)H dehydrogenase quinone 2 (NQO2) was identified as a potential off-target interactor with acetaminophen (paracetamol) based on affinity proteomics (60). Investigators have shown previously that NQO2 is a promiscuous protein, binding to several different compounds and potentially explaining their off-target effects (61–63). CETSA melting curves in cells and lysates confirm the TE of acetaminophen and its analogues to NQO2. Researchers used $ITDRF_{CETSA}$ at 72°C to study concentration-dependent binding to NQO2. A high temperature was required owing to the high T_m of NQO2, but specific cell leakage experiments confirmed the integrity of the cell membrane, even at this high temperature. The $ITDRF_{CETSA}$ experiment supports the hypothesis that acetaminophen and its analogues require a concentration two orders of magnitude higher to obtain similar cellular TE to resveratrol, the most effective NQO2 inhibitor used in the study. Superoxide dismutase 1 was highly stable throughout the temperature range used and was therefore used as a reference protein for normalization at different temperature points. Such reference proteins are likely to be of particular value for tissue experiments, in which the amounts of cells in different tissue aliquots need to be normalized.

Two studies also showed that CETSA is feasible for TE studies of lysine and arginine methyltransferases, families with important applications in drug discovery. TE of the novel inhibitor EPZ015666 to the protein arginine methyltransferase-5 produced a clear shift in lysates (64). As another example, $ITDRF_{CETSA}$ measurements, including time-dependent studies of drug effects,

successfully assessed CPI-360, an inhibitor of the histone-lysine *N*-methyltransferase EZH2 (65). Additional studies using CETSA to examine enzyme inhibitors are presented in **Table 1**. Overall, most, but not all, of the studies using CETSA to assess TE in the first year of CETSA adoption provide support for TE of the studied compounds. However, more extensive quantification of replicate measurements, parallel studies in lysates and cells, and use of negative controls are recommended in future studies to help interpret the relevance of CETSA measurements to TE.

CONCLUSIONS AND FUTURE PERSPECTIVES

CETSA constitutes a novel biophysical technique for studies of ligand binding to proteins in cells and tissues. It is likely applicable to many types of proteins, although its usefulness for larger and more complex soluble proteins and for membrane proteins remains to be determined. CETSA is likely to serve as a valuable tool at many stages of drug development. **Figure 5** depicts both how CETSA can be complementary to existing functional genomics or proteomics strategies and where CETSA-based experiments could be used to generate valuable information in a drug discovery pathway. If the aim is to generate a drug for targeted therapy, CETSA may be the ideal strategy for high-throughput screening (HTS), as it can be performed directly in a physiologically relevant context. HTS is often performed on purified proteins, cell lysates, or cells harboring overexpressed target proteins to generate initial hits. Although these assays can be relatively robust, difficulties in efficiently translating compounds originating from such screens into relevant cellular systems are common when the protein is in a different form in its physiological context (66). Implementing CETSA in a high-throughput system, such as the homogenous AlphaScreen assay mentioned above (32), constitutes a very robust and direct HTS method for identifying hits based on cellular TE. The further validation and optimization of hits according to parallel lysate and cellular CETSA TE measurements, including time-dependent $ITDRF_{CETSA}$, should facilitate the rapid initial analysis of aspects of drug transport and metabolism that are relevant to the optimization of TE. Indeed, further disease relevance can be achieved by performing screening, validation, and optimization campaigns in primary cell types, differentiated induced pluripotent stem cells, tissue arrays, or tissue samples.

In phenotypic screening, the alternative pathway for hit generation, MS-CETSA has the potential to provide novel strategies for hit deconvolution and target identification. Although drug approval does not require the identification of the drug target or targets (67), such knowledge is valuable for both addressing toxicology and selecting optimal clinical candidates. MS-CETSA will complement other strategies for deconvolution of hits from phenotypic screens (68) and potentially enable the detection of direct binding to the drug target as well as the discovery of mechanistic CETSA biomarkers indicating which pathways are affected in cells. Notably, mechanistic CETSA biomarkers could also be used as readouts in high-throughput phenotypic screens for the modulation of specific cellular processes.

During lead optimization and clinical candidate selection, CETSA can play a variety of roles. MS-CETSA in lysates and cells can serve as an alternative to other strategies for the identification of potential off-target engagement or pathway activation, which can explain polypharmacology and drug toxicity. One of the most exciting aspects of CETSA is the fact that the same principal assay is applicable to many different cells and tissue types. Knowledge generated from different cell or animal tissue systems can be integrated directly to shed light on many different aspects of drug effects. As researchers discover relevant mechanistic CETSA biomarkers for activities such as drug toxicity, such biomarkers should serve as valuable complements to other assays in

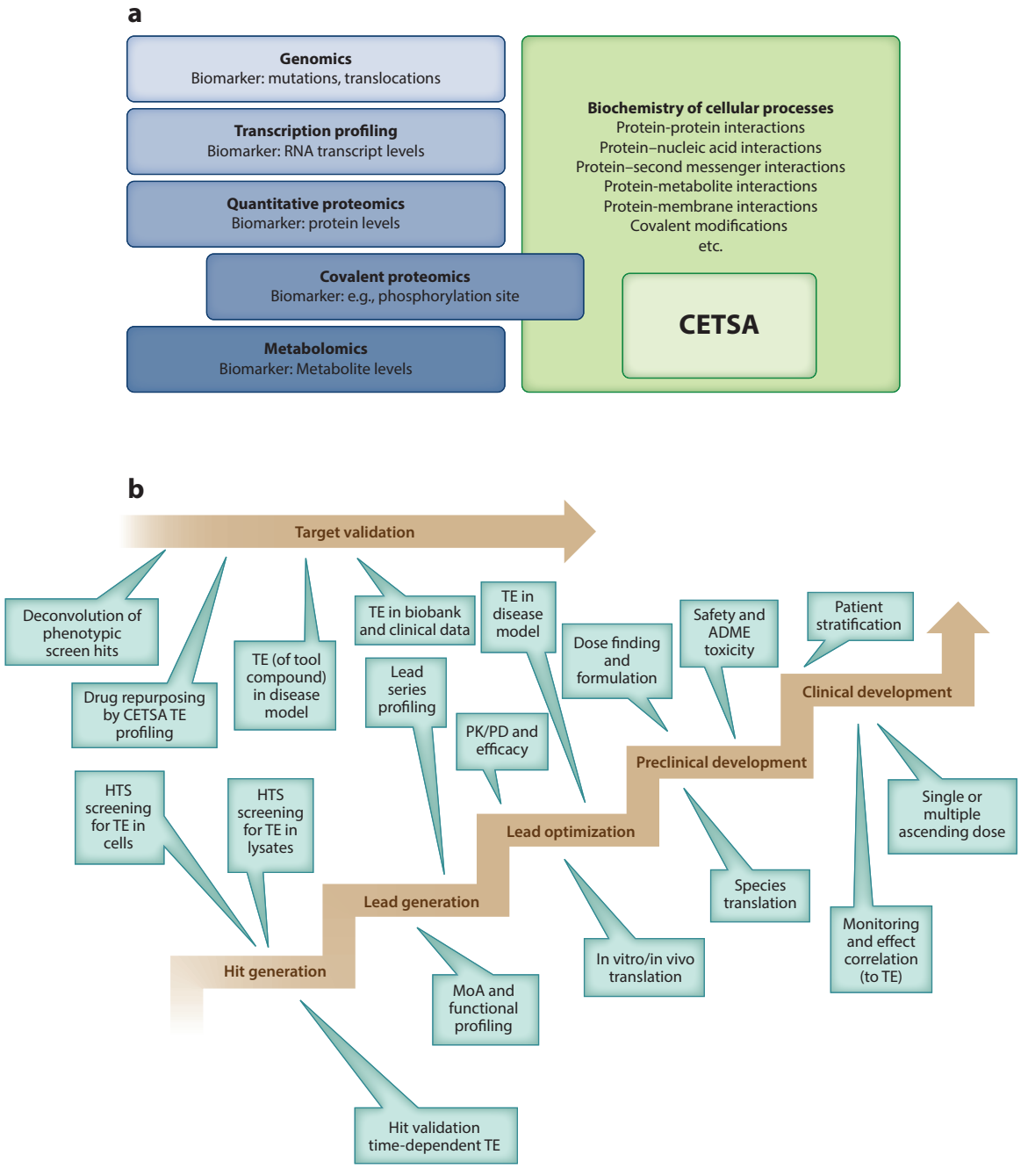


Figure 5

(*a*) MS-CETSA as a new biomarker discovery strategy complementary to existing functional genomics and proteomics strategies. (*b*) A schematic view of the processes in which CETSA could be useful during drug development. Abbreviations: ADME, absorption-distribution-metabolism-excretion; CETSA, cellular thermal shift assay; HTS, high-throughput screening; MoA, mode of action; MS-CETSA, mass spectrometry CETSA; PK/PD, pharmacokinetics and pharmacodynamics; TE, target engagement.

preclinical animal studies. Therefore, CETSA has the potential to add an extra dimension to animal pharmacokinetic and pharmacodynamic studies and to toxicology studies because TE and mechanistic CETSA biomarkers can be studied in detail in both target tissues and tissues prone to adverse effects.

Because of the high relevance of CETSA measurements, the method may also become a valuable tool for in vitro diagnostics, although the applicability and usefulness of CETSA for clinical studies remains to be established (69). CETSA studies of clinical samples from accessible tissues of patients who are in treatment are, in analogy with our mouse studies, technically feasible, although some drugs and targets may be less informative because their T_m values are greater than the temperature at which cells lyse. The use of fresh patient samples for ex vivo CETSA measurements of local effects on TE and mechanistic biomarkers will constitute an alternative means for diagnostics. This strategy could, for example, allow for rapid evaluation of multiple cancer drugs before and during therapy to establish whether the cells in the tissue are prone to innate or acquired resistance, as reflected by TE or mechanistic CETSA biomarkers.

It is encouraging to see that during its first year, researchers have already applied CETSA extensively, and there is now a continuous flow of novel studies containing CETSA data entering the literature. The dominant application to date is the direct validation of TE by novel compounds in cell culture. The fact that most of these studies examined different proteins and protein families supports the broad applicability of the assay and the ease of implementing it for novel protein targets. This is in contrast to affinity-based TE assays, which often require very significant development efforts (3). Some CETSA studies have used both cells and lysates, and some have also used $ITDRF_{CETSA}$ to establish relative TE by different compounds. However, many exciting applications and implementations of CETSA clearly remain to be explored. The next phase is likely to include more animal studies as well as the routine use of MS-CETSA as a discovery strategy for direct drug binding in cells, for identifying off-targets and polypharmacology, or for assisting when deconvoluting hits from phenotypic screens. We predict that in the long term, MS-CETSA will provide an important means of discovering mechanistic biomarkers for downstream drug effects and for toxicology-related processes. In addition to its applications to drug development, MS-CETSA has the potential to provide a broadly applicable functional proteomics strategy for studies of general cell and animal biology, although this topic is not discussed in this review. We will follow the development of novel CETSA applications in the coming years with great excitement.

SUMMARY POINTS

1. CETSA is a novel, stringent, label-free, biophysical assay that measures physical TE by drugs in cells and tissue samples directly.
2. CETSA enables the direct detection of changes in the functional states of proteins, such as alterations in PPIs and protein-metabolite interactions, and such measurements can serve as mechanistic biomarkers.
3. CETSA measurements, owing to their biophysical origin, are highly stringent and not prone to false positives and, based on their use in the initial years of CETSA adoption, are broadly applicable to different protein families.
4. MS-CETSA is a novel discovery strategy for off-target toxicity, polypharmacology, and downstream mechanistic biomarkers.

5. CETSA measurements in animals provide a novel means of addressing ADME, as TE and CETSA biomarker effects can be examined directly in target and toxicity-prone tissues.
6. CETSA has the potential to serve as a useful clinical assay either for TE and biomarker measurements in samples from treated patients or in ex vivo treated samples.

DISCLOSURE STATEMENT

The authors are cofounders of the company Pelago Bioscience AB that commercializes CETSA. P.N. is the inventor of a patent covering the CETSA method.

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