

Isothermal Titration Calorimetry – Gold Standard Information for Small Molecule Drug Discovery

Discover the Fundamentals of Biomolecular Interactions
with the Next Generation of Label-free Analysis



Introduction to Microcalorimetry

Isothermal Titration Calorimetry (ITC) 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.

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. This information provides a true picture of the biomolecular interaction.

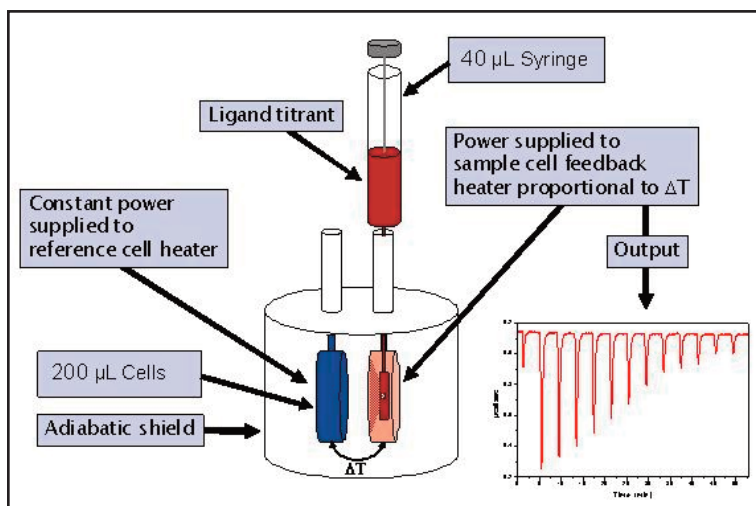


FIGURE 1: A syringe containing a “ligand” solution is titrated into a cell containing a solution of the “macromolecule” at constant temperature. When ligand is injected into the cell, the two materials interact and heat is released or absorbed in direct proportion to the amount of binding. As the macromolecule in the cell becomes saturated with ligand, the heat signal diminishes until only background heat of dilution is observed.

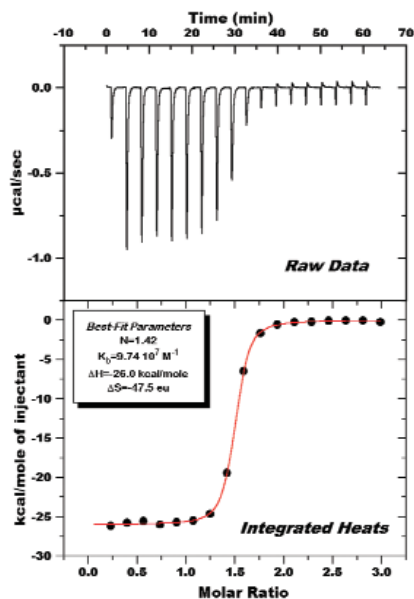


FIGURE 2: Representative ITC data. Twenty injections of ligand solution are added to protein solution in the ITC cell. The area underneath each injection peak (top panel) is equal to the total heat released for that injection. When this integrated heat is plotted against the molar ratio of ligand added to macromolecule in the cell, a complete binding isotherm for the interaction is obtained (bottom panel). The one site model was used to fit the data. The values for stoichiometry, binding constant and enthalpy are shown in the box.

The Latest Innovation in Microcalorimetry the iTC₂₀₀TM and the Auto-iTC₂₀₀TM

Isothermal Titration Calorimetry has gained wide acceptance in drug discovery and development laboratories throughout the world. Major pharmaceutical and biotech companies, as well as research institutions, are now utilizing ITC to fully characterize biomolecular interactions. Binding parameters determined by ITC are often referred to as “gold standard” values and are frequently used as a reference for other techniques.

As modern ITC instrumentation has evolved, these instruments have become more sensitive, faster and easier to use. The new iTC₂₀₀ and Auto-iTC₂₀₀ from MicroCal, use a 200 µl cell, reducing the sample required to obtain a complete binding profile to as little as 10 µg of protein. The miniaturized sample cell design and ultrasensitive electronics have addressed both the sample consumption and the time constraints that have previously limited the use this powerful technique.

The iTC₂₀₀ provides a significantly faster equilibration time than previously available from traditional ITC instrumentation, thereby increasing the experimental throughput by 2-4 fold.

For applications requiring higher throughput, the Auto-iTC₂₀₀, a fully automated version has been developed. This automated system utilizes the iTC₂₀₀ as its calorimeter core and offers unattended throughput of up to 75 samples per day.

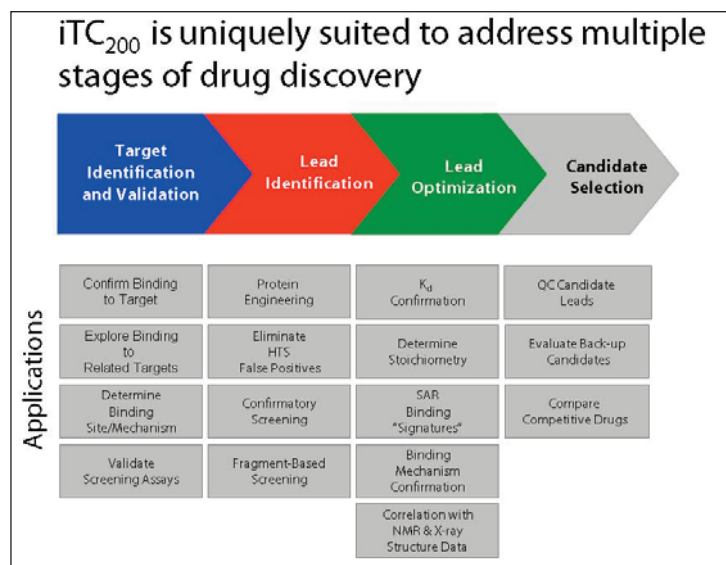


FIGURE 3: The iTC₂₀₀ and the Auto-iTC₂₀₀ now make it possible to effectively utilize isothermal titration calorimetry at earlier stages in the drug discovery and development process.

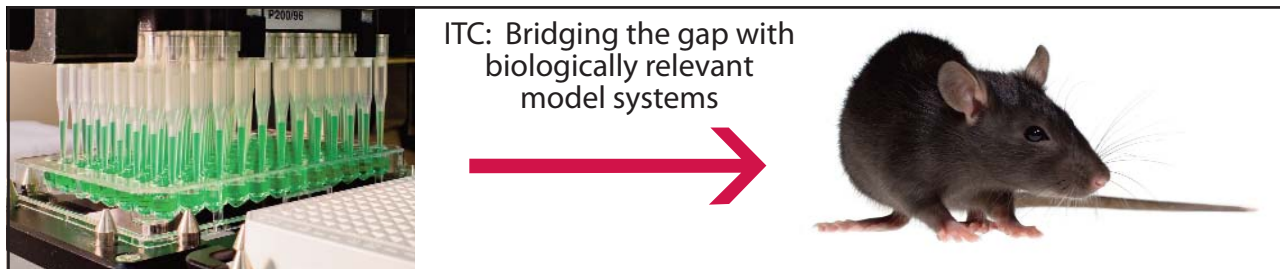


Figure 4: One of the key benefits of ITC is the unique capability to create an experiment that is biologically relevant. No other technique offers a completely label-free and in-solution assay environment requiring no immobilization of either the target macromolecule or ligand. The use of ITC can play a significant role in establishing and validating relevant model biological systems.

Mechanism of Action Studies and Assay Development¹⁻⁸

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 can play a critical role in the determination of the mechanism of action (MOA) of a specific 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. 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 proven to be a valuable tool for assay development, i.e. testing the biological relevance of engineered assay systems that utilize a surrogate readout scheme 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. Entire binding mechanisms can be systematically characterized. For example, comparison of ligand binding can be made in all phases of the catalytic process (Figure 5), with free enzyme, enzyme-substrate complex, or enzyme product, whether the enzyme is active or non-active. This can give a true picture of competitive, noncompetitive, and uncompetitive ligand binding¹.

This ITC data can yield insights into the binding mechanism. Factors such as enthalpy and entropy can suggest structural mechanisms for binding. In addition, differences in the thermodynamic profile of structurally similar inhibitors can imply different modes of binding^{2,3}. The stoichiometry can be a useful tool to assess percentage functionality of expressed proteins¹. It can also be used to better understand the mechanism of inhibition, i.e. a stoichiometry of 0.5 could indicate half site reactivity of a protein target that is a homodimer³.

ITC has also been used to study the binding of agonists versus antagonists with nuclear hormone receptors, G-coupled protein receptors and ligand-gated ion channel receptors. The thermodynamic signature can provide insights into mechanism of action for coactivation or corepression⁴. Binding studies can also be performed in conjunction with techniques like site-directed mutagenesis to determine the location of catalytic domains and other "hot spots"⁵.

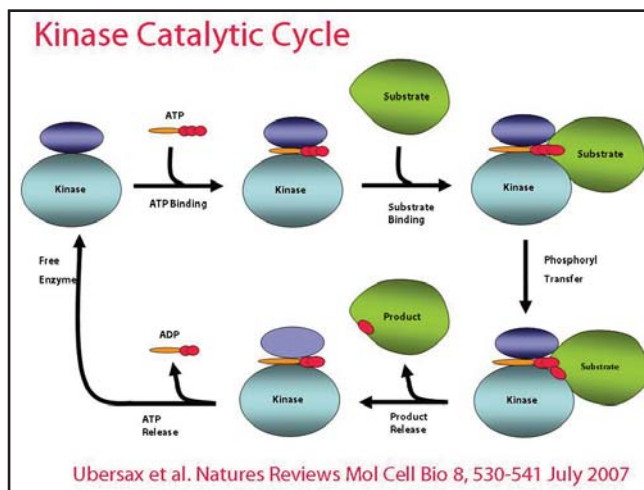


Figure 5: Kinase Catalytic Cycle

Hit Validation

It is common for approximately 1% of the compounds from high throughput screening (HTS) to demonstrate some level of antagonistic or agonistic effects during the assay campaign, hence being categorized as hits. The next step is to validate these by repeating the primary screening assay. The hits that repeat will go into a secondary screening hit selection process to help choose the best candidates to go into lead optimization.

Secondary screening will yield more detailed dose response information, allowing the comparison of the affinities of the compounds for the target. This is usually represented as IC_{50} data, with a good hit having $<10 \mu M$ potency. Among the challenges at this point, is to cull the false positives and assign a level of quantitative intelligence to help guide the decision making process. The ITC provides the only direct measurement of binding affinity (K_d) and the data correlates very well with IC_{50} 's (Figure 6). However decisions as to which hits to move forward are often based on total binding affinity only.

In addition to total binding affinity, a single ITC experiment can give insights into the binding mechanism; this includes "lock and key" forces such as hydrogen bonds, van der Waals interactions (ΔH), which are an opportunity to improve selectivity, hydrophobic interactions (ΔS) and stoichiometry (n). This information can be invaluable for rank order determination and can eliminate false positives, thereby offering an excellent compliment to traditional counterscreening techniques. The Auto-iTC₂₀₀ can provide complete binding profiles for up to 75 samples per day and offers a very compelling platform for assessing the quality of lead molecules.

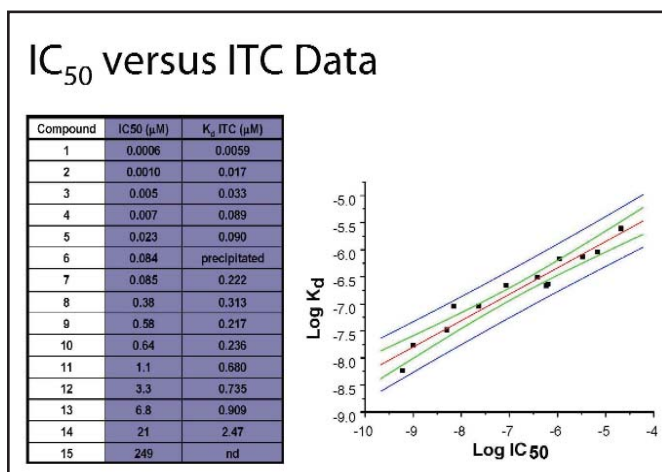


Figure 6: IC_{50} data correlates well with ITC K_d values. Data presented at the 2007 Current Trends in Microcalorimetry Conference, by Dr. Stephanie Leavitt, Gilead Sciences, San Francisco

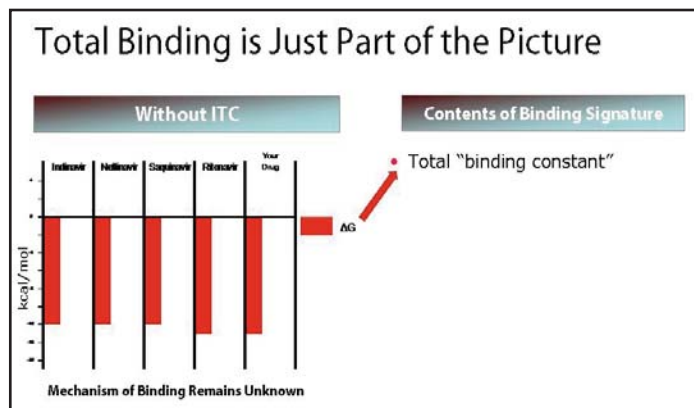


Figure 7: (ΔG) is directly related to total binding affinity, but does not give any insights into binding mechanism.

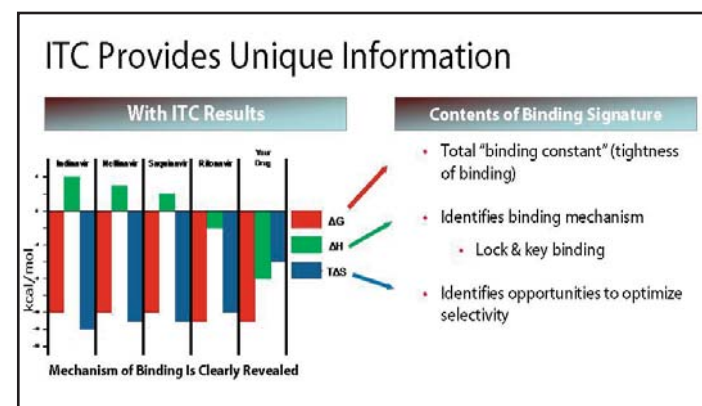


Figure 8: ITC provides (ΔG) as well as (ΔH) and (ΔS), giving a true picture of binding mechanism.

Fragment-Based Screening⁹⁻¹²

The strategy behind fragment-based screening involves probing the ligand binding domain of a protein target with low molecular weight (<200 daltons) fragments. The objective is to identify binding "hot spots" that often exist as only small regions on the target protein. Dissecting a ligand into smaller fragments can provide insights into understanding the role of key functional groups in a protein-ligand interaction.

The fragments that exhibit the best binding to the ligand binding domain still have very low affinities, in the low mM range. Fragments with the best ligand efficiencies (MW/affinity) can then be combined to form high affinity binders. In principle, fragments that occupy a receptor site simultaneously will yield a much higher affinity when linked together than just as the sum of their individual affinities, i.e. the affinities are synergistic (Figure 9). These more complex fragment molecules then go into lead optimization programs.

The Auto-iTC₂₀₀ offers several benefits for screening fragment libraries. Since it measures the heat of the binding interaction directly there is no need for chemical modification or assay design, so physiological relevant biological models can be used. It is capable of measuring a broad range of affinities in the mM to nM range and producing a full thermodynamic profile on up to 75 compounds per day. In addition, the binding enthalpy (ΔH) is of particular interest in fragment-based screening because it is a measure of very specific hydrogen bonds and van der Waals interactions. ITC is the only technique that directly measures this parameter.

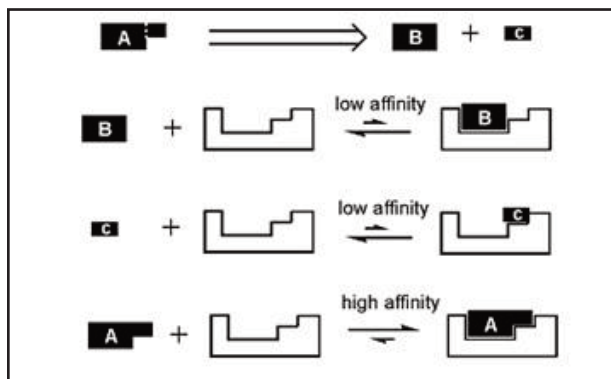


Figure 9: Schematic of fragments binding to "hot spots" where binding affinities are synergistic.¹¹

Lead Optimization¹³⁻¹⁹

Optimizing leads from hit to lead discovery usually starts off with selecting the leads from secondary screening with proven specificity and the highest binding affinities to the target of interest. Often binding affinities start off in the 1-10 μM range, requiring potency improvements of up to five orders of magnitude before they would be considered viable drug candidates. The lead optimization stage will often be accompanied with structural information from ligand-target X-ray crystallization data.

Data generated during secondary screening such as IC_{50} curves can equate chemical structures with binding affinity. This information can help infer structural modifications that have the potential to improve potency. However, very little information may be available that will give insights into how and why the hits bind to the target. Selecting the best compounds to move into lead optimization will dramatically improve the chances of candidates with desirable potency and selectivity.

Isothermal Titration Calorimetry fulfills a very important role by providing information on the binding forces of ligands to their target macromolecule. It is a direct readout technique and a single experiment can yield a wealth of information about binding. This includes a quantitative measure of attractive forces such as hydrogen bonds and van der Waals interactions (ΔH), hydrophobic interactions (ΔS) and

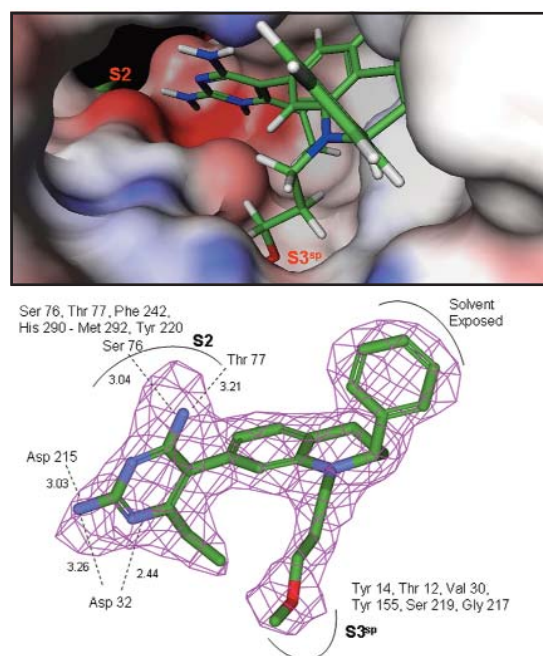


Figure 10: X-ray crystal structure of renin with inhibitor.¹³

stoichiometry (n). The information from ITC is complimentary to X-ray structural data and can be utilized help guide the medicinal chemistry process, ultimately resulting in a drug candidate that has the best potency and selectivity.

The ITC provides thermodynamic data that can be used to guide the synthesis of lead templates. In this example, a rennin inhibitor has undergone a series of four iterations of synthesis (Figure 11). The result is a lead compound with a 45-fold improvement in binding affinity from the initial hit. In addition, the ITC and X-ray data indicates that the binding is predominately due to hydrogen bonding (ΔH). This is much more desirable than affinity due to hydrophobic attraction alone.

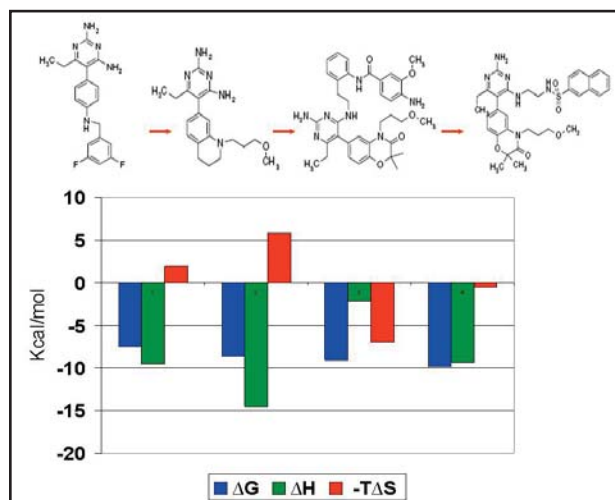


Figure 11: ITC data can be used to guide the process of lead optimization.¹³

iTC₂₀₀ Isothermal Titration Calorimeter

The iTC₂₀₀ system is the most sensitive isothermal titration calorimeter available. It is designed to address the needs of today's life science researchers and is based on over 30 years of experience developing state of the art microcalorimeters. With its reduced size and associated sample quantity requirements, the iTC₂₀₀ is significantly faster than previous models. Designed for ease of use, all functions of the iTC₂₀₀ are operated through software to facilitate fast and accurate analyses. Applications include the characterization of molecular interactions of small molecules, proteins, antibodies, nucleic acids, lipids and other biomolecules.



iTC₂₀₀

iTC₂₀₀ features

- Directly measure sub-millimolar to nanomolar binding constants (10^2 to 10^9 M⁻¹). Measure nanomolar to picomolar binding constants (10^9 to 10^{12} M⁻¹) using the competitive binding technique.
- Investigate any biomolecular interaction with high sensitivity. Experiments require only 200 μ l of sample and as little as 10 μ g of protein in the sample cell.
- True in-solution technique: No labeling or immobilization required. No buffer restrictions. Easily handles turbid solutions.
- Easy to use: Includes user friendly experimental design wizards and easy filling and cleaning procedures.
- Fast time to first result: With fast equilibration times, up to two runs per hour can be easily accomplished. Requires no assay development or dedicated user to achieve high quality results.
- Operating temperature range of 2°C to 80°C.
- Complete system: No additional accessories to purchase. No reagents are required.
- Includes the iTC₂₀₀ Washing Module for easy cell and syringe filling and cleaning.
- Automatable: iTC₂₀₀ can be upgraded to a fully automated version, the Auto-iTC₂₀₀.

Auto-iTC₂₀₀ Isothermal Titration Calorimeter

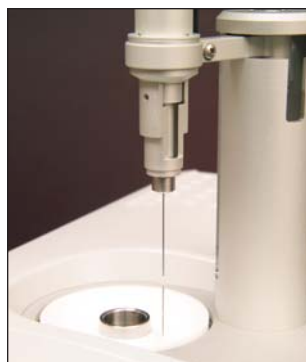
For applications requiring higher throughput, a fully automated version of the iTC₂₀₀ has been developed, the Auto-iTC₂₀₀. This automated system utilizes the iTC₂₀₀ as its calorimeter core. The Auto-iTC₂₀₀ combines all of the features of the iTC₂₀₀ along with the convenience of fully unattended automation. The Auto-iTC₂₀₀ provides, for the first time, access to complete binding profile data using samples quantities and throughput rates comparable to traditional secondary and confirmatory screening techniques.



Auto-iTC₂₀₀

Auto-iTC₂₀₀ features::

- Throughput of up to 75 samples per day with a capacity to run 384 samples unattended.
- Totally unattended operation: All filling, data collection and cell cleaning functions are fully automated.
- Capacity for up to four standard 96 well plates for loading ease. Can also accept up to five 30 ml vials.



A: At Rest



B: Filling



C: Titration



D: Washing

Manual operation A-D of iTC₂₀₀

References

1. Holdgate, G., (2007) Revealing kinase inhibitor Mechanisms: ITC leads the way. *MicroCal Applications Note*.
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.
3. Aulabaugh, A., Kapoor, B., Huang, X., et al. (2007) Biochemical and biophysical characterization of inhibitor binding to caspase-3 reveals induced asymmetry. *Biochemistry* **46**, 9462-9471.
4. Kroe R., Baker M., et al. (2007) Agonist versus antagonist induced distinct thermodynamic modes of co-factor binding to the glucocorticoid receptor. *Biophysical Chemistry*, **128**, 156-164.
5. Milne J., et al. (2007) Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* **450**, 712-716.
6. Freire, E. (2007) A new era for microcalorimetry in drug development. *European Pharmaceutical Review* **5**, 73-78.
7. Krell, T. (2008) Microcalorimetry: A response to challenges in modern biotechnology. *Microbial Biotechnology* **1**, 126-136.
8. Leavitt, S. (2007) Calorimetry, binding and thermodynamics in the drug discovery arena. *2007 Trends in Microcalorimetry Conference Review*.
9. Whitesides, G., Krishnamurthy, V. (2006) Designing ligands to bind proteins, *Quarterly Reviews of Biophysics*, 1-11.
10. Ciulli, A., Williams, G., Smith, A., Blundell, T., Abell, C., (2006) Probing Hot Spots at Protein-Ligand Binding Sites: A fragment-based approach using biophysical methods. *Journal of Medicinal Chemistry*, **49**, 4992-5000.
11. (2006) Fragment-Based Drug Discovery: Study low affinity ligands by ITC. *MicroCal Applications Note*.
12. Turnbull, W. B. (2005) Divided We Fall? Study low affinity fragments of ligands by ITC. *MicroCal Applications Note*.
13. Sarver, R.W., Peevers, J., Cody, W. L., et al. (2008) Binding thermodynamics of substituted diaminopyrimidine renin inhibitors. *2007 Trends in Microcalorimetry Conference* (E. Reese and S. Spotts, eds. MicroCal Northampton, MA), 3-24.
14. Ohtaka, H., Freire, E. (2005) Adaptive inhibitors of HIV-1 protease. *Biophysics and Molecular Biology* **88**, 193-208.
15. Carbonell, T., Freire, E. (2005) Binding thermodynamics of statins to HMG-CoA reductase. *Biochemistry* **44**, 11741-11748.
16. Velazquez-Campoy, A., Freire, E (2005) ITC in the Post-genomic Era...? Priceless. *Biophysical Chemistry* **115**, 115-124.
17. Ruben, A., Kiso, Y, Freire, E. (2006) Overcoming roadblocks in lead optimization: A thermodynamic perspective. *Chemistry Biology Drug Design* **67**, 2-4.
18. Holdgate, G. (2007) Thermodynamics of binding interactions in the rational drug design process. *Expert Opinion Drug Discovery* **2**, 1103 -1114.
19. Lee, K., Behnke, M., Foley, M., Ramarao, M., et al. (2008) Benzenesulfonamide indole inhibitors of cytosolic phospholipase A2 alpha: Optimization of *in vitro* potency and rat pharmacokinetics for oral efficacy. *Bioorganic & Medicinal Chemistry* **16**, 1345-1358.

GE Healthcare
MicroCal Products Group
22 Industrial Drive East
Northampton, MA 01060
USA
Tel: 413 586 7720/800 633 3115
Fax: 413 586 0149

www.microcal.com

GE Healthcare
MicroCal Products Group Europe
2 Warren Yard, Warren Farm Office Village
Wolverton Mill, Milton Keynes MK12 5NW
United Kingdom
Tel: +44 (0) 1908 576330
Fax: +44 (0) 1908 576339
info@microcal.eu.com



Ultrasensitive Calorimetry for the Life Sciences™

ITC-400
© 2008 MicroCal, LLC.
Printed In USA