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A combinatorial biophysical approach; FTSA and SPR for identifying small molecule ligands and PAINs

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ABSTRACT

Biophysical methods have emerged as attractive screening techniques in drug discovery both as primary hit finding methodologies, as in the case of weakly active compounds such as fragments, and as orthogonal methods for hit validation for compounds discovered through conventional biochemical or cellular assays. Here we describe a dual method employing fluorescent thermal shift assay (FTSA), also known as differential scanning fluorimetry (DSF) and surface plasmon resonance (SPR), to interrogate ligands of the kinase p38α as well as several known pan-assay interference compounds (PAINs) such as aggregators, redox cyclers, and fluorescence quenchers. This combinatorial approach allows for independent verification of several biophysical parameters such as K_D, k_{on}, k_{off}, ΔG, ΔS, and ΔH, which may further guide chemical development of a ligand series. Affinity values obtained from FTSA curves allow for insight into compound binding compared with reporting shifts in melting temperature. Ligand–p38 interaction data were in good agreement with previous literature. Aggregators and fluorescence quenchers appeared to reduce fluorescence signal in the FTSAs, causing artificially high shifts in T_m values, whereas redox compounds caused either shifts in affinity that did not agree between FTSA and SPR or a depression of FTSA signal.

Biophysical methods of interrogating the binding of putative ligands offer various advantages over their biochemical and cellular counterparts [1]. Techniques such as surface plasmon resonance (SPR)[1] [2], isothermal titration calorimetry (ITC) [3], and fluorescent thermal shift assay (FTSA) [4] measure the direct effect of ligands binding to a protein; therefore, specific reagents such as fluorescent displacement probes and artificial substrates (e.g., peptides with cleavable fluorophores for enzymatic assays) do not need to be generated. The above techniques infer compound binding only as a direct effect of the readout as opposed to a secondary effect such as enzyme inhibition or second messenger production, allowing for greater confidence in determining the affinity of weak binders. Weak binders are often screened at concentrations that may interfere with traditional fluorescence assays by mechanisms such as compound autofluorescence and quenching [5,6] as well as aggregation that may inhibit enzymatic activity [7] and cause nonspecific G-protein-coupled receptor inhibition [8]. Biophysical techniques also show utility within the validation of hits obtained using traditional biochemical or cellular screens. High-throughput screening using classical biochemical or cellular methodologies is known to identify a number of undesirable pan-assay interference compounds (PAINs) that do not match the effect of real ligands in biophysical screens [7,9]. Compounds that progress through hit-to-lead and lead optimization may also benefit from biophysical technique as further interrogation of binding than simple affinity may allow for fine-tuning of molecular interaction. Modification of molecular interactions can be included in terms of extended residence time to improve pharmacokinetics/pharmacodynamics [10]. Thermodynamic categories of interaction may also be considered; enthalpically driven hydrogen bonding or π–π stacking, rather than less specific entropically driven interactions

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[1] *Abbreviations used:* SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; FTSA, fluorescent thermal shift assay; PAIN, pan-assay interference compound; DSC, dynamic scanning calorimetry; qPCR, quantitative real-time polymerase chain reaction; DLS, dynamic light scattering; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; CAC, critical aggregation concentration; NTA, nitrilotriacetic acid; HBS, Hepes and NaCl (pH 7.4); NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

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[11–13], may present as a useful tool for querying and improving medicinal chemistry design and experiments.

Biophysical techniques are, however, not without limitations; ITC and FTSA have relatively high protein requirements when compared with traditional biochemical experiments [14], often microgram to milligram quantities per data point. FTSA can require up to 25-fold less protein than ITC [4], yet FTSA still can require micromolar concentrations of protein. Furthermore, although SPR has much lower protein requirements than ITC and FTSA [15], all three techniques have a much lower throughput than traditional biochemical assays [1]. During measurement with the above techniques, single data points can take several minutes and are often run in tandem rather than in parallel. FTSA offers a higher throughput analogous technology to dynamic scanning calorimetry (DSC) [4]. DSC detects phase transitions, such as the melting of a protein, by measuring the difference in energy required to heat a sample compared with a control [16]. Rather than a direct measurement of energy input, FTSA employs an environmentally sensitive dye, the fluorescence of which increases in hydrophobic environments such as the core of proteins that are exposed during melting. FTSA, therefore, requires lower protein concentrations (often only hundreds of nanograms per data point) (Table 1).

True ligands stabilize protein structure and impede thermal denaturation [4,16–19]. Hence, a binding interaction can be inferred when the temperature at which a protein melts across a thermal gradient increases in the presence of a ligand. FTSA experiments may be performed in a thermal cycler suitable for reverse transcription–quantitative real-time polymerase chain reaction (RT–qPCR); therefore, large numbers of samples may be run in parallel [4,17,19].

To demonstrate the utility of these techniques to the drug discovery scientist, the model protein p38α was selected. p38α has been well characterized previously in the literature and has several well-known small molecule ligands that exhibit different, well-understood molecular binding mechanisms [20–23]. These ligands include simple ATP competitive ligands in the SB prefix series (Fig. 1, compounds **5**–**7**) [20,21,23] or the tight binding allosteric BIRB 796 compound (Fig. 1, compound **1**) [22]. Three fragments of BIRB 796 were also assessed (Fig. 1, compounds **2**–**4**), the binding of which was predicted by structure similarity. These selections allowed verification of results against other methods within the literature as well as invite critique within a familiar system.

To determine the profiles of PAINs within FTSA, several compounds known to cause interference in biochemical and cellular assays were selected (Fig. 1, compounds **8**–**13**). Four common mechanisms of assay interference were chosen for study: promiscuous aggregation [24], redox cycling [9], fluorescence quenching [25], and chelation [26]. Rottlerin and Congo Red were chosen as promiscuous aggregators (Fig. 1, compounds **8** and **9**) [27,28]. These compounds form nanoparticles in solution that are able to sequester protein onto their surface, causing nonspecific inhibition of enzyme activity and protein–protein interactions [27]. To confirm formation of nanoparticles, dynamic light scattering (DLS) was employed [29]. Redox cycling compounds PR-619 and benzoquinone (Fig. 1, compounds **11** and **12**) were selected [30,31]. Redox cyclers can cause oxidation of cysteine residues on the

Table 1
Typical protein requirements for biophysical and biochemical assays within Sygnature Discovery.

Assay	Protein concentration	Volume
SPR	fM (on chip) 100–1000 nM (coupling)	100 µl (coupling)
FTSA	100–6000 nM	3–20 µl
ITC	25–75 µM	Up to 2.1 ml
FRET/AlphaScreen	10–100 nM	5–30 µl
Enzymatic	0.05–1000 nM	10–100 µl
Receptor binding	10–1000 nM	10–100 µl

Note. FRET, fluorescence resonance energy transfer.

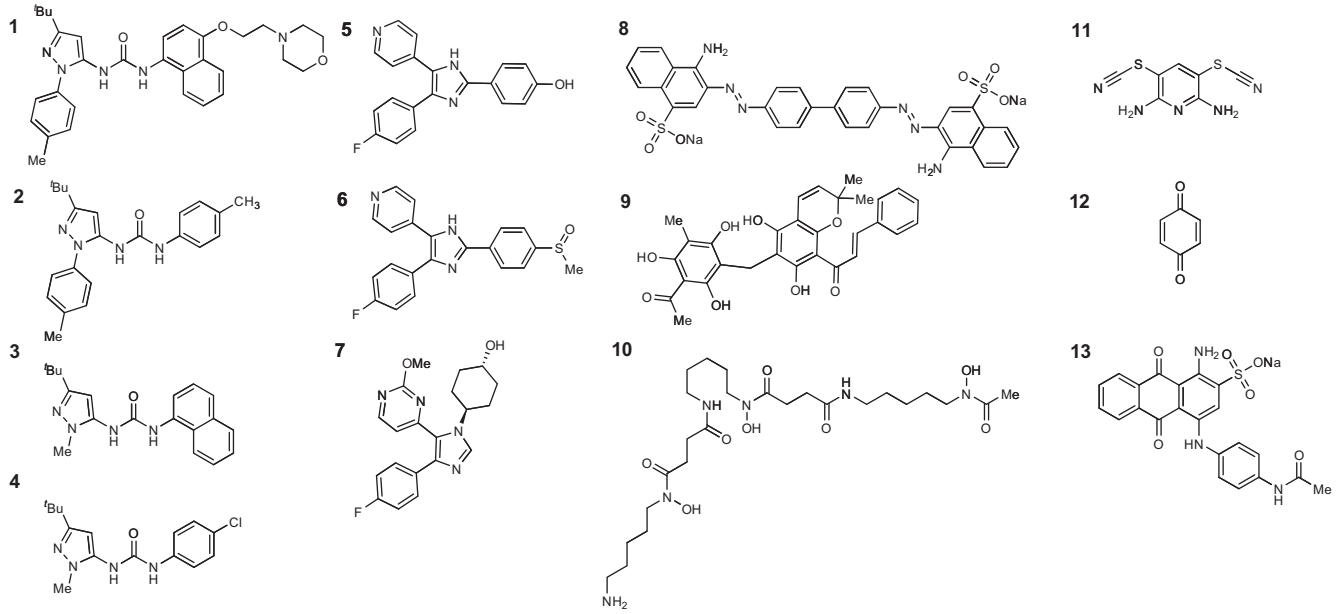

Fig.1. Structures of the compounds tested. Compounds **1** to **4** represent **1** (p38 inhibitor BIRB796) and **2**–**4** (fragments of its structure). Compounds **5** to **7** are the pyridinyl imidazole p38 inhibitors SB202190, SB203580, and SB239063. Compounds **8** to **13** are interference compounds (**8** and **9** are the aggregators Congo Red and rottlerin, **10** is the chelator deferoxamine, **11** and **12** are redox cyclers PR-619 and benzoquinone, and **13** is the fluorescence quencher acid blue 40).