# Biophysical Techniques For Target Engagement and MOI Studies During Small Molecule Drug Discovery


## AUTHORS

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## INTRODUCTION

Cancer Research Horizons - Therapeutic Innovation (CRH-TI) is committed to driving therapeutic innovations in oncology. At CRH-TI we have established a portfolio of biophysical applications for the validation of hits resulting from high throughput screening. After using a range of biochemical assays to eliminate false positives, demonstration of direct target engagement increases confidence in the hits and drives their progression to lead optimisation. Furthermore, these biophysical techniques can provide insight into specific mechanisms of inhibition.

Here, we describe biophysical techniques and how they have been applied to our current portfolio of targets to address Target Engagement and Mechanism of Inhibition.

## TECHNIQUES AND EXAMPLES

### MST

In Microscale Thermophoresis the movement of molecules across a laser induced temperature gradient is measured. Thermophoresis is influenced by changes in charge, size and the hydration shell of a molecule (Figure 1). $K_D$ values can be extracted and target engagement confirmed.

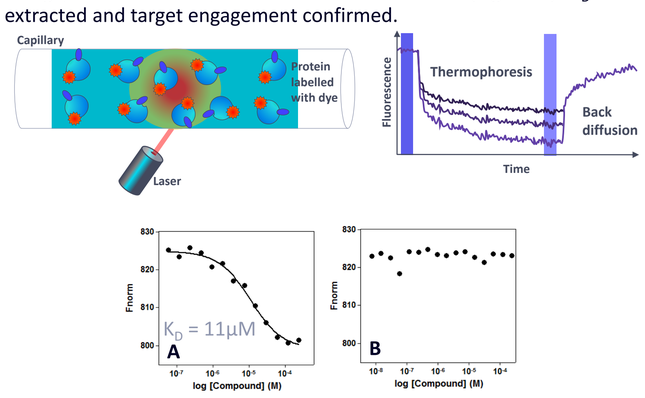



Fig 1. (A ) MST data for Compound A with a biochemical IC50 of 0.75µM, for which the X-ray structure had been solved in-house. (B) An enantiomer of Compound A, with a biochemical IC50>120µM, and for which binding could not be detected by crystallography, showed no binding by MST.

### SPR

Surface plasmon resonance measures the binding of a ligand flowing in solution over a target immobilised on a chip. This technique produces kinetic and affinity data. A change in mass on the surface confirms target engagement, with more detailed assays to look at MOI in the presence and absence of substrate.

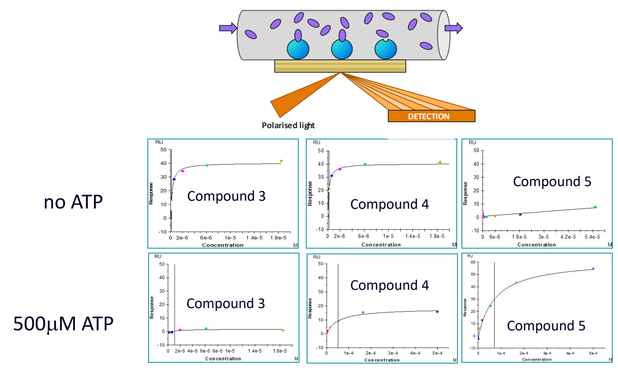



Fig 2. Equilibrium KDs were measured in the presence and absence of saturating ATP, the target's substrate. Binding of compounds 3 and 4 decreased in the presence of ATP, indicating competitive inhibition. Compound 5, however, displayed preferential binding in the presence of substrate.

### ITC

Isothermic Titration Calorimetry is a gold standard for measuring binding affinity and stoichiometry. The technique is label free, in solution and enables thermodynamic parameters (enthalpy and entropy of binding) to be directly measured. Ligand is gradually titrated into the sample cell and heat evolved or absorbed on complex formation is measured. This technique can be used to confirm target engagement.

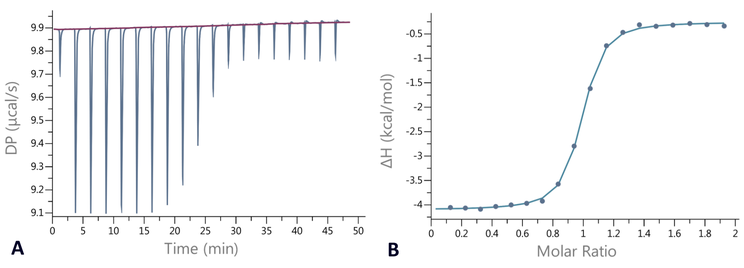



Fig 3. Ligand is titrated into the sample cell containing target and the heat given off is measured (A) The molar ratio of ligand : target is then plotted (B) and from this a $K_D$ value and stoichiometry can be calculated.

### DSF

Fig 4A. Differential scanning fluorimetry measures the temperature at which a protein unfolds using a hydrophobic dye with an affinity for hydrophobic patches on a protein (those typically exposed when unfolded). The change in fluorescence is plotted as a function of temperature, and Tm calculated as the midpoint of the melt curve. Target engagement is confirmed by an increase in $T_m$.

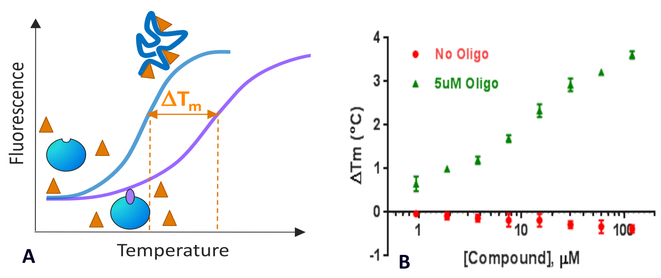


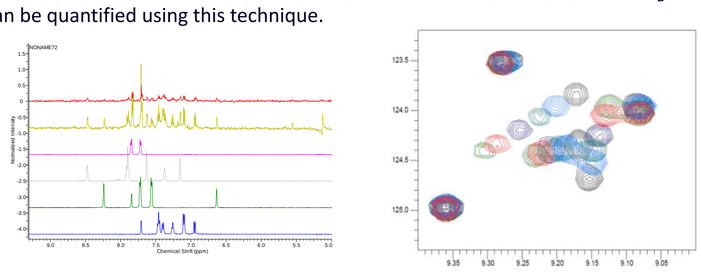

Fig 4B. $T_m$ of Target is measured with compound, in the presence and absence of Oligo substrate; confirmation of target engagement following the stabilisation of target with compound in the presence of Oligo.

### NMR

Nuclear Magnetic Resonance measures direct binding of compounds from low µM to low mM range, confirming target engagement. Insight into MOI and real time kinetics are also possible with NMR spectroscopy experiments.

**Ligand observed (A)**
Relaxation edited, water LOGSY and STD experiments compare a reference spectrum with compound alone to a response with protein present. Changes in the spectrum confirm protein binding, and therefore target engagement.

**Protein observed (B)**
$^{1}H$-$^{15}N$ HSQC NMR is extremely sensitive to changes in the environment of amide backbone residues. As ligand is titrated, peak shifts demonstrate the environment is changing and ligand is binding/interacting, to confirm target engagement. $K_D$ values can be quantified using this technique.



**(A) Ligand observed**

**(B) Protein observed**

## ORTHOGONAL VALIDATION

The techniques described above should not be used in isolation, orthogonal validation is important when understanding the detailed mechanism of an inhibitor. Detailed biochemical assays are important to demonstrate inhibition and to understand MOI, but biophysical techniques supplement these biochemical findings by demonstrating target engagement and offering further insight into MOI, again building confidence in the hit.

In the last section, both SPR and NMR are used to define the competitive action of an inhibitor on a Protein-Protein Interaction (PPI).

## PPI LIGAND COMPETITION

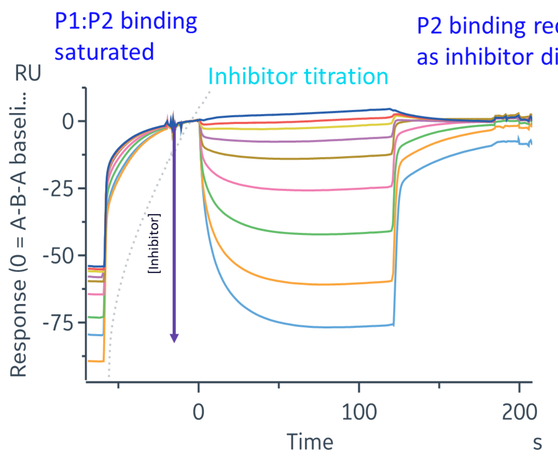



Fig 5. Competitive inhibition demonstrated by SPR. A fall in response as [inhibitor] increases indicates a reduction in mass on the chip surface, relating to the loss of P2. Once inhibitor is no longer flowed over the surface the response increases and P2 binding to P1 is recovered. This result demonstrates protein displacement by the inhibitor through competitive behaviour.

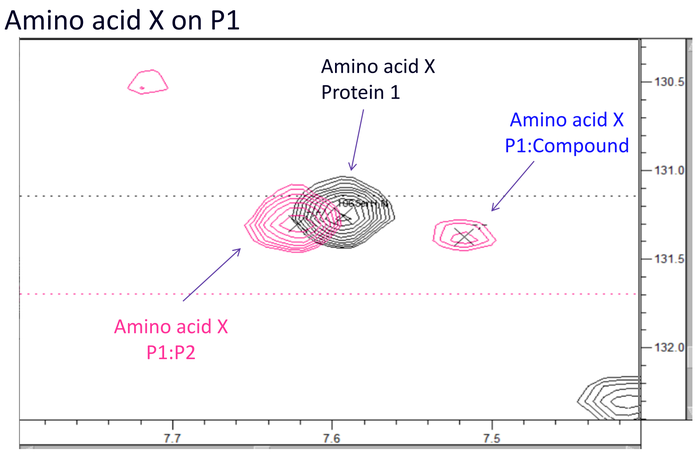



Fig 6. By SPR, the reduction in response looks to be caused by loss of P2 binding. Above is an overlay of 3 Protein observed NMR measurements following a specific amino acid identified at the protein binding interface: P1 only, amino acid X (grey), P1:P2 bound (Pink left), and compound bound to amino acid X. Compound is shown to bind amino acid X on Protein 1, at the binding interface, competing with the site of Protein 2 binding.

## AKNOWLEDGMENTS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## CONCLUSION

Biophysical techniques are an excellent tool to examine an inhibitor-target interaction. Each biophysical technique should not be used in isolation to answer a single question, and orthogonal validation is essential to determine target engagement and a detailed mechanism of inhibition. As we look to the future, our aim to is explore target inhibitor interactions in more detail using the most biologically relevant assays to understand exactly how our inhibitors function, to develop the best cancer therapeutics.