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4 **Title:**

5 Interrogating fragments using a protein thermal shift assay

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2 **Abstract**

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4 Protein thermal shift is a relatively rapid and inexpensive technique for the identification of low
5 molecular weight compound interactions with protein targets. An increase in the melting
6 temperature of the target protein in the presence of a test ligand is indicative of a promising
7 ligand:protein interaction. Due to its simplicity, protein thermal shift is an attractive method for
8 screening libraries and validating hits in drug discovery programmes. The methodology has
9 been used successfully in high throughput screens of small molecule libraries, and its
10 application has been extended to report on protein:drug-like-fragment interactions. Here, we
11 review how protein thermal shift has been employed recently in fragment-based drug discovery
12 (FBDD) efforts, and highlight its application to protein:protein interaction targets. Multiple
13 validation of fragment hits by independent means is paramount to ensure efficient and
14 economical progress in a FBDD campaign. We discuss the applicability of thermal shift assays
15 in this light, and discuss more generally what one does when orthogonal approaches disagree.

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Introduction

Protein thermal shift assays (TSA) can report on low molecular weight compound interactions with protein targets. This relatively rapid, inexpensive and high throughput method is an attractive approach for screening fragment libraries or validating initial hits in small-molecule and fragment based drug discovery (FBDD) campaigns.

The principle of a TSA experiment is simple. Protein interactions with environmental components can alter (shift) the melting temperature (Tm) of a protein relative to an apo protein reference, offering a convenient read-out of protein:compound interactions (Figure 1.) During a typical experiment, the target protein is heated in a continuous temperature ramp (typically 25 – 95 °C) with protein unfolding reported via an environmentally sensitive dye that fluoresces only when bound to hydrophobic regions exposed as the protein unfolds [1,2]. Protocols co-opt standard Real Time PCR instrumentation and use commercially available and affordable fluorescent dyes and plasticware making it relatively easy to establish the protocol in any laboratory [3,4]. Execution is straightforward, and in contrast to NMR, X-ray, SPR or mass spectrometry methods requires a greatly reduced degree of technical expertise.

Protocols have been miniaturized [1,3] and can be performed in 384 or 96 well plates, permitting final reaction volumes of ≤ 20 and 40 μL respectively. Typical final protein concentrations are in the order of 1 - 5 μM making the experiment relatively inexpensive in terms of protein usage. Using a temperature ramp rate of 1 - 3 °C min^{-1}, experiments can be completed in approximately 30 – 60 min. Furthermore, data analysis can be semi-automated with the use of instrument software or customised programs [5] allowing results to be obtained in a single day. Tm shifts arising from interactions with small molecules and fragments are frequently small in magnitude (<1 – 2 °C), generating narrow margins within which to discriminate hits from non-hits. In the absence of a known small-molecule binder with which to compare test molecules (often the case in *de novo* screens against new targets), it is accepted practice to define a hit

interaction as one that yields a Tm shifter greater than 3 times the standard deviation of the reference [6]. The solvent in which test compounds are dissolved (e.g. DMSO) can itself influence protein Tm, often in a destabilizing manner [6]. It is thus advised that solvent concentrations are kept to a minimum (2 % maximum) and imperative that comparisons of ligand-altered Tms are made to solvent-matched controls. Pivotal to the approach is that the target protein yields an unambiguous melt curve. Optimization of reaction conditions (e.g. buffer composition, protein and dye concentration) may be required to elicit a clear transition curve with a significant increase in fluorescence emission during unfolding. The approach is certainly feasible for many monomeric proteins (even membrane proteins) [1], and in theory also for oligomeric proteins if there are clearly demarcated unfolding transitions of quaternary and tertiary structure.

The stabilizing effect of a compound upon protein thermal stability is proportional to both compound concentration and affinity. Ligand binding constants can be determined by measuring the effect of increasing ligand concentration on Tm, provided that the thermodynamics of protein stability are known with accuracy e.g. from differential scanning calorimetry experiments [2]. For a given protein, different ligands with equivalent affinities can induce different Tm shifts, reflecting ligand specific differences in enthalpic and entropic contributions to binding. TSA does not distinguish between enthalpically or entropically driven interactions, although larger shifts in Tm are often observed for more entropically (e.g. hydrophobic) dominated binding interactions [3].

TSA is a simple technique, with versatile applications. It has been applied to the identification and optimization of buffer formulations that promote protein stability, and which in turn increase the likelihood of protein crystallization [7,8]. TSA also appears suitable in some cases for the detection of heavy atom binding [8] suggesting it has potential as an adjunct to experimental phasing in macromolecular crystallography. In the drug discovery arena, the potential for using TSA in small-molecule screening was the subject of excited debate more than a decade ago [1,9] and there have since been several successful applications [6,10,11,12]. Its application has since

been extended to fragment based campaigns [2,3,4]. Today, several stalwarts of FBDD programs (e.g. Astex Pharmaceuticals, Vernalis) indicate that they routinely employ TSA in their screening pipelines, and a poll of the wider fragment community suggests that it is a popular method [13]. As with all drug discovery screening, efficient first round screening with follow-up validation of fragment hits by independent means is paramount to ensure timely and economical progress in the campaign. In this light, TSA is potentially a quick and inexpensive tool that can be used for a primary screen or to complement other screening methods. In addition to its relative simplicity, TSA offers the advantage that it can identify fragments that have a destabilising effect upon the protein (indicated by a reduced rather than an increased Tm)[14]. However, despite the advantages of TSA, (and examples of its application in small molecule screening) the number of articles citing its use in FBDD, particularly as a primary screening tool, seem relatively low. In a publishing culture where negative results are not routinely reported, it is inherently difficult to gauge the extent of unhelpful or uninformative TSA experiences. However it would appear that the contribution of TSA to hit identification falls behind that of NMR and SPR, with reports from meetings indicating that "of all the methods for finding fragments, thermal shift assays seem to be the most controversial [13]."

Here we highlight recent articles that have applied TSA to FBDD and discuss some of the successes and associated challenges, with particular interest in its application to protein:protein interaction (PPI) targets.

TSA in Fragment Based Drug Discovery

TSA has been successfully employed as the primary screening tool in three recent academic FBDD campaigns against myo-inositol-3-phosphate synthase from *Trypanosoma brucei* (TbINO1) (The University of St Andrews, 2011) [15], a cytochrome P450 protein (CYP121) from *Mycobacterium tuberculosis* (University of Cambridge, 2012) [16] and a human p53 mutant Y220C (Eberhard-Karls-University, and MRC LMB, Cambridge, 2012) [17].

TSA screening of a 600 fragment rule-of-three compliant library at a concentration of 1 mM, identified 38 fragments (~ 6% hit rate) that induced a ΔTm > 1.5 °C in TbINO1 [15]. Despite the relative chemical simplicity of the molecules, several of the TSA hits exhibited trypanocidal activity when administered to cultured *T. brucei* - although for a subset of these hits there were also counter indications of mammalian cytotoxicity.

Against CYP121, TSA identified 66 fragment hits from a library of 665 (parameters: 5 mM fragment, ΔTm >0.8 °C, ~10 % hit rate), of which 56 of the most chemically diverse fragments were rescreened by ligand-detected NMR corroborating the TSA observations for 26 fragments (46 % validated hit rate)[16]. Four independent ligand-crystal structures were solved revealing that each of the fragments bound in an overlapping manner within the relatively large (1350 Å^3) active site cavity. This allowed the authors to immediately merge the overlapping structural moieties to generate a moderate affinity (28 µM) lead compound in a text book application of TSA screening, hit validation by orthogonal methods and successful SAR development.

In the case of the cancer associated p53 mutant Y220C, TSA served an elegant duality of purpose, simultaneously identifying fragment-target interactions from the library and providing a functional read-out (namely rescue of the mutant protein's reduced Tm and stability at physiological temperature [17].) Accordingly TSA was used both in the initial screening stages, and as a reporter of functionality in the subsequent SAR efforts.

In addition to the primary screening outlined above, TSA has also been employed to characterise hits identified by other methods. For example, in 2011 GlaxoSmithKline R&D applied TSA to assess the selectivity profile of two promising compounds against BET family bromodomains relative to other bromodomain containing proteins [18]. The compounds, initially identified using a fluorescence anisotropy assay, increased the Tm of BET family members by > 5 °C (consistent with other biophysical indicators of the interaction) relative to a 1.5 °C increase in the non-target bromodomain containing proteins, prompting the authors to conclude that

there may be a “window of selectivity” to exploit. The authors acknowledge that the method does not permit precise quantitation of selectivity but this is nevertheless an interesting case study in the potential for non-standard applications of the assay in fragment campaigns.

Evidently TSA can be used to interrogate fragment libraries, although the 46 % validation rate reported by Hudson *et al*. [16] raises concerns about the potential for a high false positive rate. It is of note that each of the cases described above are proteins that are considered classically “druggable” with defined concave target sites, (in the case of p53, one that is induced by the Y220C mutation). In the last decade, drug discovery attention has turned to tackling protein:protein interactions (PPI), which are highly valuable but certainly less tractable targets. PPI targets are challenging because their surfaces are typically flat and shallow, lacking the classic defined, concave cavities of what was previously considered the “druggable genome” [19]. Furthermore, existing small molecule libraries are biased towards previous targets (GPCRs, kinases and proteases.) Fragment approaches can overcome this bias, offering diverse chemotypes befitting of the new diverse range of targets [20]. Fragments may bind into small preexisting cavities on the PPI surface, or induce adaptive cavities (perhaps assisted by tethering approaches [21,22]) to provide “footholds” on the PPI surface [20] for subsequent compound development.

However the detection of fragment:PPI interactions is technically challenging. Despite this, two recent articles from academic research groups demonstrate beautifully the potential for using TSA in FBDD campaigns against PPI. This year Scott *et al* (University of Cambridge) have targeted the interaction between the tumour suppressor BRCA2 and the recombination enzyme RAD51 [23]. The surface area of the interaction is small, involving engagement of Phe and Ala side chains from a conserved FXXA motif on one BRC repeat into discrete shallow pockets on RAD51 [24]. For fragment screening, because of the instability of monomeric human RAD51 a humanized variant of the archeal orthologue RadA was used. The majority of the fragment library (> 60 %) screened by TSA had a neutral effect on the Tm (between - 0.5 and + 0.5 °C),

22 % gave a negative shift (ΔTm more negative than -0.5 °C), and 3.2 % induced a positive ΔTm of >1°C (two standard deviations) of which only two fragments (0.16 %) shifted the Tm by more than 1.5 °C. These latter hits were themselves promising, and were also successfully used as competitors in subsequent STD NMR re-interrogation of the library and evaluation of structural analogues. This paper is an excellent proof of principle in the execution of TSA for screening libraries against a challenging PPI interaction. Despite the shallow nature of the binding site, this approach identified fragments exhibiting typical binding affinities (low mM) and good ligand efficiency (LE 0.28-0.46), and crystallography confirmed these bound in a manner recapitulating the *in vivo* BRC4 Phe1524-RAD51 interaction. Notably, the authors comment that the hit rate (0.16 %) for fragments inducing Tm > 1.5 °C was very low in comparison to similar screens against enzyme active sites and indicative of the challenge of such targets [23].

In contrast, Abdel-Rahman *et al.* (University of Cambridge), in 2011 targeting the Notch ankyrin domain using TSA report a healthier hit rate (~ 3 %) for a PPI target, comparable to that obtained for other enzyme targets using the same screen [25]. Despite the rather flat and featureless surface of the ankryin domain, TSA was still able to identify fragments for which binding could be confirmed and quantified by SPR and characterized at a molecular level by crystallography.

TSA and fragments: potential challenges

Not every biophysical technique is suitable for every FBDD investigation. As with all methods, TSA has its own particular limitations and care should be taken in the interpretation of data, particularly with regards to quantifying fragment binding affinity and ranking relative potency. For comprehensive discussions on the potential errors in data analysis, the reader is referred to excellent articles describing quantitative modelling and analysis of TSA data [4,9,14]. Here we highlight briefly some of the main challenges that may be commonly encountered.

The magnitude of a Tm shift is affected by the intrinsic binding affinity of the ligand under

examination. Fragments are relatively small chemicals, that typically bind to proteins with K_D values in the millimolar range. As a consequence, high concentrations of fragments are often necessary to observe a significant shift in Tm [4]. However, the high concentrations of fragments required for screening can give rise to problems of fragment solubility and aggregation. In addition, very weakly binding fragments may be difficult to detect by TSA. For example, fragments identified as having a weakly positive but not significant, effect at 5 mM in the original TSA screen against RadA were re-assessed by STD NMR presumably because TSA was an insufficiently sensitive method to detect some interactions [23]. It is also of note that the methods available for fitting a curve to the raw experimental fluorescence data (the thermodynamic model, the Boltzman equation and higher order polynomial equations) can yield different Tm values for a given set of data; this discrepancy has been observed to result in fragments being differentially classified as a hit or non-hit depending on the model applied [5].

Moreover, fragments may bind at more than one site and may not bind only to the folded state; multiple binding events may generate falsely large increases in Tm (if they contribute to protein stability in an additive manner) or conversely result in a falsely low Tm shift, if a destabilising interaction with the unfolded state negates the stabilising interaction with the folded state. Additional technical limitations of TSA stem from the properties of the small molecules or fragments themselves. To minimise the likelihood of fluorescence interference, TSA employs a red-shifted dye (typically SYPRO-Orange) with an excitation wavelength of ~ 500 nm [3]. Despite this, optical interference can still be a problem [23,26,27], which we have also observed in our own unpublished work. A propensity for non-specific interactions with the fluorescent dye will also eliminate fragments from use in the assay. Similarly, not all proteins are compatible with SYPRO-Orange. For those cases where no folding transition is observed, or interference is apparent, alternative dyes should be investigated. Finally, fragments that induce complex melt curves (which may arise from optical interference, compound instability or aggregation [23]) must also be excluded from further analysis.

In addition to some of the potential difficulties outlined above, attempts to use TSA as a secondary interrogation method of NMR hits have on occasion yielded complex results. For example, against 3 unnamed kinases, TSA confirmed considerably fewer hits than those observed by NMR (23 %, 11 % and 8 % respectively of the initial hits) [28]. Notably, for Kinase 3, crystal structures were later obtained for protein complexes with NMR hits that were *not* validated by the follow up TSA, calling into question the value of TSA as an orthogonal reporting method in this instance. Similarly, of 15 fragments identified by a primary SPR screen against PARP15, 14/15 could be validated by NMR but only 1/15 could be confirmed by TSA [13,29].

Throwing out the baby with the bathwater?

In common with all drug discovery programmes, confirming initial fragment hits in a FBDD campaign using independent methods is an essential course of action to increase the likelihood of success by reducing the probability of pursuing false positives, with obvious advantages for time and economic efficiency. In this article we have discussed instances where secondary validation by TSA has given low hit confirmation rates - that were not necessarily indicative of the propensity for obtaining fragment bound crystal structures [28] - or produced directly conflicting results [13]. These observations raise a more general question of how one should proceed when orthogonal approaches generate inconclusive or contradictory results. Highlighting the potential for disagreement between independent approaches, parallel screening of two highly similar fragment libraries (90 % in common) against HIV-integrase 1 using NMR and SPR methods respectively, yielded two series of hits which displayed no overlap with each other [30] .The lack of overlap was attributed not - as first suspected - to differences in the experimental conditions of each screening approach, but rather to the differing stringency with which hits were accepted or rejected. Notably both approaches yielded multiple protein-ligand co-crystal structures suitable for future SAR studies, making this example a cautionary tale about how nuances in the execution and interpretation of orthogonal approaches to fragment interrogation can yield differing results.

Faced with incompatible results, stringent rejection of poorly validated fragments is intuitively the more scientifically rigorous course of action. However it carries with it a risk of binary interpretation of complex experiments that may report common but also non-redundant information, and for which subtle differences in execution or thresholds for interpretation may lead to the discarding of potentially valuable fragment hits. On the other hand, retaining poorly validated targets may result in wasted efforts (time and money) on false positives. The broad applicability of TSA as an orthogonal tool in the drug discovery toolbox is somewhat unclear, with conflicting results regarding its usefulness. As highlighted in this article, TSA has been used successfully in primary screening efforts against enzymes and PPI - but it has also been found "not suitable as a frontline hit-finding method" [28] by others. Notably, in the same article secondary characterization of TSA was reported to be generally (although not always) predictive of the likelihood of obtaining protein:fragment crystal structures. It is evident that because of the potential for optical interference, TSA is not compatible with all compounds limiting applicability in both primary screens [23] and secondary characterization [27]. Furthermore, even among successful TSA investigations there are frequently high rates of neutral Tm shifts [23,25]. These may be true negatives, but may also be false negatives arising from the technical challenges of detecting small shifts in Tm associated with low affinity fragment binding [31]. Rejection of such compounds may reduce the absolute number and chemical diversity of hits, with knock-on effects for hit-to-lead progression. The simplicity and high-throughput nature of the TSA still make it a highly attractive tool in FBDD programmes, but it is advisable to consider the limitations of its applicability before relying upon it solely as a screening method or orthogonal verification tool.

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1 **Figures**
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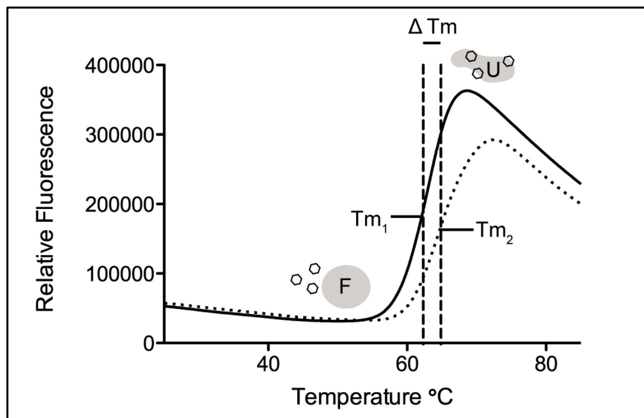

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5 **Figure 1**. **A typical thermal shift assay.** During a standard experiment the folded (F) globular
6 protein is heated in a continuous temperature ramp (typically between 25 and 95 °C) causing it
7 to unfold (U). Protein unfolding is reported via an environmentally sensitive dye (e.g. SYPRO®
8 Orange, Life Technologies, schematically represented here as a series of hexagons) that
9 fluoresces only when bound to hydrophobic regions exposed as the protein unfolds. The protein
10 melting temperature (Tm) can be calculated as the inflection of the resulting transition curve
11 using a simple Boltzmann equation. Increases in Tm (Δ Tm) between the reference protein
12 (solid line) and protein in the presence of a test compound (dashed line) occur as a result of the
13 additional free energy contribution of compound binding.

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