

Mechanism of Action Studies Tailored to Your Needs

Isothermal titration calorimetry is an ideal analytical method for MOA studies, allowing you the freedom to design your experiments the way you want.



Introduction

After a drug target has been identified, a rigorous evaluation needs to occur to demonstrate that modulation of the target will have the desired therapeutic effect. This involves intensive *in vitro*, as well as *in vivo* studies that provide information on the effects of the pharmacological intervention, leading to a valid understanding of the mechanism of action for a small molecule's biological activity. The result of these efforts is to establish sufficient knowledge so that physiologically relevant model systems can be developed into assays for downstream screening. This can have a significant impact on reducing the attrition rate of drug candidates due to clinical failures.

Isothermal Titration Calorimetry (ITC) can play a crucial role in the determination of the mechanism of action (MOA) of a small molecule's activity on a specified target pathway. Since ITC directly measures heat released or absorbed during a biomolecular binding event, no prior knowledge of the biological process is required and there is no requirement for labeling or immobilization.

Biomolecular interactions can be monitored in their natural state, often with endogenous enzymes, substrates or ligands. This has the added benefit of dramatically reducing the assay development time. ITC has also proven to be invaluable for assay development, testing the biological relevance of engineered *in vitro* assay systems that utilize a surrogate reporter to indicate biological activity. These results can be compared to the original endogenous mechanisms and give confidence that they mimic each other appropriately. This includes all forms of ligand and receptor complexes, as well as enzyme-substrate reactions and kinetics.

Introduction to Microcalorimetry

Isothermal titration calorimetry is a thermodynamic technique for monitoring any chemical reaction, initiated by the addition of a binding component and has become the method of choice for characterizing biomolecular interactions, (Figure 2).

When substances bind, heat is either generated or absorbed. Since ITC directly measures heat released or absorbed during a biomolecular binding event, it is the only technique which allows simultaneous determination of all binding parameters such as: stoichiometry (n), affinity (K_D), enthalpy (ΔH) and entropy (ΔS) in a single experiment, (Figure 1). This information provides a true picture of the biomolecular interaction.

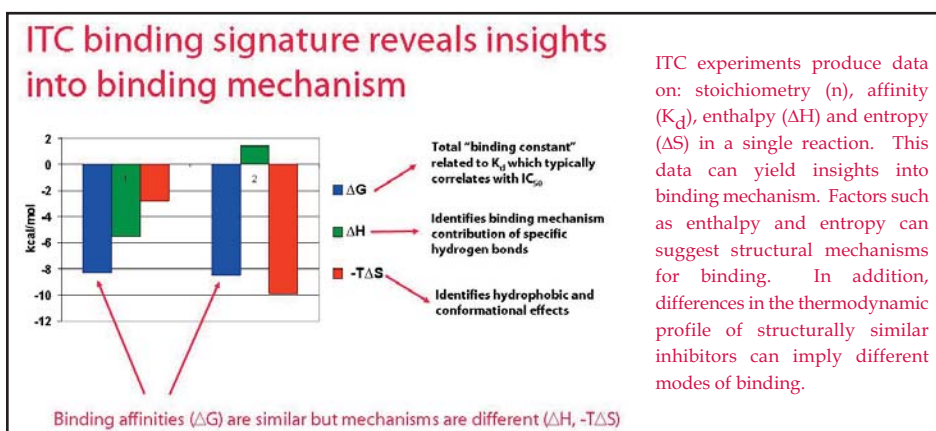


Figure 1: ITC provides much more than just binding affinity data (K_D). The thermodynamic binding signature can give insights into the mechanism of binding. In this example, both inhibitor molecules have virtually identical affinities (ΔG), but much different mechanisms

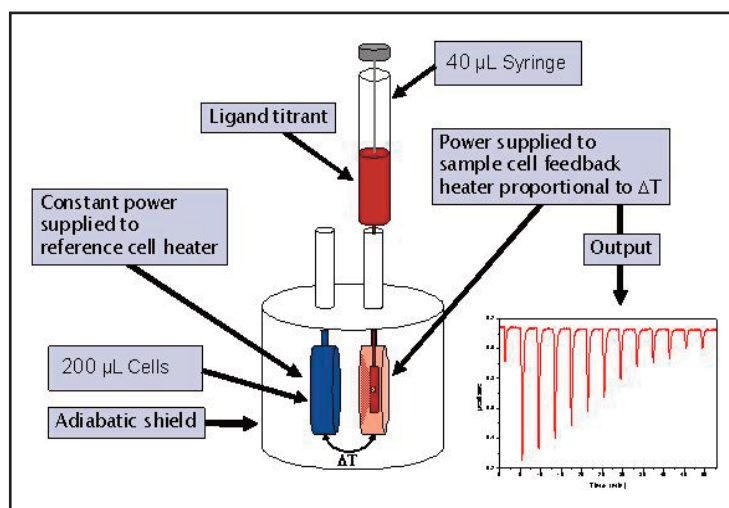


Figure 2: A syringe containing a “ligand” solution is titrated into a cell containing a solution of the “macromolecule” at constant temperature. When ligand is injected into the cell, the two materials interact, and heat is released or absorbed in direct proportion to the amount of binding. As the macromolecule in the cell becomes saturated with ligand, the heat signal diminishes until only background heat of dilution is observed.

Advances in ITC instrumentation

Advances in miniaturized sample cell design and ultra-sensitive electronics have addressed both sample consumption as well as time constraints allowing ITC to be effectively utilized at earlier stages of the drug discovery and development process. The latest microcalorimetry system from MicroCal, the iTC₂₀₀TM, reduces the quantity of protein (or other macromolecule sample) required to obtain a complete binding profile to as little as 10 µg. With its reduced size and associated sample quantity requirements, the iTC₂₀₀ provides a significantly faster equilibration time than previously available ITC instrumentation, thereby increasing the experimental throughput by 2-4 fold or up to 16 samples per eight hour day. For applications requiring higher throughput, a fully automated version the Auto-iTC₂₀₀TM has also been developed. This automated

system utilizes the iTC₂₀₀ as its calorimeter core, enabling an iTC₂₀₀ to be upgraded to a fully automated system at a later point offering throughput of up to 75 samples per day.



Benefits of ITC for MOA studies:

- Binding is measured in a biologically relevant environment
- There is no need for “activated” enzymes
- Complex processes can be systematically characterized unconstrained by assay limitations
- Thermodynamic data can provide insights into binding mechanism

ITC Applications for small molecules Drug Discovery:

- Differentiate competitive, non-competitive, and uncompetitive binding
- Assess effects of co-factors
- Assay validation
- Site-directed mutagenesis
- Works with virtually all binding reactions
 - Enzymes
 - Nuclear receptors
 - GPCR's
 - Membrane-bound proteins

Determination of kinase inhibitor binding modes¹

Enzyme kinetic assays usually are not configured to populate particular enzyme forms occurring along the reaction pathway, 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 or enzyme-phosphoprotein complexes, depending upon the mechanism of catalysis. Using ITC, entire binding mechanisms can be systematically characterized.

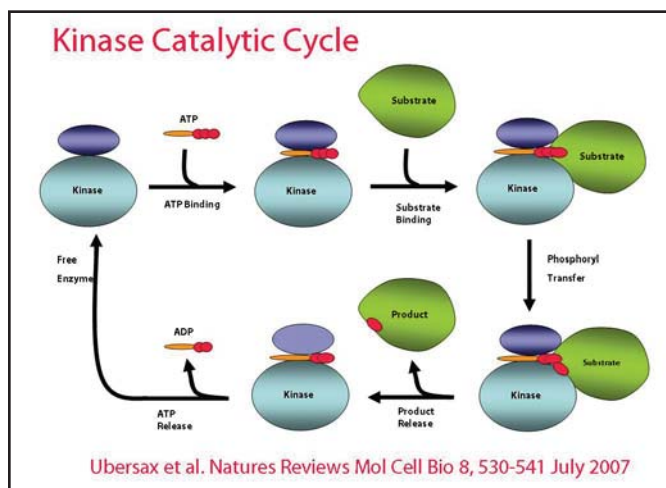


Figure 3: This schematic represents the catalytic cycle for a protein kinase reaction. ITC can be used to probe the binding mechanism of small molecule inhibitors at various stages of the process.

Scientist at AstraZeneca in Macclesfield, UK used ITC to characterize the effects of ATP on the binding of an inhibitor on a protein kinase target. This involved two simple experiments (Figure 4). The first experiment was to titrate the protein kinase target, contained in the sample cell, directly with the inhibitor solution. The resulting binding isotherm showed no heat was generated (kcal/mole of injectant), indicating no binding between the two molecules. The experiment was repeated with the protein kinase preincubated with ATP in the sample cell. The binding isotherm showed significant heat generated and a 0.7 μM binding affinity was measured for the small molecule inhibitor and the protein kinase. This clearly indicates that the inhibitor only binds in the presence of ATP, indicating that it is uncompetitive relative to ATP.

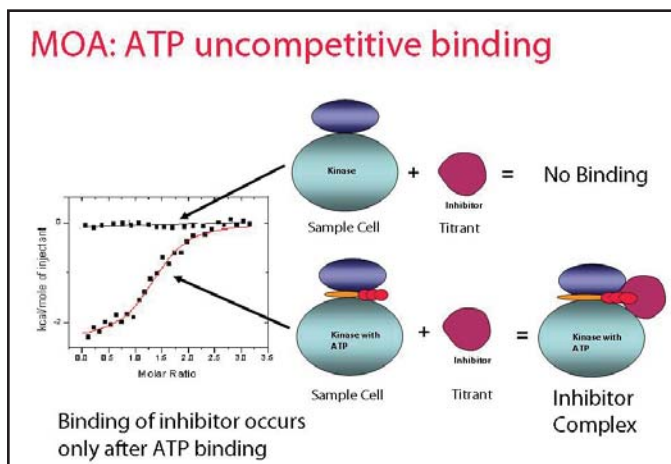


Figure 4: Illustrates the differences in the binding isotherms for a small molecule inhibitor and a protein kinase with and without ATP. The lack of binding before the addition of ATP indicates that the inhibitor is uncompetitive relative to ATP. (Data from Reference 1).

Similar experiments were carried out to characterize the effect of ATP on the binding of a non-competitive inhibitor to a protein kinase target. ITC titrations were performed with a test compound in the presence and absence of 100 μM ATP (Figure 5). The ITC results clearly show that there is no change in affinity for 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 confirm 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 that ITC is useful in characterizing mechanistic details of compound binding, as well as a determining binding affinities.

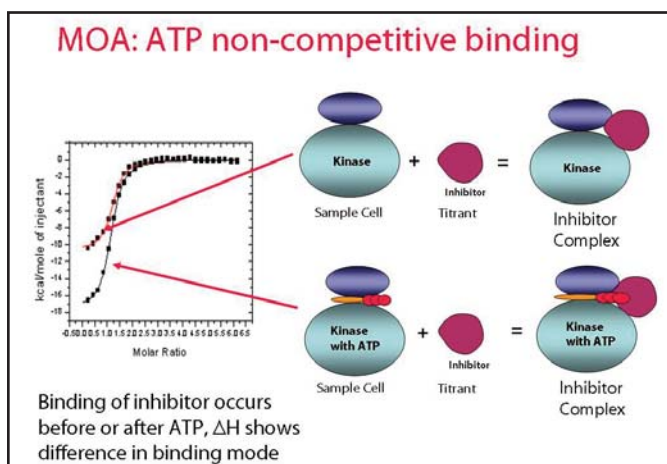


Figure 5: ITC binding isotherms for a small molecule inhibitor binding to a protein kinase with and without ATP. The inhibitor binds both before and after the addition of ATP indicating that it is non-competitive relative to ATP. (Data from Reference 1).

Establishing a full understanding of MEK1 inhibition²

The Mitogen-Activated Protein Kinase (MAPK) pathway is one of the most widely scrutinized signaling pathways in biology. This is because it plays such a fundamental role in cellular function making it a prime candidate for pharmacological intervention.

MEK1, as a member of this pathway (Figure 6), is very well characterized in terms of enzymatic activity, domain functions and inhibitor actions. There has been very little historic information available on the binding interactions between the isoform MEK1 and its ligand states with nucleotides (ADP, ATP) and small molecule inhibitors. Obtaining this knowledge is critical in establishing an understanding on the effects of inhibitors on MEK1 activation and its ability to phosphorylate its downstream substrate ERK2. Research has shown that these inhibitors are non-competitive with ATP, binding to an allosteric site.

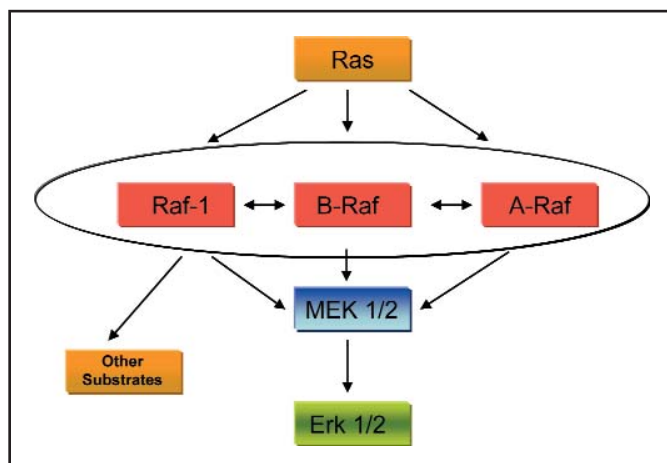


Figure 6: Mitogen-activated protein kinase cascade

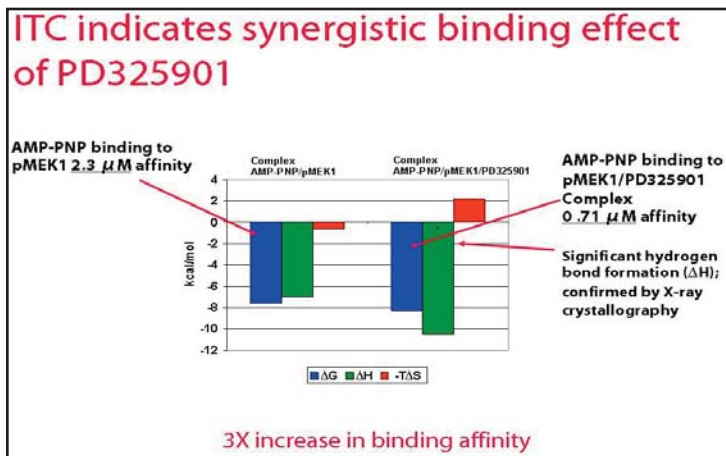


Figure 7: Bar graph comparison shows synergistic effects on affinity of nucleotide binding without and with PD325901 inhibitor. Binding mechanism shows significant hydrogen bond formation (ΔH). (Graph generated from data in Reference 2).

Scientists at Schering-Plough Research Institute in Kenilworth, NJ designed a series of experiments to evaluate the affinity and thermodynamics of two inhibitors, PD0325901 and U0126 with the various permutations of non-phosphorylated MEK1 (npMEK1), phosphorylated MEK1 (pMEK1) in the presence of ADP and AMP-PNP (ATP analog). Isothermal titration calorimetry was used because it gives a direct readout of binding affinity, as well as other important thermodynamic parameters. Traditional IC_{50} experiments are enzymatic reactions, so it is not possible to dissect the reaction down into its constituent components.

The results of these experiments yielded some very interesting observations. The binding affinity of AMP-PNP to the inhibitor PD325901/pMEK1 complex was 3-fold greater compared to the binding of AMP-PNP to pMEK1 alone (Figure 7) and similar results were obtained for npMEK1-nucleotide complex. This indicates the synergistic effect of the ternary complex on the binding affinity due to the inhibitor. There was also a significant difference increase in the enthalpy (ΔH) along with a shift in the entropy (ΔS), suggesting a much different binding mechanism in the presence of the inhibitor.

In contrast to PD325901, similar experiments run with inhibitor U0126 showed no such effect (Figure 8). Instead, the binding affinities were additive and the binding enthalpy (ΔH) was approximately 75% lower than PD325901, indicating that there was a much different binding mechanism. ITC can provide very important insights about the binding affinities and binding mechanisms which would be very difficult to determine by any other technique. The results of these experiments clearly indicate that these two inhibitor molecules act in very different ways.

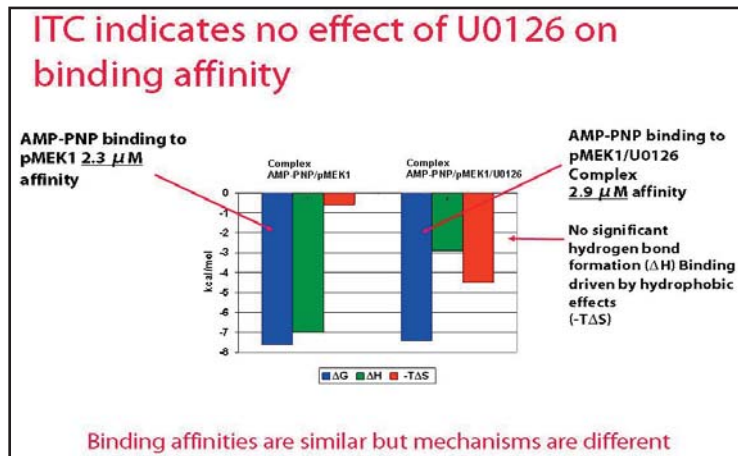


Figure 8: Bar graph comparison shows no effect on affinity of nucleotide binding without and with U0126 inhibitor. Binding mechanism shows hydrophobic effects. (Graph generated from data in Reference 2).

The modes of cofactor binding to the glucocorticoid receptor³

The glucocorticoid receptor is involved with the transcriptional regulation of genes associated with inflammation. This regulation occurs through a complex series of events, including either the binding of an agonist or an antagonist at the GR ligand binding domain (GR-LBD). This then results in the differential recruitment of a co-activator which turns on the gene expression process, or co-repressor which shuts down the gene expression process. Crystal structure data suggests that conformational changes induced in the GR-LBD by either the agonist or antagonist, are responsible subsequent selectivity for the down stream co-activator or co-repressor. Scientists at Boehringer Ingelheim in Ridgefield, CT utilized ITC as a technique to confirm this hypothesis³. A series of ITC experiments was conducted to demonstrate that it is the ligand that dictates the differential co-factor binding and that this process is driven by thermodynamic discrimination. Experiments were run using the agonist dexamethasone, which selectively bound the co-activator peptide, TIF2, and the antagonist RU486, which

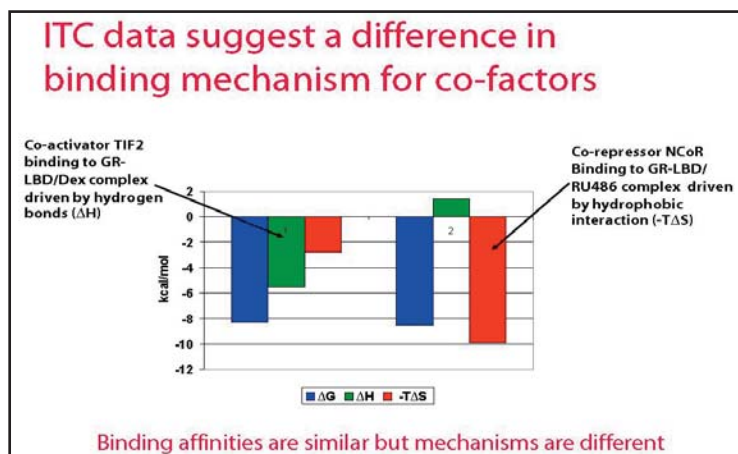


Figure 9: Bar graph comparison shows the differences in the binding mechanism for the co-activator TIF2 and co-repressor NCoR which leads to the conclusion that ligand induced conformational changes are responsible for the differential binding selectivity. (Graph generated from data in Reference 3).

selectively bound co-repressor NCoR. Both TIF2 and NCoR had very similar binding affinities (ΔG) for their respective ligated GR-LBD, yet the thermodynamic data indicated a very different mechanism of binding. TIF2 binding was primarily driven by a high degree of enthalpy (ΔH) or hydrogen bonding and NCoR binding was almost exclusively driven by favorable enthalpy (ΔS) or hydrophobic binding (Figure 9). This provides strong evidence that this process is driven by thermodynamic discrimination and that ligand induced conformational changes are responsible for the differential binding selectivity.

Understanding the mechanism of interaction for small molecule Caspase-3 inhibitors⁴

Caspases play a central role in apoptosis mediated diseases. Peptide-based inhibitors have not been very potent or selective, therefore research efforts are focused on small molecule inhibitors. Detailed structural and kinetic information is known on the overall activation and regulation mechanisms of the caspase family of cysteine proteases. However, little is understood about the specific details of caspase inhibition from a stoichiometric and thermodynamic perspective. This has made it very difficult to take a rational approach to small molecule inhibitor drug design.

Scientists in the Department of Neuroscience at Wyeth Research in Pearl River, NY have used microcalorimetry to better understand the mechanism of small molecule inhibition. Caspase-3 is a homodimer with two active sites. Only

inhibitors that complex with both active sites have been reported. Researchers at Wyeth evaluated three different classes of inhibitors: peptidomimetic, pyrimidindolones and isatins. These all have structural similarities and crystallographic results of these complexes indicated binding similarities. However, ITC stoichiometry and thermodynamic data revealed that the isatins are a new class of inhibitors with a new mechanism of inhibition. Experiments indicated that isatin binds with a stoichiometry of 0.5, or to only one of the two active sites (half-site reactivity). (Figure 10) This results in a conformational change and enzyme activity is inhibited 300-fold.

These research insights will lead to a better understanding of caspase-3 inhibition and potentially more potent and selective inhibitors.

Summary

These are just a few examples of situations where microcalorimetry has played a critical role in helping scientists address their research challenges. In order to develop novel therapeutics targeted towards regulating enzymatic processes, it is vital that we fully understand the way that enzymes behave *in vivo*. These theories then need to be applied to develop model systems that mimic these processes in a biologically relevant way. Microcalorimetry, unencumbered by the limitations of traditional techniques, is playing a valuable role in addressing these needs. Microcalorimetry has established itself as a critical path technology, as well as a gold standard for assessing the mechanisms of small molecule binding and kinetics to drug targets.

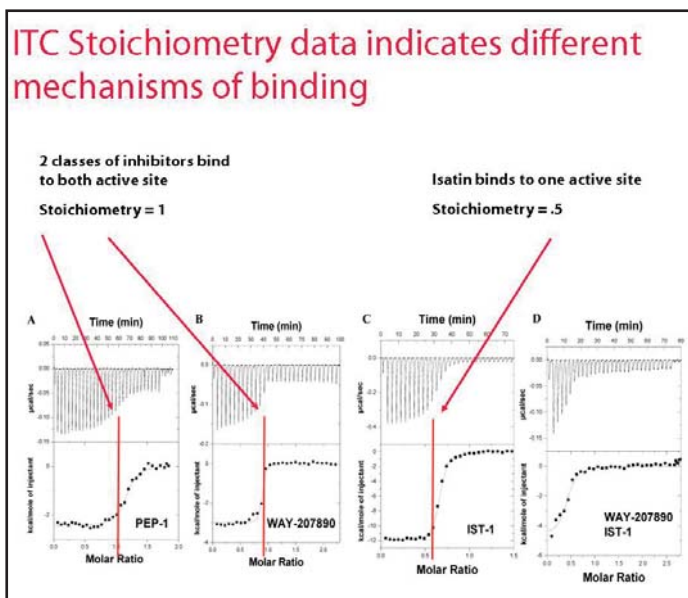


Figure 10: ITC binding isotherms for 3 classes of Caspase inhibitors. The Stoichiometry for two classes of inhibitors on the left is 1.0. However the stoichiometry value for isatins is 0.5 indicating that they only bind to one of the active sites. (Data from Reference 4).

ITC A Proven Technology for MOA Studies

- False artifacts can be eliminated
- Binding is measured directly
- No need for “activated” enzymes
- No need for surrogate readout mechanisms
- Complex processes can be simplified
- Thermodynamic profile can provide insights into binding mechanism

A complimentary CD containing over 7,900 literature citations for the use of microcalorimetry in the life sciences is available at your request. For more information, or to discuss your specific needs, please contact MicroCal.

References

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- 2 Smith, C. K. & Windsor, W.T. (2007) Thermodynamics of nucleotide and non-ATP-competitive inhibitor binding to MEK1 by circular Dichroism and Isothermal Titration Calorimetry. *Biochemistry* **46**, 1358-1367.
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