Structure-based design of AChBP ligands, new insights and applications

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Chapter 3

Thermodynamic analysis in fragment-based drug discovery

Ewald Edink[#], Chimed Jansen[#], Rob Leurs and Iwan J.P. de Esch

[#] These authors contributed equally.

Abstract

Thermodynamic analysis provides access to the determinants of binding affinity, enthalpy and entropy. In fragment-based drug discovery (FBDD), thermodynamic analysis provides a powerful tool to discriminate fragments based on their potential for successful optimization. The thermodynamic data generated by FBDD studies can in turn be used to better understand the forces that drive biomolecular interactions. In this review, the technologies that enable thermodynamic analysis of fragment-protein complexes are discussed. In addition, the available thermodynamic data on fragment-protein complexes is summarized and several key studies which highlight the role of thermodynamics in FBDD are discussed in more detail. Although, thermodynamic analysis is not yet applied widely within the FBDD field, the first success stories are starting to appear, exemplifying its value in the development of a more efficient fragment optimization process and a better understanding of ligand-protein interactions.

Introduction

Fragment-based drug discovery (FBDD) is making an impact as an efficient and effective drug discovery method.[1] With small libraries of a few thousand structures and hit rates reaching 10%, FBDD provides a valuable alternative to HTS.[2] A crucial aspect of FBDD is the efficient optimization of the binding affinity of fragment hits towards high affinity clinical candidates. Ultimately, binding affinity is governed by the changes in enthalpy and entropy (see **Glossary**) that occur upon formation of a ligand-protein complex. The small size of fragments simplifies the process of relating the thermodynamic profile of fragments to the interactions made during binding. When combined with structural data, the stepwise fragment growing process provides an ideal dataset with which to improve our understanding of the thermodynamics of binding. In this manner both FBDD, and our understanding of the thermodynamics of binding, have much to gain from the application of thermodynamic analysis in FBDD.

Thermodynamics in drug discovery

Thermodynamic analysis provides access to the constituents of the Gibbs energy of binding ($\Delta G°$), enthalpy ($\Delta H°$) and entropy ($\Delta S°$) (see **Glossary**). Enthalpy, or heat energy, is associated with direct binding forces such as hydrogen bonding, van der Waals forces and π-π interactions. Entropy, a term determined by the number of accessible states of a system, is associated with conformational freedom and the hydrophobic effect (see **Glossary**). The thermodynamic changes that occur when a ligand binds to its respective protein binding site are schematically depicted in **Figure 1**.

Improving the enthalpic contribution to binding generally involves optimizing the polar interactions made by a ligand. This may be achieved by strengthening already existing interactions within the binding site or by forming new interactions by adding additional polar groups to a ligand. However, a poorly optimized polar interaction may not simply provide a lower favorable contribution to the enthalpy change on binding, it may actually result in an unfavorable contribution to enthalpy. Desolvation of polar functionalities comes at a cost, which can only be overcome when the positioning of interacting groups obeys strict angle and distance requirements.[3] As a consequence, enthalpic optimization is a difficult route by which to improve affinity. However, due to the specificity of polar interactions, enthalpy driven ligand binding may provide a major selectivity advantage.[4] Interestingly, retrospective analysis of the thermodynamics of HIV-protease inhibitors and cholesterol lowering statins, shows that the later best in class compounds, which outperformed earlier first in class compounds, had been enthalpically optimized.[5]

While enthalpic optimization can provide highly selective high affinity drugs, medicinal chemists tend to optimize entropy, as shown in a recent study comparing synthetic and natural drugs.[6] Unfortunately, too much focus on entropic optimization by constraining ligands in their bioactive conformation, and by the addition of hydrophobic groups, may in the end result in poorly soluble compounds, with reduced selectivity and higher chances of attrition.[7,8] A further complication to thermodynamic optimization results from enthalpy-entropy compensation. As a polar interaction becomes tighter (enthalpically favorable), the binding atoms

become locked into a tighter conformational geometry (entropically unfavorable).[9,10] This means that optimizing enthalpy necessarily adversely affects entropy and vice versa. Since we are looking at interactions occurring in water, desolvation and the ordering or disordering of water surrounding the binding partners may play an important role in enthalpy-entropy compensation.[10] As a result, large favorable changes in enthalpy or entropy often result in only minor gains in binding affinity.

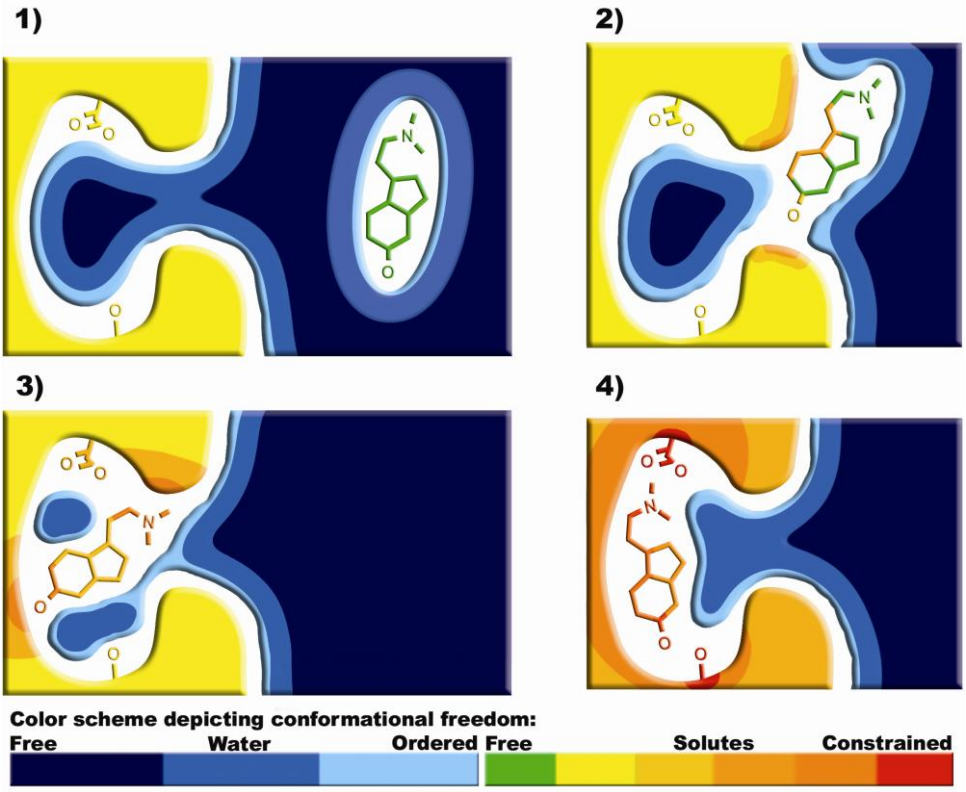


Figure 1: *In this simplified schematic diagram, four stages of ligand binding are shown. The colors indicate the degree of conformational freedom of the ligand, protein and water as indicated on the key.* ***1)*** *Prior to complex formation, the ligand and the protein binding site are solvated by water.* ***2)*** *and* ***3)*** *As the ligand approaches the binding pocket, the ligand and the protein's conformational freedom become increasingly restricted, resulting in an unfavorable entropic contribution. In addition, the polar moieties become partially desolvated, resulting in an unfavorable change in enthalpy. At the same time, the formation of enthalpic van der Waals interactions, as well as the hydrophobic effect (see* ***Glossary****), ensure that this process is favorable.* ***4)*** *Eventually, the ligand reaches a binding conformation and a significant favorable contribution to enthalpy is made as the ligand and protein become locked together, resulting in an unfavorable entropy contribution. At this crucial stage, the net effect of the polar interactions, desolvation, conformational constraint and the hydrophobic effect, will determine the final thermodynamic profile of the ligand.*

For an extensive overview on the use of thermodynamics in drug discovery, the reader is referred to the book on drug-receptor thermodynamics edited by Raffa.[11] An overview of the studies in which isothermal titration calorimetry (ITC) (see below) has been applied to attain thermodynamic data is published annually, and represents a good starting point for those interested in an overview on more recent studies.[12-18] Finally, several online databases that contain data on the thermodynamics of ligand-protein interactions exist, providing access to relevant studies.[6,19,20]

Glossary

Gibbs energy change (ΔG°): The energy change on binding can be divided into enthalpy (ΔH°) and entropy (ΔS°): ΔG° = ΔH° –TΔS°. Or looked at as a function of the binding affinity (e.g., K_D,): $\Delta G^o = RT \ln K_D$. In which R is the gas constant and T is the absolute temperature.

Van 't Hoff Equation: Combining the two Gibbs equations results in the van 't Hoff equation: $\ln K_D = \frac{\Delta H^o}{RT} - \frac{\Delta S^o}{R}$ .This equation can be used to derive enthalpy and entropy from affinity measurements at a range of temperatures.

Enthalpy change (ΔH°): A measure for the heat that is released or absorbed upon binding, often associated with polar interactions.

Entropy change (ΔS°): A measure of the change in order/disorder or in the number of configurations available to the system upon binding, associated with conformational freedom and the hydrophobic effect.

Heat capacity change ($\Delta C_p°$): A measure for the change in the ability of a system to absorb heat. The van 't Hoff equation can only be used when ΔH° does not vary with temperature ($\Delta C_p° = 0$). When ΔH° has temperature dependence ($\Delta C_p° \neq 0$), the integrated form of the van 't Hoff equation that includes a $\Delta C_p°$ term can be applied to derive ΔH°, ΔS° and $\Delta C_p°$(where α and β are constants):

$$\ln K_D = \frac{1}{R}\left[\frac{1}{T}(\Delta C_p^o T + \alpha) - (\Delta C_p^o \ln T + \beta)\right]$$

Values of ΔH° and ΔS° can subsequently be obtained using the following equations:

$\Delta H°(T) = \Delta C_p° \cdot T + \alpha$

$\Delta S°(T) = \Delta C_p° \cdot \ln T + \beta$

Determination of $\Delta C_p°$ can provide additional information about biomolecular interactions, for example a negative $\Delta C_p°$ in combination with favorable entropy changes is indicative of hydrophobic interactions.

The hydrophobic effect: A favorable change in entropy during ligand binding resulting from the displacement of water molecules as apolar regions of the binding site are brought together.[61]

Ligand Efficiency (LE): A metric that normalizes the binding affinity of molecules by their size. LE is calculated by dividing the Gibbs energy change ($\Delta G°$) by the number of heavy atoms (HA): $LE = \Delta G°/HA$.[62]

Enthalpic Efficiency (EE): A molecular size corrected measure of the enthalpic contribution to binding, calculated by dividing the enthalpy ($\Delta H°$) by the number of heavy atoms (HA): $EE = \Delta H°/HA$.[21]

Application of thermodynamics in FBDD

Determination of thermodynamic binding profiles may be especially useful in a FBDD context. In general, the identified fragment hits exhibit mM to µM affinity towards their respective protein targets, and as a consequence extensive increases in binding affinity are necessary to obtain low nM binders that can enter the clinic. As mentioned in the previous section, it is easier to improve binding affinity by optimizing entropy, with the addition of hydrophobic groups, than it is through enthalpy, which requires polar interactions to be optimized. Therefore, choosing a fragment in which binding is enthalpically driven as a starting point and adding hydrophobic groups during optimization, may provide the easiest route to a high affinity compound with favorable changes in both enthalpy and entropy and a reduced risk of attrition. The measure, enthalpic efficiency (EE, see **Glossary**), has recently been postulated as a tool for chemists and may prove useful as a hit selection criterion in FBDD, to supplement established criteria such as ligand efficiency (LE, see **Glossary**), logP, polar atom count, and synthetic accessibility to rank identified fragment hits.[21]

Measuring the thermodynamics of binding requires a suitable system. Here we describe two methods, used to acquire thermodynamic data, calorimetry and van 't Hoff, and provide examples of their application through the technologies, Isothermal Titration Calorimetry (ITC) and Surface Plasmon Resonance (SPR) biosensor analysis, respectively.

Isothermal Titration Calorimetry (ITC)

The most direct method of acquiring thermodynamic data is via calorimetry. The technology most commonly used for this purpose in drug discovery is ITC (systems available from e.g., microcal, which is now part of GE Healthcare, www.microcal.com, or TA Instruments, www.tainstruments.com). During an ITC experiment, a solution containing one of the binding partners is titrated into a sample cell containing the second binding partner. During the ligand binding event heat will either be released (exothermic) or absorbed (endothermic). This is measured through changes in the amount of energy required to retain a constant temperature in the sample cell. ITC does not require labeling or immobilization of

the protein or ligand and allows determination of the enthalpy and entropy change, binding constant and binding stoichiometry in a single experiment. Performing experiments at multiple temperatures allows determination of the heat capacity change ($\Delta C_p°$) (see **Glossary**) , which may provide further insight into the mode of binding, e.g., manifestation of the hydrophobic effect.

A problem encountered in using ITC within FBDD is the low binding affinity of the small fragments, which may preclude direct measurement of all the binding parameters. This problem can be circumvented by performing competition experiments.[22] However, two major disadvantages of ITC remain; the amount of protein required and the time taken per experiment. As a consequence, ITC is not suitable for primary fragment screening, but may be considered as a secondary screening tool, able to validate identified fragment hits and obtain the thermodynamic parameters of binding at the same time. Nevertheless, recent progress in downsizing the ITC sample cell and automation of sample handling enables a throughput of 75 samples per day with as little as of 10 µg of protein per sample.[23] However, It should be noted that the amount of protein required for analysis is dependant on the affinity and thermodynamic binding profile of the specific interaction that is under investigation. Furthermore, reducing the amount of protein per sample or the time per experiment comes at a cost to accuracy.[24]

Van 't Hoff method using SPR biosensor analysis

Any method which is able to provide affinity data over a range of temperatures can be deployed to retrieve thermodynamic data using the van 't Hoff equation (see **Glossary**). In practice this has mainly been done using radioligand binding assays, however, spectroscopic techniques such as NMR, mass spectroscopy and chromatographic methods have all been employed to obtain data for the van 't Hoff method.[11] In this review we will focus on a relatively new technology used to obtain affinity data, surface plasmon resonance (SPR) biosensor analysis, to highlight some of the differences between using calorimetry and the van 't Hoff method (systems available from e.g., Biacore which is now (also) part of GE Healthcare, www.biacore.com, Reichert, www.reichertspr.com, Ibis, www.ibis-spr.nl, Metrohm, www.metrohm.com, and Xantec, www.xantec.com). SPR biosensor analysis measures mass changes resulting from binding of an immobilized binding partner to a binding partner in solution. This is done by measuring the change in the incidence angle, of light reflected off the surface on which binding occurs, required to achieve resonance with surface plasmon.[25]

Modern SPR biosensor systems provide integrated van 't Hoff analysis software to retrieve thermodynamic data. When a limited amount of the target protein is available, SPR biosensor analysis is particularly attractive as a technique for thermodynamic analysis, since it requires small amounts of protein. Furthermore, besides determination of the thermodynamics behind a binding event, SPR biosensor analysis also enables retrieval of the kinetics (k_{on} and k_{off}) of binding, although kinetic analysis may not be possible for low affinity fragments because the kinetic rates are too fast.[26] If kinetic and thermodynamic data can be obtained, investigation of transition state thermodynamics, e.g., enthalpy of association, becomes a possibility and may provide additional insights into binding mechanisms.

Comparison between ITC and SPR biosensor analysis

Much has been written on differences in results obtained by ITC and van 't Hoff experiments.[27-34] Several studies were performed to test for consistent differences between calorimetric and van 't Hoff determinations of binding thermodynamics using ITC and SPR at different laboratories. In total 61 SPR experiments were compared with 26 ITC experiments. Results from ITC and SPR experiments were found to be highly consistent, deviation between the technologies averaged just 4%.[35-37] In principle this proves that there is no difference in accuracy between thermodynamic experiments performed by ITC and those performed by SPR. However, only two protein targets were used in the experiments, so other targets might show greater variability. The studies found significant differences across locations and experimental results from some labs were not included because of the presence of artifacts, which were presumed to have resulted from a lack of maintenance and/or thorough calibration of the equipment. Indeed, in thermodynamic studies which compare experimental conditions it has been found that, salt levels,[38] protein sources,[39,40] metal oxidation states,[41] cofactor presence,[42] species source,[43] pH,[44] and temperature[45] can all have significant influence on the measured thermodynamic profile. This presents a warning for those comparing thermodynamic data from different sources and illustrates the need for standardized protocols to be used in the measurement of thermodynamic data.

ITC is a near universally applicable technology for thermodynamic analysis, requiring no special sample preparation steps and having a broad affinity range. Since ITC is a direct method for determination of enthalpy changes, it is considered to be more accurate than the indirect van 't Hoff method.[46] However, ITC requires large amounts of sample, making its use in studies looking at hard to obtain proteins untenable. On the other hand, SPR biosensor analysis is efficient in sample consumption and analysis times. It should be realized though, that switching between measurement temperatures, which is required for thermodynamic analysis, significantly lengthens the measurement times. Furthermore, the required immobilization of one of the binding partners represents an additional initial effort, which may not always be successful and requires subsequent validation. In addition, retrieval of thermodynamic binding parameters using SPR biosensor analysis may be complicated by the manifestation of non-linear van 't Hoff plots which have to be fit over a large temperature range with a substantial amount of data-points in order to include heat capacity ($\Delta C_p°$) in the model. However, curvature in the van 't Hoff plots may be masked by experimental noise, compromising the accuracy of the determined thermodynamic binding parameters.[33] A comparative overview of both technologies is shown in Table 1.

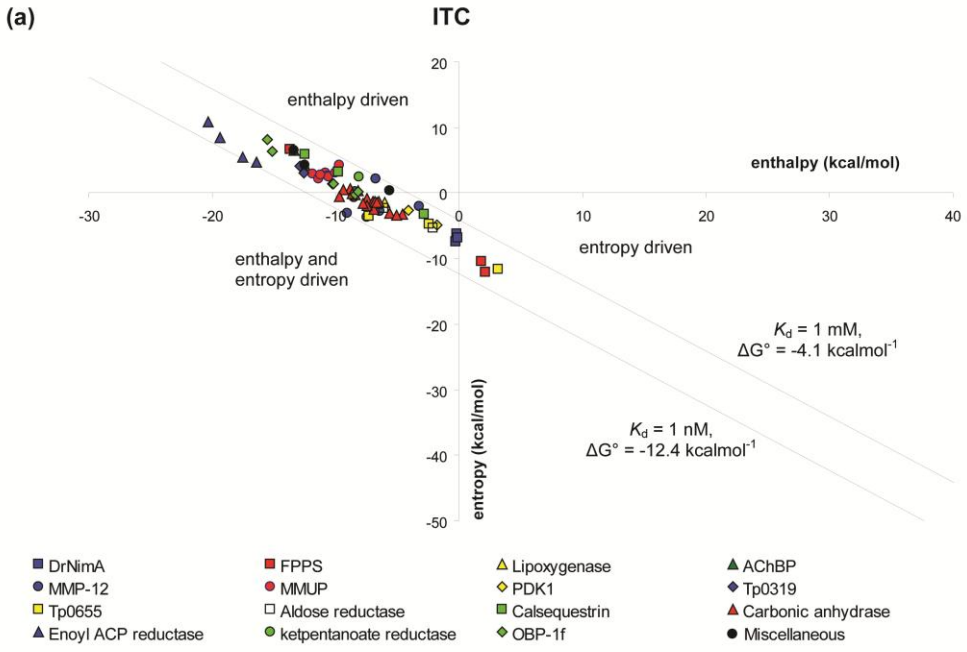
Examples of the application of thermodynamics in FBDD

Despite its potential, the use of thermodynamic analysis in a FBDD context, is currently far from common and there are only a few examples in literature in which both technologies have been combined (see below). However, since FBDD is a relatively young discipline, thermodynamic data on fragment-like molecules may be available that has not been labeled as FBDD. Inspection of literature and the databases mentioned earlier, revealed that indeed already a substantial amount of thermodynamic data on fragments is available. In order to increase our understanding of the thermodynamic aspects of fragment-protein binding, we

constructed a database containing 162 unique ligand-protein complexes in which the molecular weight of the ligand was smaller than 300 Da.[47] The thermodynamic data that was collected using ITC is summarized in **Figure 2a** and data that was obtained using van 't Hoff analysis is summarized in **Figure 2b**.

Several trends can be observed from the enthalpy-entropy plots depicted in **Figure 2**. First of all, van 't Hoff data is characterized by a greater amount of entropy driven binding. This is not a trend resulting from the techniques, but rather a trend resulting from the different targets being tested. The van 't Hoff experiments involving fragments were almost exclusively performed on membrane-bound receptors (see **Figure 2b**), while the ITC experiments tended to be performed on water-soluble enzymes (see **Figure 2a**). When applying van 't Hoff analysis on membrane-bound proteins, temperature effects on the membrane itself (e.g., membrane fluidity) may influence the determined thermodynamic binding parameters. As such, these effects may therefore play a role in the observed differences between **Figures 2a** and **2b**. Nevertheless, the trend of wider distribution of thermodynamic binding signatures in the case of membrane-bound receptors is also likely to originate from the conformational changes that are required for receptors to switch between active and inactive states. This explanation is in line with an additional trend that is visible in **Figure 2b**; the thermodynamic discrimination between agonists and antagonists in many of the tested receptors. This phenomenon, in which agonist binding to a receptor is entropy-driven and the binding of its antagonist is enthalpy-driven, or vice versa, has been observed for G-protein-coupled receptors (GPCRs), such as β-adrenoceptors, adenosine A_1 and A_{2A}, as well as for ligand-gated ion channels (LGICs), such as glycine, $GABA_A$, 5-HT_3 and nicotinic acetylcholine receptors.[48,49] Although the exact origin of thermodynamic discrimination remains unclear, for adenosine A_1 receptors it has been suggested that binding of an antagonist results in the displacement of a water network from the binding site, whereas the binding of agonists does not, providing an explanation for the distinct thermodynamic profiles of binding.[50] In the case of LGICs, the conformational change that takes place upon receptor-activation to induce channel opening, has been linked to the discrimination between agonists and antagonists.[51] Although fragments that exhibit enthalpy driven complex formation may be the preferred candidates for further optimization, **Figure 2** illustrates that this may depend on the target protein being an enzyme, or a receptor, and on what kind of functional profile (antagonist or (partial) agonist) is required to obtain the anticipated therapeutic effect. It is important to note that the van 't Hoff analysis on receptors was not performed using SPR biosensor systems, but rather by traditional affinity assays (radioligand displacement) performed at a range of temperatures.

A third trend that can be observed is that the distribution in Gibbs energy of binding ($\Delta\Delta G° = 11$ kcal·mol^{-1}) is significantly smaller than the distribution in enthalpy and entropy ($\Delta\Delta H° = T\Delta\Delta S° = 63$ kcal·mol^{-1}). These differences reflect enthalpy-entropy compensation; large favorable changes in enthalpy are compensated by unfavorable changes in entropy, and vice versa.[9,10]


(a)
ITC
enthalpy driven
enthalpy (kcal/mol)
entropy driven
enthalpy and
entropy driven
K_d = 1 mM,
ΔG° = -4.1 kcalmol^{-1}
K_d = 1 nM,
ΔG° = -12.4 kcalmol^{-1}
entropy (kcal/mol)
DrNimA
MMP-12
Tp0655
Enoyl ACP reductase
FPPS
MMUP
Aldose reductase
ketpentanoate reductase
Lipoxygenase
PDK1
Calsequestrin
OBP-1f
AChBP
Tp0319
Carbonic anhydrase
Miscellaneous

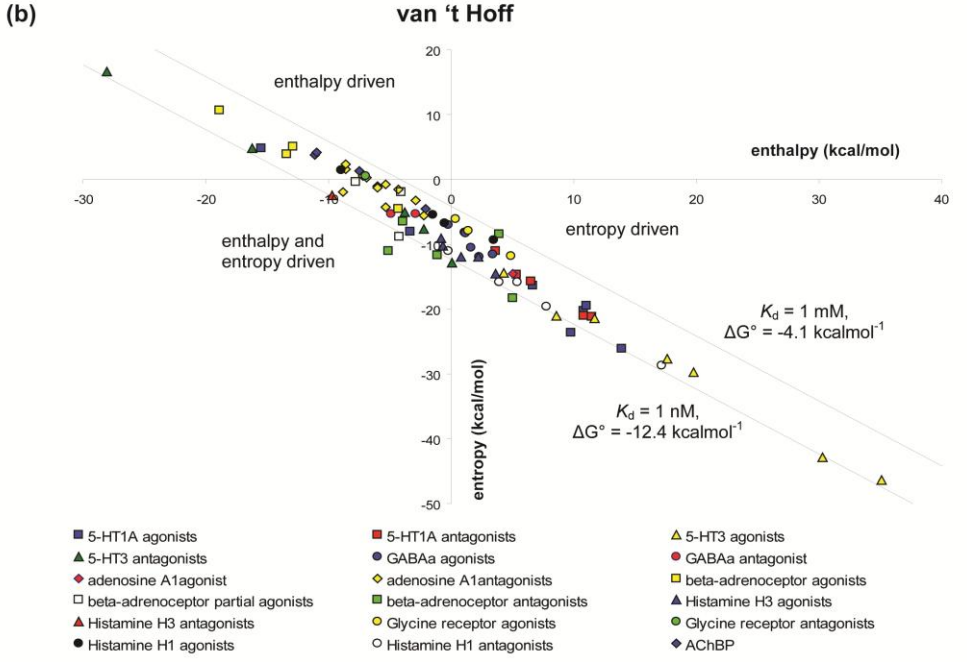

(b)
van 't Hoff
enthalpy driven
enthalpy (kcal/mol)
entropy driven
enthalpy and
entropy driven
K_d = 1 mM,
ΔG° = -4.1 kcalmol^{-1}
K_d = 1 nM,
ΔG° = -12.4 kcalmol^{-1}
entropy (kcal/mol)
5-HT1A agonists
5-HT3 antagonists
adenosine A1agonist
beta-adrenoceptor partial agonists
Histamine H3 antagonists
Histamine H1 agonists
5-HT1A antagonists
GABAa agonists
adenosine A1antagonists
beta-adrenoceptor antagonists
Glycine receptor agonists
Histamine H1 antagonists
5-HT3 agonists
GABAa antagonist
beta-adrenoceptor agonists
Histamine H3 agonists
Glycine receptor antagonists
AChBP

Figure 2: *Enthalpy vs. entropy plot of ligand-protein complexes that were obtained using ITC (**a**) and van 't Hoff analysis (**b**). The two diagonal lines show Gibbs energy changes of -4.1 kcal·mol^{-1} and -12.4 kcal·mol^{-1} equivalent to affinities of 1 mM and 1 nM, respectively.*

Several series of structurally related fragments were identified upon searching the literature and databases. This data may provide an opportunity to increase our understanding of the thermodynamic aspects of fragment optimization. By taking the smallest common scaffold as a starting point in a hypothetical fragment optimization process, the enthalpic and entropic contributions to any subsequent fragment growing or linking step could be calculated. Here, we provide a selection of examples from our analysis.

Adenosine A1 receptor

Borea and co-workers have studied the binding thermodynamics of xanthine derivatives binding to the G-protein coupled adenosine A_1 receptor.[50,52] These compounds which include theophylline and caffeine act as non-selective antagonists on the A_1 and A_{2A} adenosine receptors.[53] Measuring binding affinity constants at six different temperatures in the range from 0-35 °C, enabled van 't Hoff analysis of the thermodynamic binding parameters of 16 xanthine derivatives. In the example shown in **Figure 3**, the fragment theophylline (MW = 180) was chosen as a starting point. Theophylline exhibits a good LE and EE (see Glossary) for the adenosine A_1 receptor and its binding is completely enthalpy driven ($\Delta H°$ = -7.2 and $-T\Delta S°$ = +0.5 kcal·mol^{-1}). Binding affinity can be increased by substituting the 8-position with hydrophobic moieties such as phenyl (8-PT) and cyclopentyl (CPT) moieties, resulting in 125- and 900-fold increases in affinity, respectively. While addition of a phenyl moiety increases binding affinity solely due to a favorable increase in entropy ($-T\Delta\Delta S°$ = -4.8 kcal·mol^{-1}), growing the fragment from the 8-position with a cyclopentyl moiety results in favorable changes in enthalpy, as well as in entropy ($\Delta\Delta H°$ = -1.6 and $-T\Delta\Delta S^0$ = -2.5 kcal·mol^{-1}). An additional increase in the binding affinity of the cyclopentyl substituted fragment can be achieved by extending both methyl substituents with two carbons each (DPCPX). This modification affords a 6-fold increase in binding affinity due to a favorable increase in entropy ($-T\Delta\Delta S°$ = -2.1 kcal·mol^{-1}). Figure 3 shows that introducing or increasing hydrophobic substituents can result in increases in binding affinity due to favorable increases in entropy, illustrative of the hydrophobic effect. More interesting is the 900-fold increase in affinity upon addition of a cyclopentyl moiety to theophylline's 8-position. This extensive increase in affinity results from favorable changes in entropy as well as in enthalpy. As optimization of both thermodynamic binding parameters at the same time is the most efficient way of increasing binding affinity, this specific example may warrant further investigation. For example the generation of X-ray co-crystal structures of the starting fragment and its 8-cyclopentyl substituted derivative may provide insights into this unusual simultaneous enthalpy and entropy optimization.

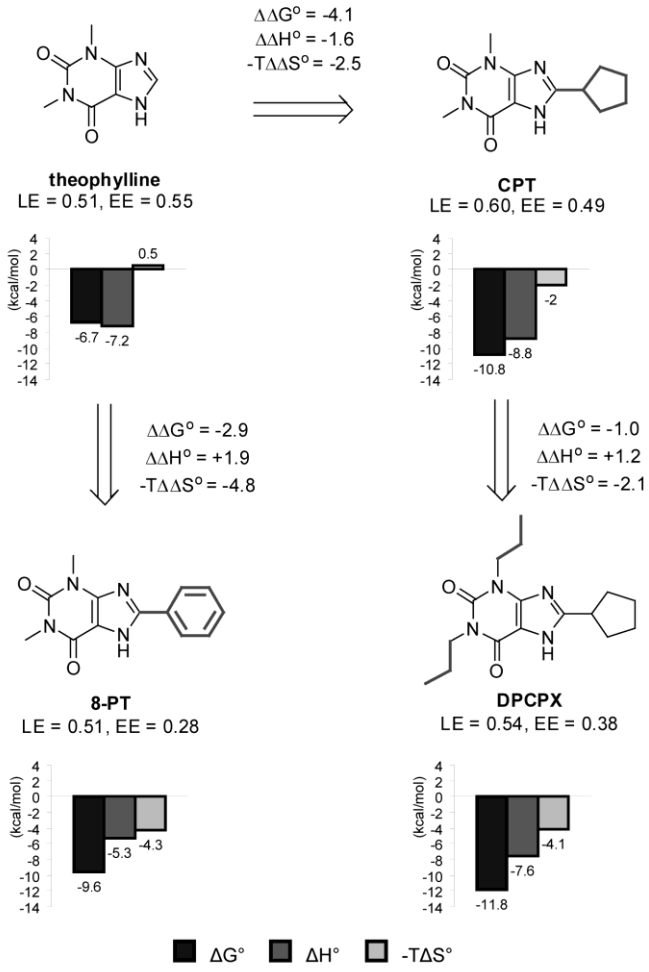


Figure 3: *Changes in Gibbs energy ($\Delta\Delta G^0$), enthalpy ($\Delta\Delta H^0$) and entropy ($-T\Delta\Delta S^0$) in kcal·mol^{-1} upon growing the fragment theophylline towards high affinity adenosine A_1 antagonists. For each compound, the LE and EE have been calculated in kcal·mol^{-1} per heavy atom.*

Matrix Metalloproteinases

Bertini and coworkers have performed a thorough study on matrix metalloproteinase 12 (MMP12) inhibitors in which they obtained co-crystal structures, as well as thermodynamic parameters of binding of a large number of inhibitors using ITC.[54] The common scaffold of all the inhibitors analyzed in this study is N-hydroxy-2-(phenylsulfonamido)acetamide (**1**). This fragment (MW = 230) binds with a K_d of 61 nM ($\Delta G°$ = -9.8 kcal·mol^{-1}) and exhibits a high LE for MMP12 (0.65 kcal·mol^{-1} per heavy atom). In a later study, the *p*-methoxy substituted analog of fragment **1** (**2**) was further deconstructed to acetohydroxamic acid (**AHA**) and *p*-methoxybenzene-sulfonamide (**PMS**), see **Figure 4**.[55] This approach allows

determination of the thermodynamic contributions to binding affinity when two separate fragments are linked. Thermodynamic analysis shows that the sum of the binding enthalpies of the separate fragments is quite similar to the binding enthalpy of **2**. The significant gain in binding affinity upon linking the fragments seems to be completely due to a gain in entropy ($-T\Delta\Delta S$ = -4.4 kcal·mol^{-1}). This favorable increase in entropy upon linking fragments arises because, upon binding to the protein binding site, ligands lose translational and rotational degrees of freedom. When the linked fragments bind, the entropy cost of restricting ligand rotation and translation only needs to be paid once. As a consequence, the linking of fragments that occupy different spots within the protein binding site can yield significant increases in binding affinity. Unfortunately, in practice the expected increases in affinity are often not obtained due to perturbation of the binding modes of the separate fragments upon linking, or because of strain in the linker that is used to connect the fragments.[56,57]

From a thermodynamic point of view, the common scaffold **1** can be considered as an ideal starting point for further optimization, as complex formation is almost exclusively enthalpy driven ($\Delta H°$ = -9.1 kcal·mol^{-1}). X-ray analysis shows that the hydroxamic group interacts with the catalytic zinc ion. Furthermore, the hydroxamic and sulfonamide moieties are involved in additional enthalpic interactions with the binding site via direct and water-mediated hydrogen bonds. Extending the 4-position of the phenyl moiety of fragment (**1**) with a methoxy (**2**) or phenyl moiety (**4**) results in additional hydrophobic interactions in the S1' pocket (see **Figure 5**) affording 3- and 25-fold increases in binding affinity, respectively. These increases in binding affinity by addition of hydrophobic moieties are in both cases due to more favorable entropy (4-MeO: $-T\Delta\Delta S°$ = -1.3 kcal·mol^{-1} and 4-Phenyl: $-T\Delta\Delta S°$ = -2.2 kcal·mol^{-1}). Analysis of the X-ray structures shows that extending the 4-position with a methoxy or phenyl moiety, worsens the interactions of the hydroxamic group with the zinc ion and the hydrogen bonds formed by the sulfonyl moiety. Bertini and co-workers suggest that this weakening of enthalpic interactions results in a more favorable entropy as the ligand and protein gain more freedom of movement. It is also likely that growing into the hydrophobic S1' pocket results in the displacement of water molecules affording additional favorable increases in entropy.

Capping the sulfonamide nitrogen of **2** with an isobutyl group (**3**) results in a 5-fold increase in binding affinity resulting entirely from a favorable increase in entropy ($T\Delta\Delta S°$ = -1.2 kcal·mol^{-1}). In contrast, a 3-fold increase in affinity by decoration of the sulfonamide nitrogen with a hydroxyethyl moiety (**5**), is established by a favorable decrease in enthalpy ($\Delta\Delta H°$ = -1.2 kcal·mol^{-1}). X-ray analysis shows that the favorable decrease in enthalpy may originate from van der Waals contacts of the ethyl spacer with Pro238 and hydrogen bond formation with a network of water molecules that is interacting with residues that align the entrance of the binding site (see **Figure 5**). In terms of binding affinity, compounds **3** and **4** can be considered the best compounds for further optimization. Nevertheless, the compound most optimized in enthalpy is the *N*-hydroxyethyl substituted derivative **5**, which may be the best choice for further optimization when EE is considered.

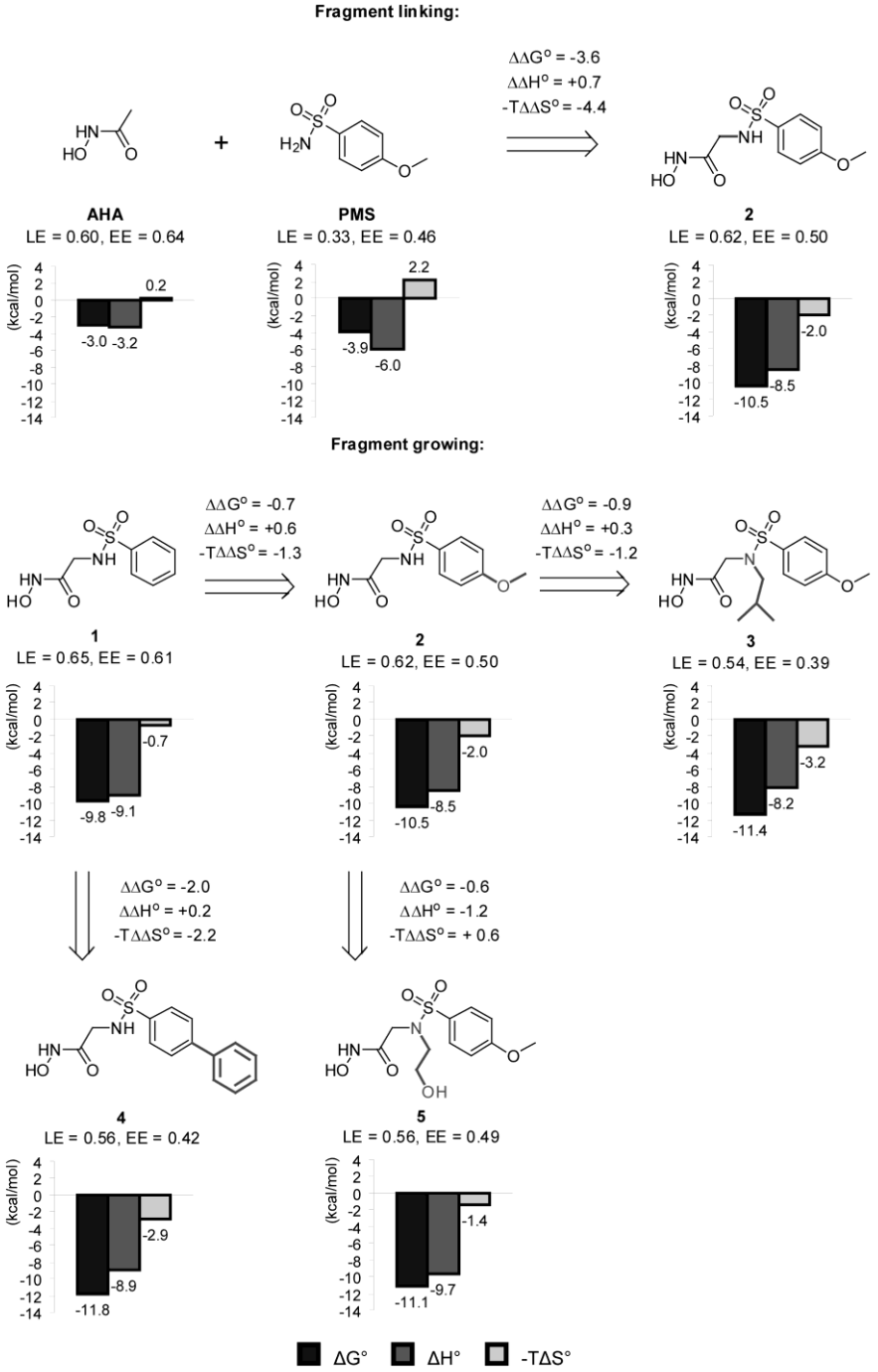

Fragment linking:
ΔΔG° = -3.6
ΔΔH° = +0.7
-TΔΔS° = -4.4
AHA
LE = 0.60, EE = 0.64
PMS
LE = 0.33, EE = 0.46
2
LE = 0.62, EE = 0.50
(kcal/mol)
0.2
-3.0 -3.2
2.2
-3.9
-6.0
-2.0
-8.5
-10.5
Fragment growing:
ΔΔG° = -0.7
ΔΔH° = +0.6
-TΔΔS° = -1.3
ΔΔG° = -0.9
ΔΔH° = +0.3
-TΔΔS° = -1.2
1
LE = 0.65, EE = 0.61
2
LE = 0.62, EE = 0.50
3
LE = 0.54, EE = 0.39
-0.7
-9.8 -9.1
-2.0
-8.5
-10.5
-3.2
-8.2
-11.4
ΔΔG° = -2.0
ΔΔH° = +0.2
-TΔΔS° = -2.2
ΔΔG° = -0.6
ΔΔH° = -1.2
-TΔΔS° = + 0.6
4
LE = 0.56, EE = 0.42
5
LE = 0.56, EE = 0.49
-2.9
-8.9
-11.8
-1.4
-9.7
-11.1
ΔG°
ΔH°
-TΔS°

***Figure 4**: Changes in Gibbs energy ($\Delta\Delta G^0$), enthalpy ($\Delta\Delta H^0$) and entropy ($-T\Delta\Delta S^0$) in kcal·mol^{-1} upon linking and growing fragments towards high affinity MMP12 inhibitors. For each compound, the LE and EE have been calculated in kcal·mol^{-1} per heavy atom.*

***Figure 5**: The binding mode of compound **5** (black ball and sticks) in complex with MMP-12 as determined by X-ray crystallography (PDB: 3NX7). The S1' pocket is indicated by the grey arrow whereas the water network which interacts with the hydroxyethyl moiety is indicated by the white arrow.*

Major mouse urinary protein

The mouse major urinary proteins (MUPs) are pheromone-binding proteins, a complex of protein isoforms encountered in mice which appear to serve as signal-modulating agents for pheromones. MUPs have been used in several studies as a model protein to investigate the thermodynamics of ligand-protein interactions.[45,58,59] X-ray crystallography and NMR studies have shown that the interior of MUPs contain a hydrophobic cavity, which forms the ligand binding site.[45] Interestingly, increasing the size of hydrophobic substituents of two identified ligands for MUP-I, 2-methyl-4,5-dihydrothiazole (**MT**) and 2-isopropyl-3-methoxypyrazine (**IPMP**), results in favorable decreases in enthalpy ($\Delta\Delta H° = -1.5$ and -1.2 kcal·mol^{-1}, resp.), see **Figure 6**. These findings are in contrast with the classical view on the hydrophobic effect, in which increased burial of hydrophobic substituents in a hydrophobic cavity results in more favorable entropy.

In an initial study by Sharrow *et al*. three possible explanations were provided for the favorable change in enthalpy that was observed upon increasing the size of the hydrophobic substituents on the 2-position of 4,5-dihydrothiazoles: (1) formation of a buried water-mediated hydrogen bond network between the ligand and protein

binding site (2) formation of strong van der Waals interactions and (3) changes in structure, dynamics, and/or hydration of the protein upon binding.[45] Bingham and co-workers studied the thermodynamics of binding to MUP-I of two methoxy substituted pyrazine derivatives (**IPMP** and **IBMP**, **figure 6**) using ITC, X-ray crystallography and NMR. As X-ray analysis showed that in contrast with the binding of 4,5-dihydrothiazole derivatives to MUP-I, no water molecules are found in the binding site when complexed to **IPMP** or **IBMP**, the formation of a water-mediated hydrogen bond network between the ligands and protein binding site cannot be responsible for the observed enthalpy-driven binding profiles of **IPMP** and **IBMP**. A subsequent study, using molecular dynamics and solvent isotopic substitution ITC measurements, suggests that solvation processes are not a major contributor to the observed favorable binding enthalpy.[58] In the same study, hydrogen/deuterium exchange experiments indicate that changes in structure or dynamics, such as "tightening" of the protein, do not contribute significantly to the enthalpy of binding. Altogether, the results of these studies on MUP-I suggest that due to poor solvation of the binding site prior to binding, the formation of strong van der Waals interactions between the ligands and protein binding site are a significant contributor to the observed favorable enthalpic contribution. The thermodynamic studies on MUP-I illustrate that depending on the topology of the protein binding site, fragments may also be optimized in terms of enthalpy by addition of or increasing the size of hydrophobic moieties and that enthalpic optimization does not necessarily prevent increases in lipophilicity. Furthermore, the 100-fold increase in affinity by increasing the size of the hydrophobic substituent of fragment **MT,** provides another example in which binding affinity is increased by simultaneous optimization of enthalpic and entropic binding parameters.

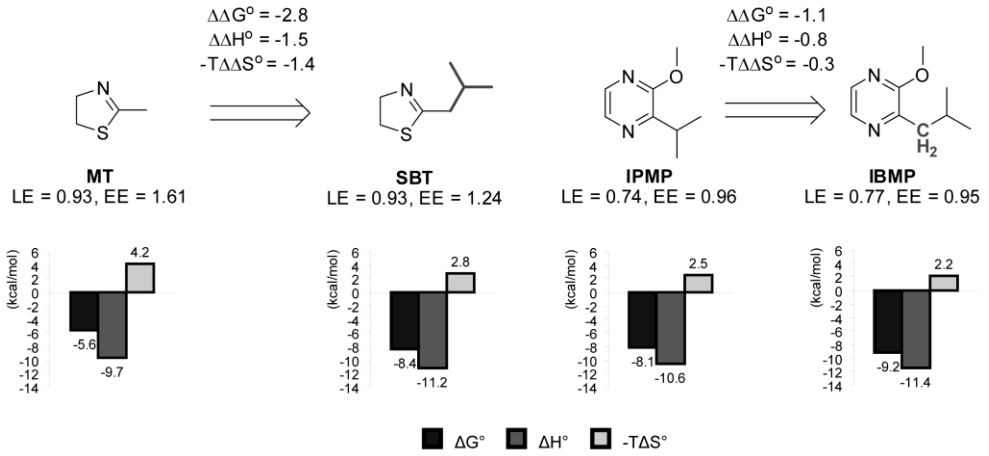


Figure 6: *Changes in Gibbs energy ($\Delta\Delta G^0$), enthalpy ($\Delta\Delta H^0$) and entropy ($-T\Delta\Delta S^0$) in kcal·mol^{-1} upon increasing the size of a hydrophobic substituent of 4,5-dihydrothiazole (**MT**) and 2-isopropyl-3-methoxypyrazine (**IPMP**). For each compound, the LE and EE have been calculated in kcal·mol^{-1} per heavy atom.*

v-Src homology (SH2) domain

The studies described above did not deliberately apply thermodynamic analysis to guide and/or monitor the fragment optimization process. However, recently two studies have been published in which thermodynamic analysis played a significant role in deciding which fragment to pursue or, monitoring the fragment optimization. The group of Ladbury has applied thermodynamic analysis on two hits that were identified using an NMR-based fragment screening on the Src SH2 domain.[60] Dissection of binding affinity into its separate thermodynamic parameters revealed that although both fragments exhibit similar affinity, one of the compounds binds with a significantly more favorable enthalpy ($\Delta\Delta H°$ = -3.3 kcal·mol^{-1}) and is therefore considered to be a better fragment hit to optimize. Unfortunately, results on further optimization of the identified fragment-hits have so far not been disclosed.

Carbonic anhydrase

The value of using thermodynamic analysis in FBDD is well illustrated by a study performed by Scott and co-workers in which the thermodynamic parameters of 20 benzene sulfonamide derivatives binding to human carbonic anhydrase were determined using ITC.[4] Substitution of benzene sulfonamide (**6**) with a fluorine atom at the 2- or 3-position affords a 3- and 7-fold increase in affinity, respectively (see **Figure 7**). Based on LE, the 3-substituted fragment **8** can be considered to be more appropriate for further optimization. However, thermodynamic analysis reveals that the increase in affinity by fluorine substitution at the 2-position (**7**) is caused by a favorable change in enthalpy ($\Delta\Delta H°$ = -1.9 kcal·mol^{-1}, $-T\Delta\Delta S°$ = + 1.3 kcal·mol^{-1}), whereas the increase in affinity for 3-fluorobenzene sulfonamide results solely from a favorable change in entropy ($\Delta\Delta H°$ = 0 kcal·mol^{-1}, $-T\Delta\Delta S°$ = -1.1 kcal·mol^{-1}). X-ray analysis of co-crystal structures shows that the fluorine atom in the 2-substituted fragment is engaged in a specific interaction with the backbone N-H of Thr200, whereas the fluorine atom of the 3-substituted benzene sulfonamide is pointing towards a hydrophobic part of the binding site. Growing both fragments with a 4-benzylamide substituent affords a 55-fold increase in affinity for the 2-fluorobenzene sulfonamide (**9**), but only a modest 6-fold increase in affinity for its 3-substituted isomer (**11**). In addition, the binding of 2-fluorobenzene sulfonamide remains entirely enthalpically driven. In this specific study, the most enthalpy efficient fragment became the most potent ligand upon optimization and retained its favorable thermodynamic signature.

Conclusion

Recent findings suggest that in drug development, compounds that have been enthalpically optimized, outperform compounds in which binding is based on favorable entropy changes. However, it is easier to improve binding affinity by optimizing entropy than it is by optimizing enthalpy. It therefore seems a good strategy to determine the thermodynamic profiles of fragment hits and include EE as one of the hit selection criteria. Choosing a fragment in which binding is enthalpically driven as a starting point and adding hydrophobic groups during optimization, may provide the easiest route to a selective high affinity compound. In addition, determination of thermodynamic profiles may be very useful in guiding and monitoring of (structure-based) fragment optimization. When combined with structural data, the stepwise fragment growing process provides an ideal dataset

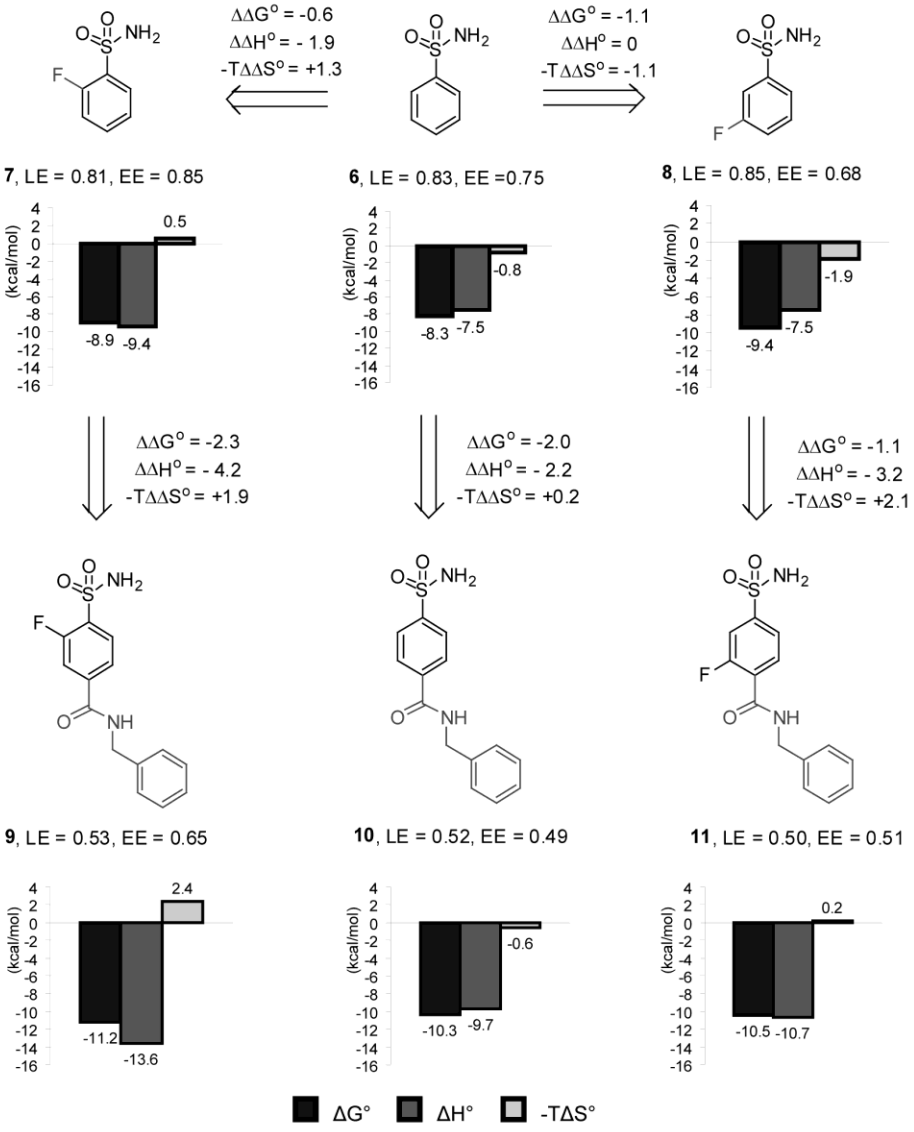


Figure 7: *Changes in Gibbs energy ($\Delta\Delta G^0$), enthalpy ($\Delta\Delta H^0$) and entropy ($-T\Delta\Delta S^0$) in kcal·mol^{-1} upon growing benzene sulfonamide **(1)** towards more potent carbonic anhydrase inhibitors. For each compound, the LE and EE have been calculated in kcal·mol^{-1} per heavy atom.*

with which to improve our understanding of the thermodynamics of binding. This is exemplified by the case on MUP that shows that extending a fragment in a poorly solvated binding pocket can afford considerable increases in binding affinity by favorable changes in enthalpy as well as entropy. Another example of the unusual simultaneous of enthalpy and entropy by fragment growing has been identified for the Adenosine A_1 receptor and may provide important lessons on how to optimize fragments in a most efficient manner. These examples emphasize that both FBDD, and our understanding of the thermodynamics of binding, have much to gain from the application of thermodynamic analysis in FBDD.

The two main techniques that are used to determine thermodynamic binding parameters are ITC and van 't Hoff analysis. The main advantage of ITC is its easy set-up, as calorimetric measurements do not require immobilization or labeling. Disadvantages are the large amounts of protein required and the relatively long time required to perform an experiment. It should be noted that progress has recently been made in resolving these issues. Measuring binding affinity at a range of different temperatures (0 – 37 °C) enables van 't Hoff analysis, providing access to the thermodynamic parameters. Although discrepancies between calorimetry and van 't Hoff analysis have been reported, more recent comparison studies show that application of both techniques result in similar values for the thermodynamic binding parameters. Nevertheless, retrieval of thermodynamic binding parameters using van 't Hoff analysis may be complicated when the heat capacity change is not equal to zero ($\Delta C_p^\circ \neq 0$). A convenient technique enabling van 't Hoff analysis is SPR biosensor analysis, since it allows accurate temperature control, requires much smaller amounts of target-protein than ITC and is a label-free technique. In addition to thermodynamic data, kinetic parameters of binding may be obtained as well, providing an additional dimension to binding affinity.

Both ITC- and SPR biosensor-based thermodynamic analysis are hampered by low throughput and are therefore not suitable for primary fragment screening. Instead, these techniques are more appropriate for secondary screening, to confirm and characterize the thermodynamics of fragment hits. If target-protein production is not limiting, one may consider ITC, since measurements can be performed without the need for immobilization and all binding parameters (n, K_B, ΔH^o and ΔS^o) are determined in a single experiment. If the target protein is more difficult to obtain, SPR biosensor analysis may be a more sensible technique to use, requiring only small amounts of protein. Although, the use of thermodynamic analysis within FBDD is still far from common, the first cases of successful application are becoming apparent, exemplifying its value in the development of a more efficient fragment optimization process.

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