



Introduction

In structure-based drug design (SBDD), full structural knowledge of the protein target molecule provides information on how a potential drug interacts with the target. Computer modeling algorithms and molecular simulations are used to predict quantitative structure-activity relationships (QSAR) and these data are used to optimize the design of new drugs. However, molecular modeling is limited by a lack of experimental thermodynamic data to verify these models.

Ultrasensitive Isothermal Titration Calorimetry (ITC) provides complete thermodynamic characterization of the binding of a drug to its therapeutic target, including enthalpy (ΔH) and entropy ($T\Delta S$), free energy (ΔG) and binding affinity (K_B). These thermodynamics parameters are related to each other by the following equations:

$$\Delta G = \Delta H - T\Delta S$$

$$\Delta G = -RT \ln K_B$$

For spontaneous reactions, ΔG is negative, and ΔG is directly related to the binding affinity. The tighter the binding, the more negative the ΔG . Enthalpy and entropy both contribute to ΔG .

Enthalpic contributions: ΔH reflects the strength of the drug-target interaction relative to those with solvent, primarily due to hydrogen bond formation, and van der Waals interactions. When ΔH is negative, binding is enthalpically favored. Favorable enthalpy requires correct placement of hydrogen bond acceptor and donor groups at the binding interface.

Entropic contributions: A positive $T\Delta S$ results in entropically favored binding. Favorable entropy changes are primarily due to hydrophobic interactions, due to an increase in solvent entropy from burial of hydrophobic groups and release of water upon binding, as well as minimal loss of conformational degrees of freedom.

Figure 1 shows different thermodynamic signatures that might be observed for three different drugs binding to the same target. Each drug has identical ΔG and binding affinity for the target. Scheme A has favorable ΔH , characteristic of hydrogen bond formation, and an unfavorable $T\Delta S$. Drugs showing this binding profile typically have large degree of flexibility, as well as high polarity, which could cause problems with membrane permeability *in vivo*. Scheme B has a favorable $T\Delta S$, indicating that binding is driven by hydrophobic interactions, and an unfavorable ΔH . Drugs showing this binding profile are very hydrophobic and are poorly soluble, and there are also conformational restraints leading to lack of adaptability and consequently a high

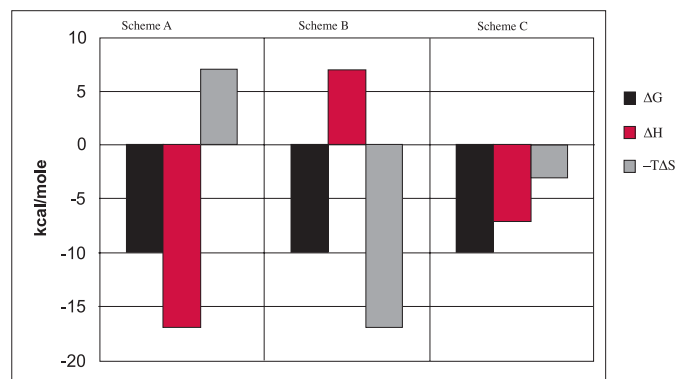


FIGURE 1. Thermodynamic signatures for three different drugs binding to same target. Scheme A: good hydrogen bonding and a conformational change. Scheme B: binding dominated by hydrophobic interactions. Scheme C: Favorable hydrogen bonding and hydrophobic interactions.

susceptibility to mutations that can cause drug resistance. Scheme C has favorable ΔH and ΔS , the most favorable thermodynamic profile for tight binding.

Effective drugs are expected to have high binding affinity and selectivity for target. Drugs for infectious diseases also should be adaptive, and bind to a broad spectrum of targets, such as protein mutants and families of targets. ITC is utilized for the following:

- **Optimization of binding affinity:** ITC determines thermodynamic signatures of lead compounds binding to target. Choose lead compound(s) with favorable enthalpy and further optimize them by the addition of hydrophobic regions to the core drug. Drugs with high binding affinities have lower dosage requirements and consequently fewer side effects.
- **Binding adaptability:** Design drugs which can bind to mutant forms of target proteins, thereby reducing drug resistance. Also design drugs which bind to a family of related targets.
- **Improvement of binding selectivity:** Design drugs which bind selectively to a specific target, bind less selectively to serum proteins or other non-specific targets.

Optimization of Binding Affinity

One goal in drug design is to make drugs which bind to their target with the highest binding affinity. Higher affinity results in lower dosage requirement, greater specificity, better drug efficacy, reduced side effects, and less drug resistance. Research by Ernesto Freire's group at Johns Hopkins University has shown that thermodynamics from ITC data can be used to characterize HIV-1 protease inhibitors, and binding affinity is optimized by overcoming enthalpy-entropy compensation.¹⁻⁵

Figure 2 shows the thermodynamic signatures of binding of several inhibitors to wild-type HIV-1 protease. Binding of indinavir, nelfinavir, and saquinavir are driven by entropy, due to hydrophobic interactions between the drug and the target. Binding of these inhibitors have unfavorable enthalpy. Ritonavir, amprenavir, lopinavir, and two experimental inhibitors KNI-272 and KNI-764 have favorable binding enthalpy, as well as a favorable entropic contribution. Binding interactions with these inhibitors have both favorable hydrogen bond formation as well as hydrophobic interactions. These inhibitors are second and third generation inhibitors. Loprinavir has the highest binding affinity of these inhibitors (K_b of $1.2 \times 10^{11} \text{ M}^{-1}$), measured by displacement ITC.

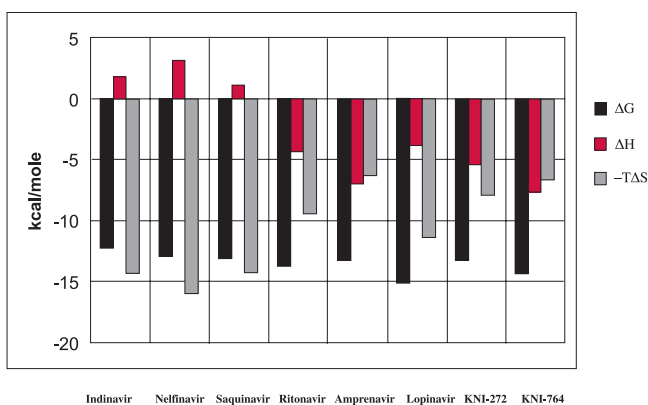


FIGURE 2. Thermodynamic signatures of binding of inhibitors to wild-type HIV-1 protease. Indinavir, nelfinavir, saquinavir, ritonavir, amprenavir, and lopinavir are in clinical use. KNI-272 and KNI-764 are experimental protease inhibitors. Data from References 4 and 5. Refer to Reference 5 for design of ITC experiments.

Hydrogen bond formation and favorable van der Waals interactions between drug and target results in favorable enthalpy. Hydrogen bond formation is a result of optimal placement of hydrogen bond donor and acceptor groups on the drug and target, and is highly directional and specific. Hydrogen bond donors and receptors may be in close proximity in a crystal structure but may have little effect on binding affinity, due to conformational effects.

A common strategy in drug design is the addition of non-polar groups to increase hydrophobic interactions between target and compound. However, hydrophobic interactions are non-specific compared to hydrogen bonding. Freire's research demonstrates that drugs with favorable enthalpy AND entropy have highest affinity, due to specific hydrogen bond formation and van der Waals interactions as well as hydrophobic interactions with the target.

In typical lead optimization, compounds are initially screened for binding affinity to target, with no information about thermodynamics. Compounds with high affinity continue through the discovery pipeline, but may not lead to a successful drug. Freire's research indicates that a better strategy is identifying lead compounds with highly favorable ΔH , and optimizing these drugs by addition of hydrophobic groups. This could result in a highly-specific drug with tight binding affinity. Preliminary work has been done on the design of SARS drugs using thermodynamic signatures.⁶ The first generation inhibitors of SARS 3CL^{pro} protease have enthalpically favorable binding, and these drugs are being optimized to increase binding affinity.

This ITC-based QSAR has also been used to develop new drugs which bind to DNA gyrase, by researchers at AstraZeneca.⁷ They observed that triazine inhibitors of DNA gyrase tend to have similar binding energetics, and they looked for triazine derivatives which had a markedly different thermodynamic signature, especially in ΔH , and studied these different derivatives for their structural differences and binding modes. This kind of screening can be useful in the discovery of new drugs.

Binding Adaptability

Drug resistance is a serious side effect associated with antiviral therapies, due to the appearance of viral strains with mutant forms of target protein. Mutants with reduced binding affinity for inhibitor typically maintain affinity for substrate. Freire's group has studied the thermodynamics of inhibitor binding to mutant strains of HIV-1 protease.³⁻⁵ One multi-drug resistant mutant with six amino acid mutations of HIV-1 protease (MDR-HM) had binding affinity for inhibitor 2 to 3 orders of magnitude lower than wild-type.⁵ Study of these mutations demonstrate individual effects of each individual mutation, as well as cooperative interactions between distal mutations.

The determination of enthalpic and entropic components of the drug resistant HIV-1 protease mutants relative to wild type, along with structural studies, was used to develop guidelines in the design of inhibitors more adaptable to drug target mutations.⁵ These guidelines include:

- Maximize ΔH contribution to ΔG for enthalpically favorable inhibitors.
- Design drug-target interactions with highest binding affinity and specific hydrogen bonding between core of the drug and highly-conserved region of the target's binding site.
- Introduce non-constrained functional groups on inhibitor facing mutation-prone regions of binding site of target. Since this will make ΔS of binding less favorable by loss of conformational degrees of freedom of inhibitor, inhibitor ΔH must be highly favorable to maintain high affinity.

Thermodynamics and ITC have also been used to design adaptive inhibitors. This is achieved by designing an inhibitor with a tight and specific binding interaction with the conserved region of the binding site of the primary target, and introduces flexible asymmetric functional groups to be able to bind the other related targets. This approach has been used to design anti-malarial drugs against proteases of *Plasmodium falciparum*, plasmepsin I, II, IV, and histo-aspartyl protease (HAP).^{8,9} A tight-binding inhibitor to plasmepsin II also inhibited secondary targets, due to the adaptive group on the inhibitor.

Binding Selectivity

Binding selectivity is dependent on the affinity of drug to both desired and unwanted targets, as well as the K_b ratio of the two binding events. Drugs can bind to non-specific targets, such as serum proteins, or to other target proteins with different binding affinities. When a drug binds serum proteins, the drug's bioavailability and efficacy are reduced, requiring a higher dosage. ITC has been used to study the binding of HIV-1 protease inhibitors to human serum proteins.¹⁰ HIV-1 protease inhibitors had a significant binding affinity for α 1-acid glycoprotein (AAG) and a relatively low binding affinity for human serum albumin (HSA). Using AAG and HSA concentration simulating *in vivo* conditions, up to 10 times higher concentration was needed to inhibit HIV-1 protease relative to the amount needed to inhibit in absence of serum proteins. When drug-resistant HIV-1 protease mutants were used, up to 2000-fold more inhibitor was required to inhibit protease, and this is beyond the solubility limit of the inhibitor.

ITC and structural studies can be used to develop new drugs to maximize selectivity and specific binding to intended target by optimization of enthalpy and entropy.

Summary

ITC studies on HIV-1 protease inhibitors, anti-malarial drugs, SARS drugs, and DNA gyrase inhibitors have generated thermodynamic signatures of drug-target interactions. These data, used with structural analysis, provide valuable information in the design of new drugs which bind with higher affinity and selectivity, as well as adaptability. The introduction of ITC instrumentation with increased throughput allows use of microcalorimetry earlier in drug discovery pipelines.

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