ELSEVIER

Can biochemistry drive drug discovery beyond simple potency measurements?

Patrick Chène

Biochemistry Unit, Oncology Research, Novartis Institutes of BioMedical Research, Basel, Switzerland

Among the fields of expertise required to develop drugs successfully, biochemistry holds a key position in drug discovery at the interface between chemistry, structural biology and cell biology. However, taking the example of protein kinases, it appears that biochemical assays are mostly used in the pharmaceutical industry to measure compound potency and/or selectivity. This limited use of biochemistry is surprising, given that detailed biochemical analyses are commonly used in academia to unravel molecular recognition processes. In this article, I show that biochemistry can provide invaluable information on the dynamics and energetics of compound–target interactions that cannot be obtained on the basis of potency measurements and structural data. Therefore, an extensive use of biochemistry in drug discovery could facilitate the identification and/or development of new drugs.

Drug discovery is a long process that covers many different fields of activity. It starts with the identification of a new drug target and ends with the launch of a new molecular entity that efficiently treats a human disease and saves lives. Throughout this difficult endeavor, many different skills (scientific or otherwise) are needed at specific steps in the drug discovery process. One such 'skill' is biochemistry. This relatively 'old' field in the natural sciences is used at a very early stage in drug discovery during the identification of the initial hit compounds and their optimization to lead compounds. It therefore holds an essential position in pharmaceutical research, because no drug discovery program[1] can progress to its successful end without any such compounds.

However, the contribution of biochemistry in some drug discovery areas seems to be relatively modest. This is reflected by an examination of 288 research articles published in the field of protein kinases between 2000 and 2010 in the *Journal of Medicinal Chemistry*. Among these articles, 260 were communications in which biochemistry was only used to estimate compound potency and/or selectivity, and 28 were communications in which biochemistry was used to provide more-detailed information on the mode of action of the tested compounds. This analysis reveals that, in many research articles, the drug discovery teams who describe new molecular entities consider that potency and/or selectivity measurements – in most cases combined with structural data (structure of compounds bound to the target) – are sufficient to gain a precise insight into the mode of action of these new molecules. This is rather surprising, given that the scientific literature contains many examples of studies, conducted mainly in academia, where a precise understanding of ligand–target interactions is obtained only when structural analyses are combined with detailed biochemical studies. This suggests that either this level of understanding is not required when developing new kinase inhibitors or it is not well appreciated by the pharmaceutical industry that valuable information could be gained if additional biochemical studies were carried out.

In this review, I show what information can be gathered from biochemical studies and how it contributes to a better understanding of target–compound interactions. Given that drug discovery is a very large field, I focus on one target class: the protein kinases. These proteins were chosen because an enormous amount of work has been done in this area over the past 25 years, and many publications in biochemistry, chemistry and structural biology are currently available. This offers the possibility of evaluating the impact of biochemistry in a mature drug discovery area. As the field of protein kinases is so huge, not all articles involving the

E-mail address: patrick_chene@yahoo.com.

[1] This of course only applies to drug discovery programs where small synthetic molecules have to be generated and so not to programs aimed at the identification of therapeutic antibodies or other protein therapeutics.

1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2012.01.022

successful use of biochemistry are covered here, so this review is not exhaustive. To complement the cited studies on protein kinases, examples taken from other target classes are also cited. Again, these articles represent a few selected studies from the literature. This review is divided into three parts: enzymatic studies, binding kinetics and thermodynamics. These topics cover areas where biochemistry reveals information on molecular recognition that can be used by drug discovery teams to better analyze the properties of their compounds.

Enzymatic studies

Potency and selectivity determination

Although some researchers involved in drug discovery might consider that enzymatic assays are not as relevant as cell-based assays for studying the properties of compounds, these assays are at the root of many research programs in the pharmaceutical industry. Indeed, for many such programs, the primary screening assay that should provide the initial hits is an enzymatic assay. A poorly designed assay can lead to poor (if any) validated hits[2] and consequently result either in the program being terminated or in painstaking alternative activities to identify starting points for medicinal chemistry. Given that enzymatic assays are so essential for the identification of initial hits, I spend some time discussing them.

It is important to use the most appropriate assay in the most appropriate conditions to identify modulators of the studied target protein. This calls for an essential step in the development of any enzymatic assay[3]: the determination of the kinetic properties of the studied enzyme. Kinetic properties are taken to mean the kinetic mechanism and kinetic parameters of the enzymatic system. Determination of the kinetic mechanism enables the rate equation that will be used to obtain the kinetic parameters of the studied enzymatic system to be defined. This is key information for establishing 'balanced assay conditions'. In such conditions, the likelihood that the inhibitors will be identified following diverse modes of actions is enhanced [1]. These activities might appear to be academic; however, they are of great help for characterizing compounds.

Enzymatic assays are primarily used in drug discovery to estimate compound potency, which is commonly expressed as IC_{50} (i.e. the concentration of compound required to inhibit 50% of enzymatic activity under the assay conditions). Given that this parameter is widely used, it is important to recall what it means. IC_{50} does not measure the affinity of compounds for a target protein, as the inhibition constant (K_I) does, but expresses the ability of a compound to compete with a substrate (or a labeled tracer) present in the assay at a defined concentration. For most inhibitors (except noncompetitive inhibitors), any change in the substrate concentration found in the assay will also result in a change in the IC_{50}, but the K_I will remain the same. This is understood when looking at Cheng–Prusoff's equation (Eqn 1; Fig. 1) for competitive inhibitors of monosubstrate enzymes [2]. It can be deduced from Eqn 1 that IC_{50} increases linearly with [*S*]. Therefore, comparing IC_{50} values can be misleading (e.g. when [*S*] is not the same in different assays), but comparing K_I values is better practice. The situation is more complex with bisubstrate enzymes, such as protein kinases. Yang *et al.* presented equations that can be used for different kinetic mechanisms and various types of inhibitors [3]. Brockhoff *et al.* [4] proposed an extended equation (Eqn 2; Fig. 1) for ATP competitive inhibitors of bisubstrate enzymes following a random Bi–Bi mechanism (for a better understanding of kinetic mechanisms [5]), the most common kinetic mechanism described for protein kinases [6]. This equation reveals a complex relationship between IC_{50} and K_I, and, because it is common practice to calculate K_I values from IC_{50} values, Eqn 2 reveals that, without a precise knowledge of the kinetic parameters, accurate K_I values cannot be calculated from IC_{50} values.

Biochemical assays are commonly used in the field of protein kinases to estimate compound selectivity, and IC_{50} values are often used for this purpose.[4] However, compound selectivity should be assessed by comparing K_I values and not IC_{50} values. To illustrate this, I consider competitive inhibitors of both monosubstrate and bisubstrate enzymes. For monosubstrate enzymes tested in assay conditions such that $[S] = K_{m(S)}$, using Eqn 1, $IC_{50} = 2K_I$. Therefore, the ratio between the IC_{50} measured with

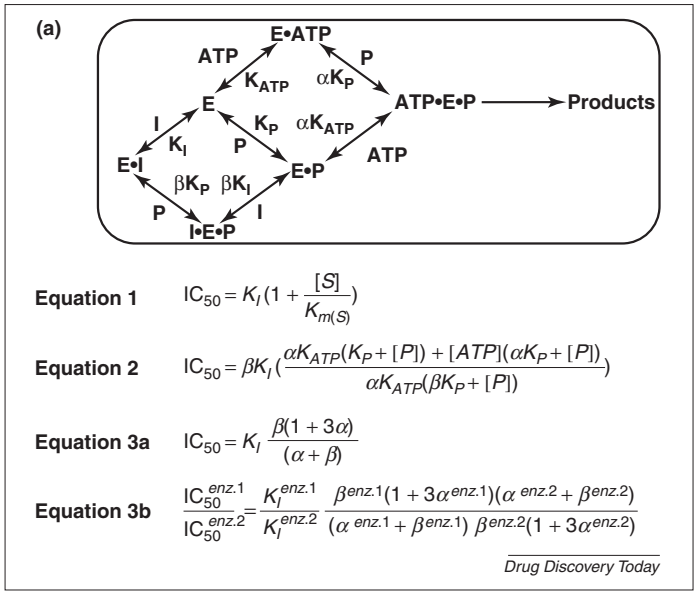

FIGURE 1

Enzymatic studies. Schematic representation of a random Bi–Bi mechanism where the kinase, E, is inhibited by the ATP competitive inhibitor, I. P corresponds to the protein and/or peptidic substrate. The meaning of the different kinetic parameters and of the different equations is given in the main text. Note that for Eqn 2 at [ATP] ~ 0 and $[P] = \alpha K_P$, $IC_{50} = K_I[\beta(1 + \alpha)/(\alpha + \beta)]$. Therefore, unless $\alpha = \beta = 1$, IC_{50} is different from K_I.

[2] Pharmaceutical companies have been engaged in the field of protein kinases for many years. Therefore, their compound libraries should contain many molecules that will score as potent hits in primary screens even though these assays have not been fully optimized. In the case of new targets for which compound libraries might contain few (potentially weak) modulators, the design of well-optimized assays is essential, otherwise these weak starting points will be missed.

[3] For nonenzyme targets, a kinetic mechanism cannot be determined, but a detailed understanding of the studied biological system is needed. For example, for protein–protein interactions, it is important to measure the strength of the interaction and to identify the key residues at the interface. This latter activity can be carried out for example by site-directed mutagenesis and/or using synthetic peptides.

[4] It should be kept in mind that several factors might also affect compound selectivity in cells.

two enzymes: $(IC_{50})^{enz.1}/(IC_{50})^{enz.2}$ is $K_I^{enz.1}/K_I^{enz.2}$. In this case, the IC_{50} ratio provides an estimate of compound selectivity because it is directly linked with the K_I ratio. For protein kinases following a random Bi–Bi mechanism, when assays are run at $[ATP] = \alpha K_{ATP}$ and $[P] = \alpha K_{Pep}$ (P protein or peptide substrate), Eqn 2 becomes Eqn 3a (Fig. 1) and the IC_{50} ratio for a compound tested with two different enzymes is given by Eqn 3b (Fig. 1). In this case, the relationship between IC_{50} and K_I depends on two kinetic parameters: α, which represents the interaction between both substrates, and β, which reflects the interaction between the protein substrate and the inhibitor I (an ATP competitive inhibitor in the above example) (Fig. 1). Values ranging from 0.04 to 130 have been measured for α with protein kinases [6] and values ranging from 1 to 49 have been determined for β with protein kinase inhibitors [4,7,8]. Given that these two parameters vary to such a large extent, the IC_{50} ratio (Eqn 3b) might differ substantially from the K_I ratio, and accurate selectivity cannot be obtained without knowing α and β.

This shows that, even for the most basic information (potency and selectivity) that can be obtained from enzymatic assays, a precise knowledge of the kinetic parameters is required to compare compounds accurately.

Mode-of-action studies

Enzymology can provide more information than the measure of compound potency and selectivity, and can lead to a better understanding of the mode of action of compounds. Such studies can easily be done and, in the absence of structural information, can help to confirm and/or validate hypotheses made by medicinal chemists. Mode-of-action studies are typically used to determine whether an inhibitor is competitive, noncompetitive or uncompetitive. However, using examples, I show that additional knowledge can be gained from such studies. Liu *et al.* [9] showed that low-molecular-weight inhibitors of cdk5/p25 target four different forms of this enzyme and they estimated the affinity of these inhibitors for each of these forms. Making such findings using other methods can be painstaking. Burke *et al.* [10] studied the mode of action of BMS-345541, an inhibitor of the IκB kinase. They performed various kinetic measurements, which led them to propose that BMS-345541 binds to similar sites of nuclear factor kappa-B kinase subunit alpha and beta (IKK-1 and IKK-2) and affects the active sites of these two subunits in a different manner. Brockhoff *et al.* [4] studied the properties of NVP-AEW541, a selective inhibitor of insulin-like growth factor 1 receptor (IGF1R). Their kinetic studies enabled them to propose a model in which this ATP competitive inhibitor binds to a conformation that is intermediate between the active and the inactive conformation of the enzyme. Vanderpool *et al.* [8] identified low-molecular-weight allosteric inhibitors of checkpoint kinase 1 (CHK1) and showed that the potency of some of these molecules is reduced in the presence of ATP. These authors propose that the formation of a complex between ATP and CHK1 affects the compound binding site and that the volume of the allosteric pocket occupied by the compound is reduced in the presence of the nucleotide.

Therefore, classic enzymology can be used to identify compounds, to measure their potency and/or selectivity and also to provide information on how they interact with their target. These mechanistic studies, which can easily be implemented, can be used by medicinal chemists during compound optimization to validate hypotheses on the mode of action of the synthesized compounds.

In many cases, the only information used to optimize compounds, aside from potency measurements, comes from the structures of these molecules bound to their target. Although there is no doubt that structural knowledge is invaluable, this does not necessarily provide an insight into two essential aspects of molecular recognition: the kinetics and energetics of the interaction. Here, when used together with structural data, classic biochemistry can produce information that enables a deeper understanding of the mode of action of compounds.

Binding kinetic studies

Binding kinetic parameters

Over the past few years, several reviews (e.g. [11–14]) have led to increasing interest in binding kinetic studies in the drug discovery community. This interest is motivated by the fact that the *in vivo* pharmacological behavior of compounds can be linked to their binding kinetic properties.

To introduce the essence of binding kinetics in a few words (interested readers are directed to [15]), the simple case of a one-step binding is presented in Fig. 2. To form the T·C complex, the target T and the compound C associate at the rate k_1 commonly called k_{on}. The T·C complex dissociates to release T and C at the rate k_2, usually called k_{off}. At equilibrium, as much T·C complex forms as dissociates. The equilibrium is reached at the rate called k_{obs} (apparent first-order rate constant), which is defined by Eqn 4 (Fig. 2). These different rates enable several parameters to be calculated: the dissociation constant of the complex (K_d; Eqn 5, Fig. 2), the dissociative half-life of the complex ($t_{1/2}$; Eqn 6, Fig. 2) and the time required to reach 95% of equilibrium ($t_{eq}^{95\%}$; Eqn 7, Fig. 2). Each of these parameters provides specific information on the interaction under study.

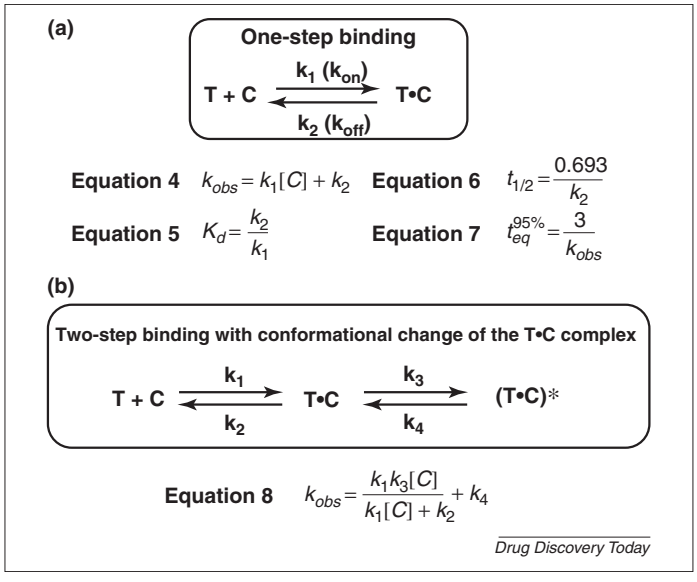

FIGURE 2

Kinetic binding studies. Schematic representation of a one-step **(a)** and a two-step **(b)** binding mechanism. The meaning of the different equations is given in the main text. T and C represent the target and the compound, respectively.

When potency has been substantially improved, compounds often bind slowly to their target protein, and are called 'slow-binding inhibitors' [16–18]. Given that maximum inhibition is only obtained at equilibrium, any measurement carried out with such compounds before equilibrium will give lower potency values. This is experimentally observed when increased pre-incubation times between a compound and its target lead to a reduction of the measured IC_{50} values (e.g. [19,20]). $t_{eq}^{95\%}$ can be used to provide an estimate of how long it takes to be close to equilibrium (full equilibrium is in theory reached at $t = \infty$) and thus to reach maximum potency.

The dissociation constant K_d measures the strength of the interaction between a compound and its target. Therefore, it is the parameter most closely comparable to IC_{50}. However, K_d values are independent of substrate concentration, which is not the case for IC_{50} values. To form a complex, T and C have to diffuse in an assay solution to come into contact. Diffusion, hence association, has an upper limit in aqueous solution (in the $10^9\ M^{-1}\ s^{-1}$ range [11,21]). Therefore, the association rate of compounds often reaches a limit (frequently $<10^9\ M^{-1}\ s^{-1}$) and additional improvements in potency can only be obtained if compounds with lower k_{off} are synthesized (Eqn 5, Fig. 2). This explains why, for many series of compounds, there is a good correlation between K_d (or IC_{50}) and k_{off}. A decrease in k_{off} is linked to an enhanced stability of the compound–protein complex, which is commonly obtained by increasing the number and/or strength of the interactions made between the compounds and their target. Therefore, compound optimization should lead to molecules with lower k_{off}. On-rates are more difficult to control than off-rates, but, in some cases, k_{on} can be modulated by changing the properties of the interacting partners. On-rates can be influenced by ligand flexibility; for example, flexible antagonists of *N*-methyl-D-aspartic acid (NMDA) receptors exhibit faster k_{on} than do more rigid analogs [22]; similarly a linear peptidic inhibitor of a bacterial collagenase shows larger k_{on} than does a more rigid cyclic analog [23]. By contrast, stapled peptides (i.e. peptides that are chemically constrained to keep a defined conformation in solution) bind faster to estrogen receptors than do unstapled ones [24]. Studies in the area of protein–protein interactions have also shown that on-rates can be changed by modifying the charge of the binding partners [25,26].

Transition state studies

Structural and thermodynamic data provide information on the system at equilibrium, but kinetic measurements enable the intermediates that exist between the free and bound states to be studied. Two compounds binding to the same target that show similar free and bound states might have distinct transition states or follow a distinct kinetic path with different intermediates. The transition state theory links k_{on} to the activation energy, which is the energy barrier between the reagents and the products. The lower this barrier is, the faster k_{on} will be. Early work on transition state theory has shown that it is possible to estimate the position of the transition state on the reaction coordinate between the reagents and the products [27]. These tools were first used to study chemical reactions where covalent bonding occurs, but this has been extended to noncovalent bonding in the area of protein folding [28] and, based on these findings, to protein–DNA interactions [29] and protein–protein interactions (e.g. [30–35]). Given that the discovery of protein–protein interaction inhibitors is an active field in the pharmaceutical industry, it would be interesting to establish whether better knowledge of the properties of transition states could help in designing inhibitors. In the case of enzymes, it was demonstrated many years ago that transition state analogs can be potent inhibitors.

Binding kinetic parameters and pharmacological profiles

The dissociative half-life of the complex, $t_{1/2}$, provides an estimate of the stability of the T·C complex. This is important information because it is directly linked to the length of target inhibition. The greater the $t_{1/2}$, the longer the target is inhibited. Eqn 6 (Fig. 2) shows that $t_{1/2}$ depends only on k_{off}(k_2) and that slow dissociating compounds will have a long $t_{1/2}$. It has been proposed that designing compounds with a long $t_{1/2}$ is advantageous because it is beneficial in terms of the duration of the pharmacological effect and target selectivity (e.g. [11]). This is a valid argument, but there are two points that need to be considered. The first concerns the experimental measure of kinetic parameters. Biochemists use simplified biological systems and experimental conditions that are often a far cry from physiological conditions. Therefore, verifications should be carried out under more physiological conditions to ensure that the measurement in biochemical assays holds true. For example, 'wash out' experiments in cell-based assays (e.g. [36]) can be used as initial tests to determine whether the length of target inhibition in a cellular environment correlates with biochemical k_{off}. *In vivo*, many other parameters (e.g. volume of distribution, clearance, protein binding) in addition to k_{off} can affect the pharmacological profile of compounds. The second point to be considered concerns a phenomenon called 'on target' toxicity. In some cases, excessively long inhibition of the target leads to toxicity in healthy tissues, and compounds with longer k_{off} might not be well tolerated. This type of issue can be minimized, for example, if the compounds preferentially accumulate more in the target tissue (e.g. tumor) than in healthy tissues.

A similar potency (K_d) does not necessarily mean similar k_{on} and k_{off}. Indeed, compounds with different k_{on} and k_{off} can have the same potency because K_d is the ratio between k_{off} and k_{on} (Eqn 5, Fig. 2). For example, the two closely related inhibitors NVP-AEW541 and OSI906, which bind to the same binding site on IGF1R, have a similar K_d (6.8 nM NVP-AEW541 and 2 nM OSI906) but distinct k_1 ($1.3 \times 10^7\ M^{-1}\ s^{-1}$ NVP-AEW541 and $5 \times 10^4\ M^{-1}\ s^{-1}$ OSI906) and k_2 ($9 \times 10^{-2}\ s^{-1}$ NVP-AEW541 and $10^{-4}\ s^{-1}$ OSI906) [37].

I show below that, in addition to providing indications on the possible pharmacological behavior of compounds, binding kinetic studies are also useful for studying their mode of action.

Mode-of-action studies

Several protein kinase inhibitors described in the literature (e.g. [19,38]) show a more complex binding behavior than a one-step binding mechanism. These inhibitors follow a two-step binding process where compound C binds first to target T and then the T·C complex undergoes a conformational change to form the $(T{\cdot}C)^{\#}$ complex (Fig. 2). For this mechanism, the expression of k_{obs} is more complex (Eqn 8, Fig. 2) than it is for a one-step binding mechanism (Eqn 4, Fig. 2). This difference in k_{obs} is useful for differentiating a two-step binding mechanism from a one-step binding mechanism. For the latter, k_{obs} increases linearly with [*C*], whereas for the

former, there is an hyperbolic increase of k_{obs} [e.g. 19,38].[5] The formation of $(T{\cdot}C)^{\#}$ depends on the rate constant k_3 and, if it is smaller than k_2, the binding process is slow. Given that $(T{\cdot}C)^{\#}$ must accumulate to be observed, k_4 is smaller than k_3, so the overall second step (the isomerization step) in this mechanism is slow and compounds following such a process are slow binders. The release of C from the $(T{\cdot}C)^{\#}$ complex, which is rate limited by k_4, leads to an extended $t_{1/2}$ [17]. Therefore, compounds following such a mechanism are interesting, because they will exhibit a long $t_{1/2}$ and, consequently, a sustained pharmacological effect.

Another binding mechanism can be envisaged. Protein kinases are flexible and can adopt various conformations in solution. A compound might only be able to form a stable complex with protein kinase molecules present in solution in a high energy conformation. Given that only a few molecules adopt this conformation (most molecules occupy the low energy conformation), the binding of the compound will induce a progressive change in the conformation of the molecules present in the solution. This conformational shift is energetically disfavored because the protein molecules have to pass through an elevated energy barrier to adopt the conformation recognized by the compound. This mechanism is called 'conformational selection' [39–42]. In such a mechanism, the expression of k_{obs} predicts a hyperbolic decrease of k_{obs} when $[C]$ increases [40]. This binding process was not found for protein kinases in the literature used for this review. However, the possibility cannot be excluded that it occurs with some inhibitors. For example, p38 mitogen-activated protein kinase (MAPK) must undergo a conformational change to enable the binding of some inhibitors [20,43].

Taken together, these discussions show that binding kinetic studies can bring valuable information to drug discovery. The parameters obtained from such studies could help identify compounds with different pharmacological profiles and they might provide additional information on their mode of action.

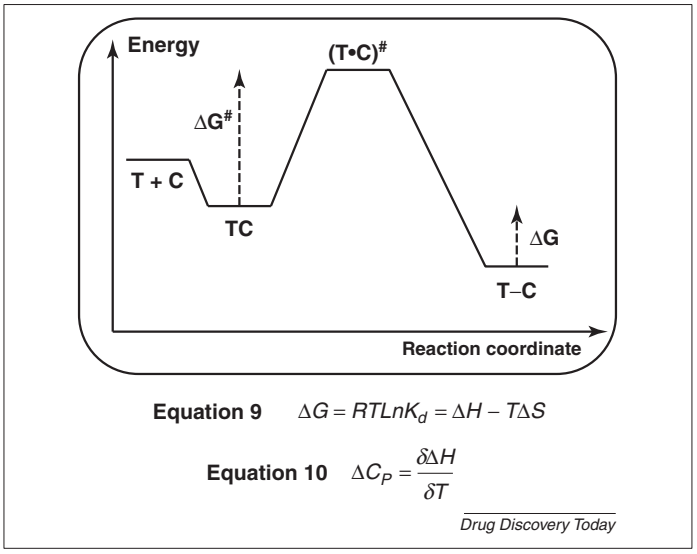

FIGURE 3

Thermodynamic studies. The cartoon is a simplified energy diagram in which different binding steps between a compound C and a target T are represented along the reaction coordinate. The two ligands interact to form an initial complex, TC. To reach the final complex, T–C, the binding process has to progress through the transition state species, $(T{\cdot}C)^{\#}$. The difference in free energy between the unbound and the bound species is ΔG. This parameter can be obtained from ITC studies. $\Delta G^{\#}$ is the free energy of activation, which measures the energy barrier between TC and $(TC)^{\#}$. $\Delta G^{\#}$ is linked to k_{on} by the Eyring equation. Therefore, binding kinetic measurements provide information on the transition state, whereas ITC measures the difference in energy between the initial and final states of that process.

Thermodynamic studies

Thermodynamic parameters

Thermodynamics is an essential aspect of molecular recognition because it reveals the energetics of the interaction between a compound and its target. Unfortunately, this type of analysis is rarely carried out. There are several possible reasons for this. The standard method used to realize thermodynamic measurements – isothermal titration calorimetry (ITC) [44] – is a low-throughput procedure, requiring large amounts of proteins and compounds with good solubility. It is also often difficult to rationalize ITC measurements because the observed changes in enthalpy (ΔH) and entropy (ΔS) reflect binding and/or conformational events that involve the target, compound and solvent. Reaching conclusions based only on thermodynamic measurements is difficult and ITC studies should be carried out in parallel with structural work that helps rationalize the observed effect on ΔH and ΔS (e.g. [45–47]). As a consequence, ITC is sometimes only used to measure compound affinity (K_d) and the stoichiometry (n) of the interaction (e.g. [48,49]). However, several authors have advocated the use of thermodynamics in drug discovery [50–53].

Mode-of-action studies

Thermodynamic measurements reveal molecular events that cannot be easily guessed at by just measuring compound potency and/or looking at structural data. Two compounds might bind to the same target with distinct ΔH and ΔS but with the same final ΔG (Gibbs free energy) and, consequently, they will show the same affinity (this can be seen from Eqn 9, Fig. 3). Therefore, without ITC measurements, it is not possible to distinguish these compounds. Given that enthalpy-driven compounds have a greater potential for affinity improvement [50,51], only ITC enables the identification of this type of ligand. In addition to measuring ΔH, K_d and n, thermodynamic studies can estimate variation in heat capacity (ΔC_P). Changes in ΔC_P have different origins [54], but they are often linked to modifications in the solvation of regions involved in

[5] Note that Eqn. (8) Fig. 2 is obtained from a more complex expression of k_{obs} for two-step binding mechanisms assuming $k_2 \gg k_3$ (equilibrium assumption). Furthermore, when $[C] < k_2/k_1$ (k_2/k_1 corresponds to dissociation constant K_d for the formation of the T·C complex), $k_{obs} \sim k_4 + (k_3/K_d)\ [C]$. In such a condition, a linear relationship between kobs and $[C]$ is obtained as for one-step binding mechanisms. A curvature in kobs versus $[C]$ plots will only be observed at high $[C]$ (e.g. [19]). Experimentally it may not be possible to reach such conditions, for example, when C has a limited solubility. One could imagine that two-step binding mechanisms are more widespread than currently observed because of conditions in which a curvature in k_{obs} versus $[C]$ plots was not reached. Conformational changes after compound binding might be frequent events since they may help both to exclude solvation water molecules trapped at the contact region and to tighten interactions between the two partners.

[6] Differential scanning calorimetry is also used to measure ΔC_P. This technique is also useful for studying the energetics of conformation transitions of proteins.

the interaction [55,56]. ITC experiments,[6] where ΔH is measured at different temperatures, enable ΔC_P to be estimated using Eqn 10 (Fig. 3). This information can be used to show whether hydrophobic [57] or polar [58] regions have a role in the interaction. Variations in ΔC_P have also been used to propose mechanisms where compound binding induces a conformational change of the target protein [59]. Many ITC studies have revealed that compound modifications do not affect ΔG (K_d, i.e. potency) because of similar variation of ΔH and ΔS (in fact $T\Delta S$; Eqn 9, Fig. 3) in opposite directions. This phenomenon is called 'enthalpy–entropy compensation' [60,61]. Some authors have suggested that this phenomenon is artefactual (e.g. [62]) and others have argued that it is not a general feature of weak association [63]. Although some studies based on the use of the van't Hoff equation are misleading because it has to be used with caution [64,65], various reports where ITC measurements and structural data are combined show clear enthalpy–entropy compensation mechanisms (e.g. [57,66–68]). Briefly, enthalpy–entropy compensation can be explained as follows: enhancing the number and/or efficacy of the compound–target contacts leads to a favorable enthalpic effect but to an unfavorable entropic contribution because of the reduced mobility of the bound compound (ligand ordering). The binding of the compound also affects the residual mobility of the protein, which also leads to entropic changes (protein ordering) [69]. Enthalpy–entropy compensation can conceal successful attempts to modify compounds to create better contacts, because no gain in potency is observed in assays. Knowing from ITC measurements that better contacts have been created (more negative ΔH) could trigger medicinal chemistry activities to design compounds that will pay a reduced entropic penalty upon binding, for example, modifying their flexibility or the H-bonds[7] created upon binding.

Thermodynamics can lead to interesting observations that can be useful for compound design. It is common practice to design rigid molecules because they should show a decreased entropic penalty upon binding and so more favorable free energy. However, this might not always be the case, because the flexibility of the target protein should also be considered. For example, Edwards *et al.* studied the binding of two transition state analogs that bind to purine nucleoside phosphorylase [70]. The most rigid analog binds to the protein with a more favorable enthalpy than does the flexible one ($\Delta H = -21.2$ kcal/mol versus -17.5 kcal/mol) but it pays a higher entropic penalty ($-T\Delta S = 7.1$ kcal/mol versus 2.1 kcal/mol) and, overall, its free energy (potency) is reduced ($\Delta G = -14.1$ kcal/mol versus -15.2 kcal/mol). On the basis of deuterium exchange experiments, the authors proposed that the most flexible compound is a stronger inhibitor, because it enables a higher conformational flexibility of the protein, thereby reducing the entropic penalty. Similar interplay between protein flexibility and ligand binding has also been studied with galectin-3 [71]. These different findings suggest alternative strategies to design compounds that should bind to flexible regions of proteins.

Cooperative systems

Thermodynamic studies also enable the analysis of an important mechanism in molecular recognition: cooperativity. This is a widespread phenomenon in nature [72] and occurs in many systems where noncovalent interactions are involved. Below, I only consider positive cooperativity, because it is likely to be more relevant to drug discovery. In an additive world, the creation of two new interactions between a compound and its target translates into an increase in free energy corresponding to the sum of the free energy of each individual new interaction. In a cooperative world, this might not always be the case and an increase in binding energy can be higher than the sum of the free energy of each new interaction. Different models have been proposed to explain the origin of cooperativity. Williams and collaborators [73] suggested a mechanism based on progressive tightening of the interactions between the compound and the target, Hunter and Tomas [74] proposed a model based on the existence of different partially bound states that can be shifted upon the introduction of new interactions. Both models shed light on the mechanisms at the origin of cooperativity and also provide an explanation as to the phenomenon of enthalpy–entropy compensation. Cooperative binding occurs with low-molecular-weight compounds and has been particularly well studied with thrombin inhibitors [66,75], for which subtle (in some cases long-range) cooperative effects have been observed. A deeper understanding of the mechanisms leading to cooperativity is a promising avenue for drug design.

Thermodynamic studies reveal many aspects of molecular recognition that would remain unnoticed when only relying on potency and structural data. A better understanding of the energetics of the mechanisms taking place during compound binding should help in the design of more effective drugs.

Concluding remarks

Drug discovery has to evolve constantly because there is a perpetual need to develop more efficient new drugs. Therefore, all the areas required to get compounds into the clinic are permanently changing. In this environment, biochemistry can make a significant contribution to drug discovery. At the interface between medicinal chemistry, structural biology and cellular biology, biochemistry holds a key position in drug development. Although this review focuses on a single family of proteins and gives limited coverage of what biochemistry has to offer drug discovery, I have shown that biochemical studies can provide much valuable information on the mode of action of compounds. A deeper knowledge of the way in which compounds interact with their target is key to improving their quality and to designing modulators of the challenging new targets that are currently emerging in the pharmaceutical industry.

Acknowledgements

I would like to thank: A. De Pover, P. Furet, J.C. Hau and T. Valat for their comments and suggestions.

[7] H-bonds are directional and need a precise geometry to be formed, leading to a substantial ordering and entropic penalties. Given that ligands often displace H-bonded water molecules upon binding, the solvent should also be taken into consideration in the design of H-bonds.

References

1 Copeland, R.A. (2005) *Evaluation of Enzyme Inhibitors in Drug Discovery*. Willey-Interscience
2 Cheng, Y.C. and Prusoff, W.H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* 22, 3099–3108
3 Yang, J. *et al*. (2009) Defining balanced conditions for inhibitor screening assays that target bisubstrate enzymes. *J. Biomol. Screen.* 14, 111–120
4 Brockhoff, M. *et al*. (2011) Factors influencing the inhibition of protein kinases. *J. Enzyme Inhib. Med. Chem*. 10.3109/14756366.2011.583922
5 Segel, I.H. (1975) *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems*. John Wiley & Sons
6 Chène, P. (2008) The new challenges of biochemistry in identifying the next generation of protein kinase inhibitors. *Drug Discov. Today* 13, 522–529
7 Gao, X. and Harris, T.K. (2006) Steady-state kinetic mechanism of PDK1. *J. Biol. Chem.* 281, 21670–21681
8 Vanderpool, D. *et al*. (2009) Characterization of the CHK1 allosteric inhibitor binding site. *Biochemistry* 48, 9823–9830
9 Liu, M. *et al*. (2008) Kinetic studies of Cdk5/p25 kinase: phosphorylation of Tau and complex inhibition by two prototype inhibitors. *Biochemistry* 47, 8367–8377
10 Burke, J.R. *et al*. (2003) BMS-345541 is a highly selective inhibitor of IκB kinase that binds at an allosteric site of the enzyme and blocks NF-κB-dependent transcription in mice. *J. Biol. Chem.* 278, 1450–1456
11 Copeland, R.A. *et al*. (2006) Drug-target residence time and its implications for lead optimization. *Nat. Rev. Drug Discov.* 5, 730–739
12 Swinney, D.C. (2006) Can binding kinetics translate to a clinically differentiated drug? From theory to practice. *Lett. Drug Des. Discov.* 3, 569–574
13 Tummino, P.J. and Copeland, R.A. (2008) Residence time of receptor–ligand complexes and its effect on biological function. *Biochemistry* 47, 5481–5492
14 Vauquelin, G. and Van Liefde, I. (2006) Slow antagonist dissociation and long-lasting *in vivo* receptor protection. *Trends Pharmacol. Sci.* 27, 355–359
15 Goodrich, J.A. and Kugel, J.F. (2007) *Binding and Kinetics for Molecular Biologists*. Cold Spring Harbor Laboratory Press
16 Golicnik, M. and Stojan, J. (2004) Slow-binding inhibition. *Biochem. Mol. Biol. Educ.* 32, 228–235
17 Morrison, J.F. and Walsh, C.T. (1988) The behavior and significance of slow-binding enzyme inhibitors. *Adv. Enzymol.* 61, 201–301
18 Szedlacsek, S.E. and Duggleby, R.G. (1995) Kinetics of slow and tight-binding inhibitors. *Methods Enzymol.* 249, 144–180
19 Iwata, H. *et al*. (2011) Biochemical characterization of TAK-593, a novel vEGFR/PDGFR inhibitor with a two-step slow binding. *Biochemistry* 50, 738–751
20 Pargellis, C. *et al*. (2002) Inhibition of p38 MAP kinase by utilizing a novel allosteric site. *Nat. Struct. Biol.* 9, 268–272
21 Schreiber, G. *et al*. (2009) Fundamental aspects of protein–protein association kinetics. *Chem. Rev.* 109, 839–860
22 Benveniste, M. and Mayer, M.L. (1991) Structure–activity analysis of binding kinetics for NMDA receptor competitive antagonists: the influence of conformational restriction. *Br. J. Pharmacol.* 104, 207–221
23 Yiotakis, A. *et al*. (1994) Cyclic peptides with a phosphinic bond as potent inhibitors of a zinc bacterial collagenase. *J. Med. Chem.* 37, 2713–2720
24 Philipps, C. *et al*. (2011) Design and structure of stapled peptides binding to estrogen receptors. *J. Am. Chem. Soc.* 133, 9696–9699
25 Schreiber, G. and Fersht, A.R. (1996) Rapid, electrostatically assisted association of proteins. *Nat. Struct. Biol.* 3, 427–431
26 Selzer, T. *et al*. (2000) Rational design of faster associating and tighter binding protein complexes. *Nat. Struct. Biol.* 7, 537–541
27 Leffler, J.E. (1953) Parameters for the description of transition states. *Science* 117, 340–341
28 Fersht, A.R. (2004) Relationship of Leffler (Brønsted) α values and protein folding Φ values to position of transition-state structures on reaction coordinates. *Proc. Natl. Acad. Sci. U. S. A.* 101, 14338–14342
29 Ferreiro, D.U. *et al*. (2008) Transition state for protein–DNA recognition. *Proc. Natl. Acad. Sci. U. S. A.* 105, 10797–10802
30 Bachmann, A. *et al*. (2011) Mapping backbone and side-chain interactions in the transition state of a coupled protein folding and binding reaction. *Proc. Natl. Acad. Sci. U. S. A.* 108, 3952–3957
31 Goldberg, J.M. and Baldwin, R.L. (1999) A specific transition state for S-peptide combining with folded S-protein and then refolding. *Proc. Natl. Acad. Sci. U. S. A.* 96, 2019–2024
32 Horn, J.R. *et al*. (2009) Principal determinants leading to transition state formation of a protein–protein complex, orientation trumps side-chain interactions. *Proc. Natl. Acad. Sci. U. S. A.* 106, 2259–2264
33 Kiel, C. *et al*. (2004) Electrostatically optimized Ras-binding Ral guanine dissociation stimulator mutants increase the rate of association by stabilizing the encounter complex. *Proc. Natl. Acad. Sci. U. S. A.* 101, 9223–9228
34 Taylor, M.G. *et al*. (1998) Kinetic epitope mapping of the chicken lysozyme HyHEL-10 Fab complex: delineation of docking trajectories. *Protein Sci.* 7, 1857–1867
35 Turjanski, A.G. *et al*. (2008) Binding-induced folding of a natively unstructured transcription factor. *PLoS Comput. Biol.* 4, e1000060
36 Wood, E.R. *et al*. (2004) A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res.* 64, 6652–6659
37 Chène, P. *et al*. (2010) Study of the selectivity of insulin-like growth factor-1 receptor (IGF1R) inhibitors. *Open Enzyme Inhib. J.* 3, 27–37
38 Johnson, E.F. *et al*. (2007) Pharmacological and functional comparison of the Polo-like kinase family: insight into inhibitor and substrate specificity. *Biochemistry* 46, 9551–9563
39 Boehr, D.D. *et al*. (2009) The role of dynamic conformational ensembles in biomolecular recognition. *Nat. Chem. Biol.* 5, 789–796
40 Hammes, G.G. *et al*. (2009) Conformational selection or induced fit: a flux description of reaction mechanism. *Proc. Natl. Acad. Sci. U. S. A.* 106, 13737–13741
41 Weikl, T.R. and Deuster, V.C. (2008) Selected-fit versus induced-fit protein binding: kinetic differences and mutational analysis. *Proteins* 75, 104–110
42 Zhou, H.X. (2010) From induced fit to conformational selection: a continuum of binding mechanism controlled by the timescale of conformational transitions. *Biophys. J.* 98, L15–L17
43 Regan, J. *et al*. (2003) The kinetics of binding to p38 MAP kinase by analogues of BIRB 796. *Bioorg. Med. Chem. Lett.* 13, 3101–3104
44 Ward, W.H.J. and Holdgate, G.A. (2001) Isothermal titration calorimetry in drug discovery. *Prog. Med. Chem.* 38, 309–376
45 Rickert, K.W. *et al*. (2011) Structural basis for selective small molecule kinase inhibition of activated c-Met. *J. Biol. Chem.* 286, 11218–11225
46 Stroba, A. *et al*. (2009) 3,5-Diphenylpent-2-enoic acids as allosteric activators of the protein kinase PDK1: structure–activity relationships and thermodynamic characterization of binding as paradigms for PIF-binding pocket-targeting compounds. *J. Med. Chem.* 52, 4683–4693
47 Bullock, A.N. *et al*. (2005) Structural basis of inhibitor specificity of the protooncogene proviral insertion site in Moloney murine leukemia virus (PIM-1) kinase. *J. Med. Chem.* 48, 7604–7614
48 Breitenlechner, C.B. *et al*. (2005) Design and crystal structures of protein kinase B-selective inhibitors in complex with protein kinase A and mutants. *J. Med. Chem.* 48, 163–170
49 Seeliger, M.A. *et al*. (2007) c-src binds to the cancer drug Imatinib with an inactive abl/c-kit conformation and a distributed thermodynamic penalty. *Structure* 15, 299–311
50 Ferenczy, G.G. and Keseru, G.M. (2010) Thermodynamics guided lead drug discovery and optimization. *Drug Discov. Today* 15, 919–932
51 Freire, E. (2008) Do enthalpy and entropy distinguish first in class from best in class? *Drug Discov. Today* 13, 869–874
52 Ruben, A.J. *et al*. (2006) Overcoming roadblocks in lead optimization: a thermodynamic perspective. *Chem. Biol. Drug Des.* 67, 2–4
53 Velazquez-Campoy, A. *et al*. (2001) The application of thermodynamic methods in drug design. *Thermochim. Acta* 380, 217–227
54 Sturtevant, J.M. (1977) Heat capacity and entropy changes in process involving proteins. *Proc. Natl. Acad. Sci. U. S. A.* 74, 2236–2240
55 Gallagher, K. and Sharp, K. (1998) Electrostatic contributions to heat capacity changes of DNA–ligand binding. *Biophys. J.* 75, 769–776
56 Spolar, R.S. *et al*. (1992) Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. *Biochemistry* 31, 3947–3955
57 Talhout, R. *et al*. (2003) Understanding binding affinity: a combined isothermal titration calorimetry/molecular dynamics study of the binding of a series of hydrophobically modified benzamidinium chloride inhibitors to trypsin. *J. Am. Chem. Soc.* 125, 10570–10579
58 Phenix, C.P. and Palmer, D.R.J. (2008) Isothermal titration microcalorimetry reveals the cooperative and noncompetitive nature of inhibition of *Sinorhizobium meliloti* L5-30 dihydrodopicolinate synthase by (*S*)-lysine. *Biochemistry* 47, 7779–7781
59 Anderka, O. *et al*. (2008) Thermodynamic characterization of allosteric glycogen phosphorylase inhibitors. *Biochemistry* 47, 4683–4691

60 Dunitz, J.D. (1995) Win some, lose some: enthalpy–entropy compensation in weak intermolecular interactions. *Chem. Biol.* 2, 709–712
61 Searle, M.S. and Williams, D.H. (1992) The cost of conformational order: entropy changes in molecular associations. *J. Am. Chem. Soc.* 114, 10690–10697
62 Cornish-Bowden, A. (2002) Enthalpy–entropy compensation: a phantom phenomenon. *J. Biosci.* 27, 121–126
63 Ford, D.M. (2005) Enthalpy–entropy compensation is not a general feature of weak association. *J. Am. Chem. Soc.* 127, 16167–16170
64 Keleti, T. (1983) Errors in the evaluation of Arrhenius and van't Hoff plots. *Biochem. J.* 209, 277–280
65 Weber, G. (1996) Persistent confusion of total entropy and chemical system entropy in chemical thermodynamics. *Proc. Natl. Acad. Sci. U. S. A.* 93, 7452–7453
66 Baum, B. *et al.* (2010) Non additivity of functional group contributions in protein–ligand binding: a comprehensive study by crystallography and isothermal titration calorimetry. *J. Mol. Biol.* 397, 1042–1054
67 Krishnamurthy, V.M. *et al.* (2006) The paradoxical thermodynamic basis for the interaction of ethylene glycol, glycine, and sarcosine chains with bovine carbonic anhydrase II: an unexpected manifestation of enthalpy/entropy compensation. *J. Am. Chem. Soc.* 128, 5802–5812
68 Lafont, V. *et al.* (2007) Compensating enthalpic and entropic changes hinder binding affinity optimization. *Chem. Biol. Drug Des.* 69, 413–422
69 Wilfong, E.M. *et al.* (2011) A multidisciplinary approach to probing enthalpy–entropy compensation and interfacial mobility model. *J. Am. Chem. Soc.* 133, 11515–11523
70 Edwards, A.A. *et al.* (2010) Conformational states of human purine nucleoside phosphorylase at rest, at work and with transition state analogues. *Biochemistry* 49, 2058–2067
71 Diehl, C. *et al.* (2010) Protein flexibility and conformational entropy in ligand design targeting the carbohydrate recognition domain of galectin-3. *J. Am. Chem. Soc.* 132, 14577–14589
72 Whitty, A. (2008) Cooperativity and biological complexity. *Nat. Chem. Biol.* 4, 435–439
73 Williams, D.H. *et al.* (2004) Understanding noncovalent interactions: ligand binding energy and catalytic efficiency from ligand-induced reductions in motion within receptors and enzymes. *Angew. Chem. Int. Ed.* 43, 6596–6616
74 Hunter, C.A. and Tomas, S. (2003) Cooperativity, partially bound states, and enthalpy–entropy compensation. *Chem. Biol.* 10, 1023–1032
75 Muley, L. *et al.* (2010) Enhancement of hydrophobic interactions and hydrogen bond strength by cooperativity: synthesis, modeling, and molecular dynamics simulations of a congeneric series of thrombin inhibitors. *J. Med. Chem.* 53, 2126–2135