



Revealing Kinase Inhibitor Mechanisms: ITC Leads the Way

Application Note

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Introduction

Studies to characterise the human kinome, as well as the explosion of available kinase crystal structures over recent years has led to increased focus on kinases as potential targets for pharmaceutical intervention in a number of therapeutic areas.

Most kinase inhibitors developed since the pharmaceutical industry became interested in kinases in the late 1980s target the ATP site.

However, the development of Gleevec, which induces a structural rearrangement leading to the ABL kinase adopting an inactive conformation has re-invigorated the pharmaceutical industry, and has led to innovative ideas for tackling kinase inhibition, including binding outside of the ATP site and attempting to prevent kinase activation.

In order to characterise the mechanism of action of compounds based on novel ideas, detailed enzyme kinetic studies are often performed. However, a limitation of this approach is that, often, kinetic experiments cannot be performed on non-activated kinases showing little activity. It is in the rapid characterisation of binding mechanism for compounds binding to both active and non-active forms of kinases that ITC has its utility. ITC provides a complete thermodynamic profile of the binding of a compound to the target protein, allowing comparison of affinity measurements for compounds binding to different enzyme forms (for example free enzyme, enzyme-substrate complex, enzyme-product complex, active and non-active enzyme).

Protein Kinases as Therapeutic Targets

The status of protein kinase inhibitors has changed somewhat over the last few years, with the emergence of clinically validated kinase inhibitors. Inhibition of kinase signalling cascades is thus a proven therapeutic approach for the treatment of diseases in both the areas of oncology and inflammation. Many major pharmaceutical companies have resources committed to the development of kinase inhibitors, leading to strong competition. It is expected that this competition will lead to an increase in the number of compounds registered for treatment of disease, especially in oncology. This will increase intellectual property restrictions and increase focus on novel mechanisms, improved mechanistic understanding and a move toward best in class compared to first in class compounds.

There are currently more than 250 kinases available for testing and the pressures described above will ensure that rapid characterisation of inhibitor mode of action is essential.

Overview of ITC

ITC measures in a single experiment several characteristics of a binding interaction including the affinity (K_d), number of ligand binding sites (n), and the enthalpy of the binding reaction (ΔH).

The technique is rapid and requires no physical modification of the target protein, such as immobilisation or fluorescent labelling and can be used with proteins having no catalytic activity, which precludes study in enzyme kinetic assays.

The ITC experiment usually involves titrating the test compound against the target protein at a constant temperature, with the ITC instrument measuring the heat released or taken up during the binding event.

ITC can be used in several ways during protein kinase focused drug discovery. These include: characterisation of protein constructs and preparations (not only in terms of correct affinity for model ligands, but also for correct stoichiometry, allowing estimation of the amount of functional protein, without the need for catalytic activity); evaluation of assays (particularly important in the pharmaceutical industry where the requirement for high throughput may, for pragmatism, enforce less rigorous assay methodology); identification of the intermolecular complex giving biological activity (ITC can give information on whether the presence of another ligand has an influence on the biological activity of a test compound). It is on this application of ITC that this application note will focus.

Protein Quality Control

Before mechanistic studies are carried out it is useful, if not essential, to undertake a quality control investigation of the target protein. This should take the form of verifying the identity, purity, concentration, functionality and stability of the protein.

Calorimetric methods can be applied in two of these important areas.

ITC has been used to validate the functionality of target proteins by comparing the affinity and stoichiometry obtained on titration of a known ligand with literature values for a range of target proteins. DSC is a 'sister' technology that has been used to verify that the protein has a melting temperature, T_m , significantly above the temperature of the experiment. Often isolated kinase domains can be only partially stable, having T_m values around 40°C, (Figure 1). Application of techniques such as these to characterise a target protein prior to embarking on detailed mechanistic studies, can be time and cost effective in the long term, helping to avoid artefactual or misleading results due to poor protein quality.

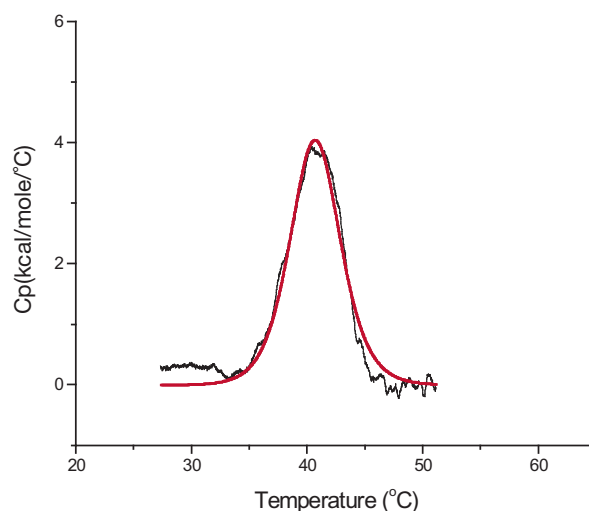


Figure 1

Figure 1: Use of DSC to investigate protein stability prior to ligand binding studies. Often isolated kinase domains have low melting temperatures, potentially indicating limited stability and highlighting the need for improved purification protocols, storage or assay conditions. The black line is the DSC data. The red line is a line of best fit to an unfolding model.

Understanding Mechanism of Action

Information on whether the presence of another ligand influences the biological activity of a test compound is crucial to several aspects of drug discovery. A second ligand may have no effect on the activity of the test compound, may compete either directly or indirectly with the binding of the compound, or may actually be required for the test compound to exert its effect. Understanding of the mechanism of action of the test compound can be useful in interpreting or predicting cellular activity at substrate concentrations different to that used to measure IC_{50} values. It can also give insight into the relevance of 3-D structures, which may be solved for different intermolecular complexes. Information on mechanism can also be used to devise subsequent assays, which can target a particular intermolecular complex. Recently, it has become clear that kinase inhibitors may preferentially bind to, or induce non-active conformations of the kinase protein. Comparison of the binding affinity of a test compound for each of these forms is also valuable in deciding whether to pursue compounds binding to the active or non-active form of the protein. Decisions of this type will affect the subsequent processes involved in kinase drug discovery.

Elucidation of Mechanism

As with all therapeutically important enzymes, kinases are not just a single molecular target for compound intervention. During the catalytic cycle the kinase binds protein substrate, ATP, intermediates and products, (Figure 2). These different enzyme forms may also exist in many different conformations. Thus there are different forms of the enzyme to which compounds may be directed, and to which different biochemical assays may be biased. Probing different mechanisms of inhibition is therefore useful in order to access different active pharmacophores providing potentially valuable intellectual property advantages. Alongside this, the physiological concentration of ATP is around 2mM, meaning that it is able to compete, often very effectively with compounds binding at the ATP site. Thus the search for compounds which bind before or after ATP, so called non-competitive compounds, or those that bind only after ATP, so called uncompetitive compounds are attractive approaches for kinase drug discovery. However, many historic kinase inhibitors target the ATP site of the enzyme, and are expected to compete with ATP for binding to the enzyme. Other compounds may target allosteric sites, which may be expected not to show this competition with ATP. Characterising

the mechanism of action of compounds allows identification of whether the presence of ATP, or indeed protein substrate increases, decreases or has no effect on the affinity of the test compound. Studies of this nature are invaluable in understanding SAR at a molecular level, and key to the search for novel pharmacophores.

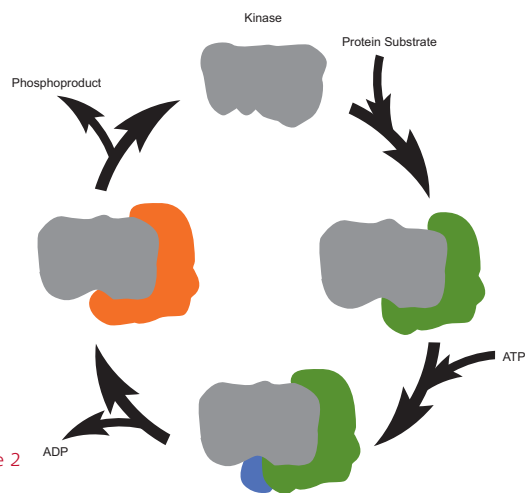


Figure 2

Figure 2: Putative catalytic cycle for a protein kinase. In this simplified scheme, the enzyme binds substrates and products during the catalytic cycle, and hence at least 4 enzyme forms are populated during the phosphotransfer reaction. Each of these different enzyme forms will be dynamic and may access many different conformations, illustrating that each kinase enzyme may represent several different targets for compound intervention. ITC allows measurement of binding affinity to at least some of these isolated enzyme forms facilitating understanding of inhibitor SAR.

Enzyme kinetic assays usually are not configured to populate particular enzyme forms occurring along the reaction pathway, and so information on the relevant enzyme form for maximal affinity is sometimes difficult to obtain directly. ITC may overcome this limitation by measuring binding affinities to different, predetermined enzyme forms. For example, binding to free enzyme is the simplest approach, but ITC conditions can be arranged to probe other enzyme forms, such as enzyme-protein substrate, enzyme-ATP, enzyme-ADP, enzyme-phosphoprotein complexes, depending upon the mechanism of catalysis. The use of non-hydrolysable ATP analogues can also be valuable in probing potential compound binding to the ternary complex of enzyme with both substrates.

Experiments to characterise the effect of ATP on the binding of a test compound were carried out for a protein kinase target. ITC titrations were carried out for a test compound in the presence and absence of 100 μM ATP (representing approximately $60 \times K_d$ for ATP), (Figure 3).

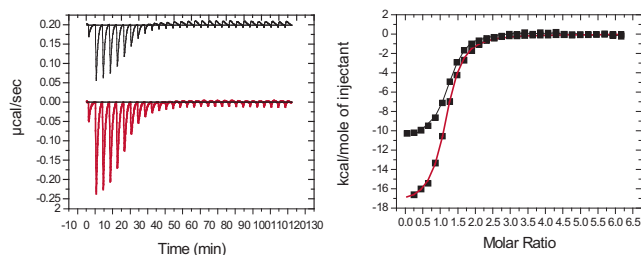


Figure 3

Figure 3: Non Competitive Inhibitor Binding. Titration of a test compound against a protein kinase target in the absence (red) and in the presence (black) of 100 μM ATP. The parameter values were, in the absence of ATP: $K_d = 0.19 \mu\text{M}$, $\Delta H = -17.4 \text{ kcal/mol}$, $n = 0.9$, and in the presence of ATP: $K_d = 0.17 \mu\text{M}$, $\Delta H = -9.4 \text{ kcal/mol}$, $n = 1.1$

The ITC results show clearly that there is no change in affinity for the compound binding to the kinase when ATP is included with the protein in the cell. The enthalpy values, which would not be obtained from any other technique, indicate that although there is no effect on the affinity, there is a significant effect on the enthalpy of binding. These results therefore suggest that the compound is non-competitive with respect to ATP binding, but that there may be some change in binding mode in the presence of ATP. This highlights not only that ITC is useful in characterising mechanistic details of compound binding, but also the dual probe nature of the technique in the measurement not only of affinity but also of binding enthalpy.

In a similar experiment, uncompetitive kinetics with respect to ATP, were observed for a different test compound binding to the same protein kinase. The K_d in the absence of ATP was $> 50 \mu\text{M}$ (not measurable in the standard ITC run) whereas in the presence of ATP it was measured at $0.7 \mu\text{M}$. (Figure 4).

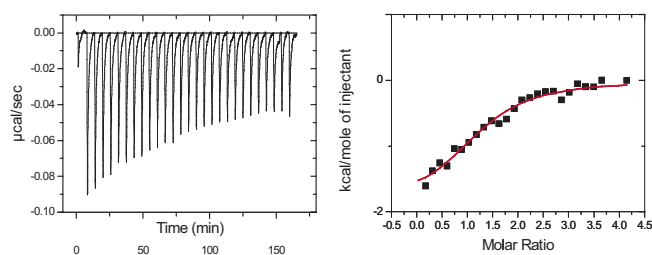


Figure 4

Figure 4: Uncompetitive Inhibitor Binding Titration. Titration for a test compound binding to a complex of protein kinase and ATP. Parameter values were $K_d = 0.56 \mu\text{M}$, $\Delta H = -2.3 \text{ kcal/mol}$, $n = 1.4$. No binding was observed in the absence of ATP.

The advantage of being able to monitor binding directly to individual protein complexes, rather than following binding occurring during the catalytic cycle, which may involve many complexes or intermediates formed during substrate turnover, is shown for another system, in which high affinity binding of a compound was suspected for a complex of two consecutive kinases in a signalling pathway. ITC allowed the binding of the compound to be studied to the upstream kinase alone and also to a complex of the upstream and downstream kinases, after first demonstrating that this complex did in fact form, again using ITC. A five-fold increase in affinity was demonstrated for the compound binding to the complex (Figure 5).

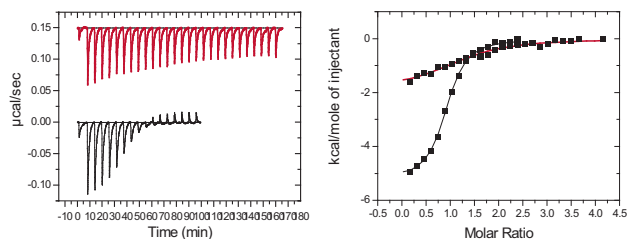
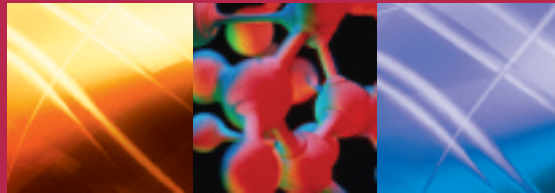


Figure 5

Figure 5: Comparison of compound binding to upstream kinase (red) and to a complex of this kinase with its downstream substrate kinase (black). A five fold increase in affinity is demonstrated for binding to the complex.

Summary

Calorimetric methods have proved to be invaluable in the study of protein kinase inhibition, facilitating protein quality control checks as well as contributing to the dissection of inhibitor binding mechanisms. The advent of higher throughput and automated instruments has added value to these applications, and the drive toward lower reagent consumption will ensure that calorimetric methods remain embedded in the process of rational drug design.



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