PERSPECTIVES

INNOVATION

Adding calorimetric data to decision making in lead discovery: a hot tip

John E. Ladbury, Gerhard Klebe and Ernesto Freire

Abstract | Recognition of the limitations of high-throughput screening approaches in the discovery of candidate drugs has reawakened interest in structure-based and other rational design methods. Here, we describe how isothermal titration calorimetry can be used to obtain thermodynamic data on the binding of compounds to protein targets. We propose that these data — particularly the change in enthalpy — could provide a valuable, complementary addition to established tools for selecting compounds in lead discovery and for aiding lead optimization.

Heavy investment in high-throughput screening (HTS) methodology has provided the pharmaceutical industry with large numbers of 'hit' compounds. However, making a decision about which compounds should be taken into later stages of the process of candidate drug identification for a given target is not straightforward. Post-screen decision making on hit compounds is crucial. It has a substantial effect on the temporal and financial burden of lead discovery and optimization, and on the likelihood of clinical success of the eventual candidate drugs. It therefore seems advisable to include as much complementary data in this process as possible.

Isothermal titration calorimetry (ITC) is an approach for measuring the heat energy associated with a molecular interaction[1–4] (BOX 1). In less than two decades, it has developed from a technique based on instrumentation built by a small number of devotees in their own laboratories to become a widely used method for which instruments capable of measuring the heat of interactions in the nanojoule range are commercially available. This sensitivity is sufficient to enable essentially all biological equilibrium interactions to be measured at a given constant temperature. The wide availability of high-sensitivity and automated ITC instrumentation has enhanced the opportunity to provide additional information for decision making in lead discovery and optimization.

A single ITC experiment provides an evaluation of the change in free energy (ΔG) and its component quantities: the change in enthalpy (ΔH) and change in entropy (ΔS) (BOX 1). The ΔG value provides quantification of the affinity of binding between two interacting molecules, although in general this information can be derived from alternative methods that are cheaper and more widely accepted by pharmacologists (for example, the half-maximal inhibitory concentration (IC_{50}) value). However, the evaluation of the compound affinity or potency alone often does not provide a clear indication of which compounds to select for further progression as, at the most difficult points in the process, the compounds will often have similar values for these parameters. In this article, we discuss how measuring contributing enthalpic and entropic terms — in particular, ΔH — could provide valuable information for decision making in lead discovery and optimization, especially when high-resolution structural data are available.

ΔH as a descriptor of an interaction

In a single experiment, ITC provides a measure of the heat energy (ΔH_{obs}) associated with forming a macromolecular complex at a given temperature. This enthalpic term is a direct measure of the net change in the number and/or strength of the non-covalent bonds on going from the free to the bound state. So, what value can be added by including these thermodynamic data in the lead discovery process?

First, it should be noted that the ΔH for an interaction has not been shown to correlate with any of the Lipinski parameters for oral bioavailability[5,6]. This is to be expected, as this characteristic is not affected by the enthalpy or entropy balance of a compound for its target protein, but rather by the interactions of the compound with other proteins and lipids that affect drug pharmacokinetics. Therefore, ΔH does not substitute for established inputs to the lead discovery process, but does provide information on the balance of forces that control the mode of binding of a compound to its target and can be used to provide a comparison of these forces between series of compounds.

Numerous attempts to correlate ΔH with the structural changes involved in the transition from the free to the bound state have been made but, for the most part, these have not proved rigorous. For example, although ΔH and surface area burial correlate in studies of protein folding[7–9], this relationship is often not seen in investigations of the binding of small molecules. The reason for this is that, on folding, most or all of the potential hydrogen-bond donor and acceptor functionalities that are buried in the protein are satisfied. However, in drug design, there is a high probability that a polar group is found buried in a position that cannot establish a non-covalent interaction. Desolvation of that polar group will incur an enthalpic penalty that is not compensated for by the enthalpically favourable formation of a bond. Indeed, unfavourable (that is, positive) enthalpies usually arise from incorrectly positioned polar groups[8,10].

A recent survey of the thermodynamic parameters generated by ITC of over 250 distinct protein–ligand interactions[11] revealed that interactions involving compounds from medicinal chemistry programmes have on average a proportionately greater favourable entropic contribution to the ΔG than natural, biological ligands. This is likely to reflect the ease with which ΔS can be improved compared with ΔH. Thus, drug designers seem to have 'over

Box 1 | **Isothermal titration calorimetry**

Isothermal titration calorimetry (ITC) allows the highly accurate determination of thermodynamic parameters to be made with no requirement for chemical modification, labelling, immobilization or limit on the size of interacting species. ITC directly measures the heat of interaction (change in enthalpy, ΔH_{obs}) at a constant temperature on titrating two compounds — for example, a protein and a small-molecule ligand — of known concentration that form an equilibrium complex. This provides a probe with which to determine the extent of interaction over the course of the experiment.

The ITC measurements include contributions from all equilibria that occur as the interacting molecules go from the free to the bound state, including those associated with solvent interactions and macromolecular conformational changes. The thermodynamic terms are therefore considered as observed rather than absolute values, as denoted by the subscript 'obs'. At any point in the titration, the amount of free or bound ligand can be determined, establishing the equilibrium binding constant, K_B, which is the inverse of the dissociation constant, K_D. The change in free energy, ΔG_{obs}, can be calculated from $\Delta G_{obs} = -RT\ln K_B$, in which R is the gas constant and T is the absolute temperature. The change in entropy, ΔS_{obs}, is calculated with the knowledge of ΔH_{obs} and ΔG_{obs}, as $\Delta S_{obs} = (\Delta H_{obs} - \Delta G_{obs})/T$.

If only the ΔH term is required, this can be determined from one or a small number of additions of a ligand in which the concentration regime is set up such that all of the molecules added in an aliquot of ligand bind to molecules of the protein in the calorimeter cell.

There are no specific classes of proteins to which ITC experiments are most suited; however, issues such as aqueous solubility, potential conformational changes and membrane localization will dictate the protocol adopted and the required control experiments to be done. To gain the most from thermodynamic data, high-resolution structural detail is important. As this is a prerequisite for rational drug design, empirical thermodynamic data can be valuable in this endeavour.

used' the strategy of adding hydrophobic functionalities to an initial lead skeleton or making its framework more rigid, resulting in a more favourable ΔS, to increase potency rather than optimizing ΔH. Indeed, it is well established that, over the course of lead optimization, the entropic term usually improves as the programme proceeds. However, there is a limit to the improvement that can be gained from increasing the hydrophobic nature of a compound, as solubility is ultimately compromised.

The reason why lead optimization is characterized by an increasing ΔS contribution is clear: *de novo* design of non-covalent bonds between a protein and a lead compound in a binding site to enhance ΔH is exceptionally difficult. Nevertheless, to achieve high-affinity binding, favourable entropic and enthalpic contributions are necessary. Interestingly, studies of two drug classes with several approved members — the statins and the HIV protease inhibitors — have shown that enthalpically optimized compounds are best-in-class rather than first-in-class compounds[12] (FIG. 1). It might seem simple in principle to look at a crystal structure of a complex and, for example, identify potential hydrogen-bonding groups in the protein towards which functional groups in the compound might be extended. However, in practice, obtaining bonds of the optimal length and angle is difficult and often achieved only serendipitously[13]. Furthermore, it is difficult to estimate the effect of the solvent on the interacting components of polar interactions. The energy required to desolvate a polar group is substantial. This means that simply adding potential bonding moieties with the aim of establishing non-covalent interactions can considerably reduce the overall affinity.

In addition, an improvement in the binding enthalpy does not necessarily produce a higher binding affinity. The ubiquitous phenomenon of enthalpy–entropy compensation means that entropy losses often negate enthalpy gains, resulting in no affinity gains. So, to achieve affinity gains, it is necessary to overcome enthalpy–entropy compensation. During the process of affinity optimization, two aspects seem to be responsible for most of the compensation of enthalpic gains: the structuring of protein regions by newly formed hydrogen bonds or other polar interactions, resulting in a loss in conformational entropy; and the overexposure of non-polar groups, resulting in a loss in solvation entropy[12,14,15].

Structural information on several compounds of a given class allows the number of hydrogen bonds and charge–charge interactions to be estimated. Based on theoretical considerations, this estimate can be quantified to indicate the contribution of these features to the ΔG of binding. However, although this approach based on 'docking' and additive group contributions has proved its worth on numerous occasions and forms a mainstay of many structure-based approaches to ligand design, it lacks empirical input. Using the directly measured calorimetric ΔH values on these related compounds provides a more rigorous quantification.

Using ΔH to aid decision making

The ΔH term is governed by the number of non-covalent bonds that are made (or broken) between the molecules involved in the complex, and so a favourable ΔH indicates a net increase in the number or overall strength of bonds on forming an interface. Therefore, knowledge of the heat derived from the net change of non-covalent interactions on forming a complex between a therapeutic target and a small molecule is useful as, in principle, compounds with more favourable ΔH values make additional and/or stronger bonds.

Taking an idealistic example, the thermodynamic data for the binding of two similar compounds to a protein target that results in the burial of similar hydrophobic surface area could be used to decide which compound should be taken to the next stage of development, based on their ΔH values. The most favourable ΔH value indicates the compound that is forming the most effective non-covalent interactions in the protein-binding site. So, given that it is harder to improve the ΔH contribution than to improve the ΔS contribution, as discussed above, this compound (in the absence of other data on compound performance) would be selected. Extrapolation of this principle to situations in which the structures of the compounds differ substantially from one another increases the need for high-resolution structural input to assess the probable solvent effects. Nonetheless, the general assumption is that more favourable ΔH values signify better non-covalent bond complementarity between the protein and the compound.

As mentioned above, optimization of the enthalpic contribution can be seen retrospectively over the time course of development of the statin class of cholesterol-lowering drugs, which inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. The progression to the best-in-class compound (from left to right in FIG. 1a) is associated with considerable improvement in the ΔH contribution, suggesting that increasing the net favourable bonding was beneficial to the optimization of the drug. A similar improvement is also apparent when retrospectively

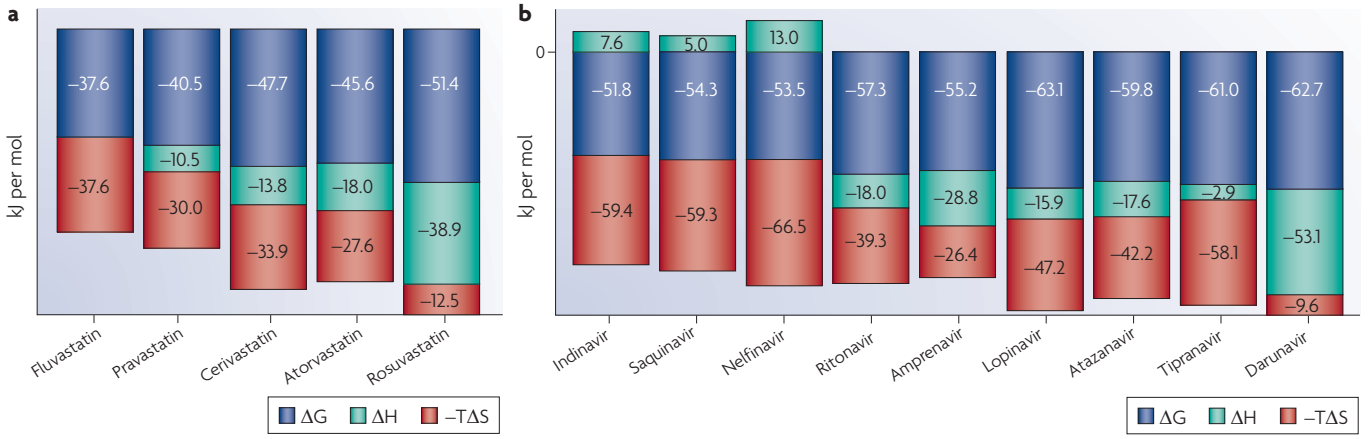

Figure 1 | **Examples of enthalpic optimization towards best-in-class compounds. a** | Thermodynamic profile of the binding of a series of statins to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. The sum of the change in enthalpy (ΔH; shown in green) and the change in entropy (ΔS) multiplied by the absolute temperature (T) (shown in red) gives the change in free energy (ΔG; shown in blue). Analysing the various compounds approved in this class over time (left to right) reveals enhancement of the ΔH contribution. **b** | Thermodynamic profile of the binding of a series of small-molecule inhibitors to HIV protease, with the same colour coding as in part **a**. The contribution of ΔH to the binding energy increases considerably from the first drug to be approved to the most recently approved drug. Adapted from REF. 12.

analysing the various HIV protease inhibitors that have been developed (FIG. 1b). In this case, the enthalpic contribution to binding from early molecules, such as indinavir (Crixivan; Merck), is actually unfavourable (positive). The progression towards the best-in-class compound is associated with a gradual (if intermittent) improvement in the ΔH contribution (that is, it becomes more negative). These two examples of enhanced enthalpic contributions to improving drugs in a given class illustrate the potential value of determining thermodynamic data. It suggests that, had initial compounds been selected based on the criterion of ΔH, best-in-class compounds could have been identified more quickly.

Enthalpic efficiency

Similar to the efficiency indices that have previously been proposed[16–18], ΔH provides a viable measure with which medicinal chemists can rank compounds. In the optimization process, increasing the potency of a lead compound is usually accompanied by an increase in molecular mass[19]. Screening for potency alone can therefore result in compounds of lower molecular mass being discarded. This strategy could be particularly problematic in the assessment of fragment molecules.

The use of efficiency indices (such as 'ligand efficiency' $\Delta g = \Delta G/N_{\text{non-hydrogen atoms}}$) has been suggested as a tool for medicinal chemists[16]. This term provides a useful quantification, improvement of which does not require simply increasing the potency of the compound, but focuses the decision making or synthetic-chemistry strategy on the atom types or the molecular mass. The enthalpic efficiency (EE)[20] (for example, EE = ΔH/Q; in which Q is the number of non-hydrogen atoms or molecular mass) provides a measure that relates the changes in non-covalent bonding with the size of the molecule. A perhaps more relevant evaluation could be derived from the specific EE (that is, $EE = \Delta H/N_{pol}$; in which N_{pol} is the number of polar atoms), which directly indicates the contributions of hydrogen-bond donors and acceptors to the binding enthalpy and therefore reflects the strength of those bonds. Comparison of the EE values of a series of compounds with similar structural templates could have a substantial impact on the decision-making process.

The use of a ΔH term also focuses the chemist on what type of interactions the compound might make. For example, the EE is not likely to improve dramatically during lead optimization if the chemist uses the strategy of simply adding more hydrophobic surface area, as the thermodynamic effect of this would be largely entropic in nature. However, it has been shown that there is a limit to the number of non-covalent interactions that can be included in a binding site[11,21]. So, for example, a different strategy may be taken for a lead compound or low-molecular-mass precursor with a high EE than for one with a low EE, to avoid synthesis of compounds that have more groups with the potential to form non-covalent interactions than there are potential counterpart groups available in the protein binding site.

Application to fragment screening

One potential application of EE in decision making is in the area of fragment-based lead discovery. The fragment-based approach involves identifying (through various screening protocols) compounds of low molecular mass (M_r < 100–250 or 8–18 non-hydrogen atoms[22]) that bind to an appropriate site on a target protein. These fragments will typically bind with lower affinity than compounds from screening protocols that use larger entities; however, they provide initial starting points for developing lead compounds. This can be done by building out from the fragment by adding chemical moieties to interact with proximal bonding groups on the target. Alternatively, in the case in which multiple low-molecular-mass entities bind in different but proximal positions in the target binding site, they can be linked to produce a higher-affinity lead compound[23,24].

Usually, the fragments bind with low affinity (mM to 30 μM), which in some cases results in K_D values that preclude the use of ITC unless a competition binding assay is used. However, determination of the EE requires that only the ΔH is measured. This can be obtained from an ITC experiment even in the absence of a K_D determination. The small molecule can be added to the protein target under a concentration regime whereby all the ligand binds, which provides a direct readout of the enthalpy of binding.

Although data are currently limited, it seems that initial ITC screening of small fragment molecules often results in a favourable ΔH_{obs}, making ITC detection a realistic proposition (G. Williams, Astex

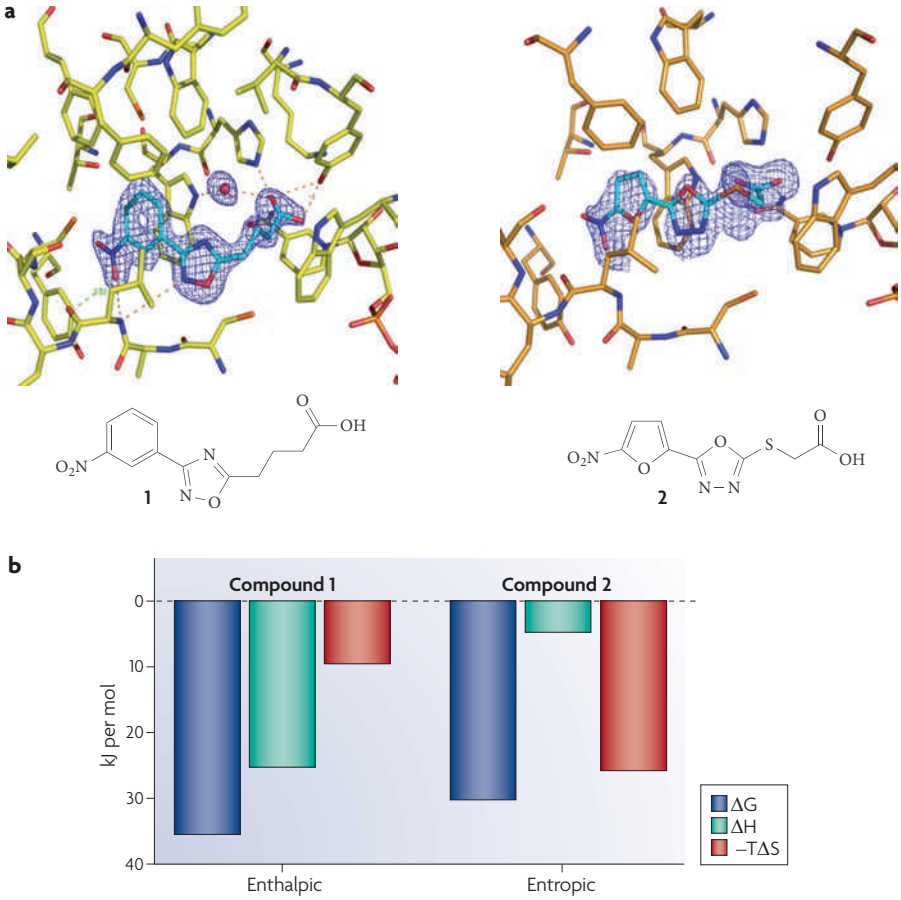

Figure 2 | **Effect of inclusion of a water molecule on thermodynamic parameters. a** | The two similar ligands shown differ considerably in the extent to which entropy and enthalpy contribute to their respective thermodynamic signatures for binding to the protein aldose reductase. As the X-ray crystal structural analysis in part **a** shows, the complexes formed by the two ligands with aldose reductase differ by the acquisition of one water molecule. **b** | The inclusion of the water molecule in the complex on the left of part **a** results in a less favourable entropy contribution (shown in red) but a more favourable enthalpy contribution (shown in green), owing to the additional hydrogen bonds. ΔG, change in free energy; ΔH, change in enthalpy; ΔS, change in entropy; T, absolute temperature. Adapted from REF. 26.

Therapeutics, personal communication). This enthalpic driving of small-molecule interactions is perhaps not surprising as, for complex formation to occur in the absence of substantial release of water from the small-molecule binding site, there has to be sufficient enthalpic contribution to overcome the entropic penalties of rigid-body binding. In addition, the initial fragment is expected to have a more polar than apolar character, as specific non-covalent interactions are based on hydrogen bonds or charge–charge interactions. At this stage, the evaluation of the EE of compounds could provide a guide as to which compound should be selected. This has been described for fragment screening of the Src SH2 domain[25]. In this example, one of the fragment hits derived from a ^{19}F nuclear magnetic resonance screen showed better EE characteristics than the physiological ligand (phosphotyrosine) that it was to inhibit (hit-specific EE = –2.38 kJ per mol; phosphotyrosine-specific EE = –1.0 kJ per mol). Further development of the hit could also be guided by the EE at each stage of synthesis as described above for lead development.

Other contributions to binding enthalpy

It is necessary to determine the enthalpy for the interaction in the protein binding pocket to obtain an accurate EE of a drug candidate. A number of factors can impinge on this determination. Differences in the residual solvation structure of two similar ligands can considerably alter the characteristics of the thermodynamic profile. A superimposed release or acquisition of a water molecule into the binding interface shifts the thermodynamic properties towards an entropic or enthalpic advantage, respectively[26]. FIGURE 2 shows the effect of inclusion of a water molecule on the thermodynamic parameters. Compound 1, which includes an interfacial water molecule in the complex, has a less favourable ΔS term than compound 2, but the increase in ΔH owing to the additional hydrogen bonds with the water molecule have a substantial effect on the affinity (and hence ΔG)[26]. Structural information about the corresponding complexes is required to make an assessment of this effect.

A second overlaid effect that can affect the thermodynamic profile is potential changes in the protonation state of the functional groups of the ligand or the protein. Protonation of an atom on a functional group is accompanied by a ΔH term that is strongly dependent on the chemical environment of the atom; for example, the heat of ionization is considerably larger for a nitrogen-containing group than an oxygen-containing group[27]. Protonation states depend on the pK_a (the logarithm of the acid dissociation constant) values of the groups involved; however, the pK_a value can vary considerably depending on the chemical environment of the functional group, for example, during complex formation. Thermodynamic data recorded from different buffer conditions provide a rapid way of assessing and correcting for the number and enthalpic contribution of accompanying protonation events[28–30].

Finally, differences in residual mobility contributions, involving the protein or the ligand, also have an effect on the net enthalpic change that occurs on complex formation. High residual mobility of chemical moieties of the protein and/or ligand in the final complex is entropically beneficial. By contrast, a more firmly fixed ligand contributes a stronger enthalpic response and a reduced entropic contribution[31]. These differences should be taken into account to allow for a conclusive interpretation of the thermodynamic binding profile with respect to enthalpy. In favourable cases, such properties are indicated by the temperature factors or the quality of the electron density that is available from the crystallographic analysis or from molecular dynamics simulations performed in parallel.

Conclusion

The ultimate goal of predicting the affinities of compounds for highly resolved, structurally defined binding sites on target proteins has not yet been achieved. However, thermodynamic-based approaches provide accurate experimental guidelines for the

optimization of drug–target interactions. In an ideal scenario, high-resolution structural information on the free and bound interacting molecules would permit some assessment of the bonding in the binding site, the positioning of water molecules and conformational changes. In combination with available structural detail, the input of thermodynamic data can aid the decisions about which compounds to select for further investigation, as well as their optimization. The throughput of ITC instrumentation precludes its use as a primary screening tool, and the strength of thermodynamic data is greatest when comparing a small number of similar compounds at the secondary or tertiary screening stage. Here, we have highlighted the use of the ΔH term and proposed EE in particular as a measure of the quality of a molecule that could be a valuable complement to the data typically used in lead discovery and optimization.

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Competing interests statement
The authors declare no competing financial interests.

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