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# Is there a link between selectivity and binding thermodynamics signatures?

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**Abstract**

Thermodynamics of ligand binding is influenced by the interplay between enthalpy and entropy contributions of the binding event. The impact of these binding free energy components, however, is not limited to the primary target only. Here we investigate the relationship between binding thermodynamics and selectivity profiles by combining publicly available data from broad off-target assay profiling and corresponding thermodynamics measurements. Our analysis indicates that compounds binding their primary targets with higher entropy contributions tend to hit more off-targets compared to those ligands that had enthalpy-driven binding.

High on-target affinity and designed selectivity against off-targets are usually the key points in the target product profile of many discovery programs and consequently these are among the most desired objectives of multiparameter medicinal chemistry optimizations. Potency optimizations are generally carried out by introducing apolar or polar substituents and subsequently monitoring the binding affinity (expressed in $K_i$ or $IC_{50}$ values). High specificity, however, does not demand high affinity [1]. Improving the binding affinity can be achieved by both enthalpy and entropy driven optimization that covers substantially different thermodynamic profiles. Apparently enthalpy and entropy changes are linked due to the widely observed enthalpy-entropy compensation, although its impact has been recently challenged [2]. Binding affinity shows the quantity of the ligand-protein interactions via the Gibbs free energy of binding while the corresponding thermodynamic profile describes the quality of the interactions.

The relationship between the knowledge encoded in Gibbs free energy of binding and its components, enthalpy and entropy can be explained by the analogy of the projection. Constellations of stars such as the Cassiopeia are plane projections having a graph pattern. Stars, however, are not located in the same distance, some stars are much closer than the others and therefore the projection has a hidden dimension. Taking this distance dimension into account makes the plane to a three-dimensional object. Constellations were used for efficient navigation for hundreds of years, as improvement of the binding free energy drove medicinal chemistry programs in the last decades. Space travellers, however, should use the information from the third dimension for successful navigation and similarly thermodynamics profiles provide beneficial information on the interactions for medicinal chemists.

Ligand-protein interactions involve attractive forces and hydration-effects. Properly positioned polar groups contribute to specific interactions, such as H-bonds, salt-bridges, polar-polar interactions and non-classical interactions such as σ-hole mediated halogen bonding that result in enthalpy gain. In order to exploit this enthalpy reward the binding partners should be in optimal orientation, since the binding energy is highly sensitive to both the distance and the angle of the interacting atoms [3, 4]. Non-polar groups typically form weaker, less oriented and less specific interactions such as van der Waals contacts and π-π stacking [5]. Changes in desolvation entropy are favourable in both cases, but the desolvation of polar groups is associated with unfavourable desolvation enthalpy. For example the desolvation enthalpy of OH and NH functionalities are 36.4 kJ/mol and 33.0 kJ/mol, respectively - that is in the range of the enthalpy gain realized with polar interactions - while for a methyl group the corresponding value is only 2.4 kJ/mol [6, 7]. If the interactions with binding site water molecules do not override the primary ligand-protein interactions the affinity gain achieved by the introduction of polar groups is generally enthalpy biased. In contrast, introduction of non-polar substituents typically results in entropic reward that is mainly mediated by desolvation effects. Suboptimal positioned polar moieties would not be exploited in terms of enthalpy gain. The positional sensitivity of enthalpic optimization can be exemplified with a HIV-1 protease inhibitor pair. Saquinavir and TMC-126 have the same number of polar groups, however Saquinavir binding is associated with unfavourable ~+5 kJ/mol binding enthalpy while TMC-126 binding is significantly more enthalpic (ΔH~-50 kJ/mol) due to the better orientation of its polar groups [7]. It should also be noted that binding-site water molecules have complex influence on thermodynamics signatures [8-10]. Orientation of polar groups largely influences their specific interactions compared to non-polar functional groups that are introduced to fill apolar cavities. The latter types of interactions show less dependence on distance and are less sensitive to orientation. As a result, optimization of binding affinity is more straightforward by hydrophobic moieties. Non-interacting or suboptimal positioned polar atoms are charged by the unfavourable desolvation enthalpy and thus generally results in decreased affinity. Accordingly, enthalpy-driven optimization is considered to be significantly more challenging compared to the entropy driven process. Favourable binding energy can be achieved by entropy driven approaches such as the introduction of non-polar groups around apolar protein surfaces.

Replacement of unstable water molecules within hydrophobic pockets is mostly driven by entropy changes, although enthalpy gain coupled with water replacement by apolar moieties had also been reported [8-9]. The effect of binding site waters has been recently reviewed by using WaterMap for solvation energetic calculations [11]. Selectivity between dopamine D2 and D3 receptors and kinase targets were also successfully rationalized by the analysis of binding site water molecules [12-13]. Therefore, computational approaches can significantly facilitate the design of selective compounds, if high-quality crystal structures are available. Furthermore, the combination of experimental and computational approaches is able to rationalize unique cases where apolar contacts contribute to the favourable binding enthalpy, in a protein binding site occluded from solvent water [14].

The quality of interactions and the accompanying binding thermodynamics profile impact selectivity against off-targets [15]. Enthalpically optimized compounds possess carefully positioned ligand-binding site atom pairs to

achieve the desired gain in binding enthalpy. Considering a different binding pocket presented in an off-target protein the designed interactions will not be able to yield the enthalpic contribution to binding free energy, due to the improper orientation of the ligand. Since the very same desolvation penalty of the polar atoms must be paid the off-target affinity of the ligand will be limited. In contrast, entropically optimized compounds have less positional constraints and desolvation of the apolar moieties can result in entropy gain due to the lower dependence from the binding environment. These compounds have therefore higher propensity to form attractive interactions with off-targets. In this paper we investigate this hypothesis by analysing the thermodynamic and selectivity profiles of optimized compounds and marketed drugs.

**Binding thermodynamics and selectivity optimization**

*HIV-1 protease*

The relationship between the binding thermodynamics properties of a closely related pair of compounds published by Kawasaki and Freire serves as an illustrative example for the impact of thermodynamics on selectivity [15]. The thermodynamic profile was measured on the primary target HIV-1 protease, while cathepsin D and pepsin were monitored as antitargets. In the first case, as subtle change as the introduction of two methyl groups into a phenyl moiety resulted in -11.2 kJ/mol gain in binding free energy due to the more favourable enthalpy contribution of the methylated derivative [Figure 1.].

This effect is a result of the optimal occupancy of a small cavity around the aryl moiety that is well oriented and the methyl groups can form desirable contacts. The selectivity towards pepsin and cathepsin D increased from 12 to 157 and 72 to 2464, respectively. In the second pair the thioether moiety was replaced by the sulfonyl-methyl group that resulted in 1.2 kJ/mol decrease in binding free energy. However, the binding enthalpy improved from -34.3 kJ/mol to -50.6 kJ/mol, and the entropy contribution decreased by 11.2 kJ/mol. The introduced sulfonyl group establishes a strong hydrogen bond with Asp30 of the protease, as evident in the crystal structure. The selectivity against pepsin and cathepsin D increased by 7 and 9 fold. The authors suggested that maximal selectivity can be achieved by introducing a few very strong hydrogen bonds towards the primary target protein. H-bonds have very rigorous distance and angular constraints. Consequently suboptimal H-bonds formed with the off-target protein are penalized and this result in a larger decrease in the corresponding binding free energy. The overall picture of the four compounds suggests that as the enthalpy contribution to binding free energy is increased, the compounds are more specific to the primary target. It is interesting to note that among these four compounds, not the highest affinity one has the highest selectivity, but the one with the most favourable binding enthalpy. Although there is no theoretical background to support the linear correlation between these quantities linear correlation coefficients ($r^2$) between $\Delta H_{protease}$ and $\Delta\Delta G$ values obtained for pepsin and cathepsin D were significant (0.9 and 0.93, respectively). $\Delta G_{protease}$ and $\Delta\Delta G$ values were somewhat lower being 0.87 and 0.77, for pepsin and cathepsin D, respectively.

*Matrix metalloproteinase*

Matrix metalloproteinase 12 (MMP12) inhibitors were optimized by using X-ray crystallography and thermodynamics measurements [16] while monitoring selectivity against matrix metalloproteinase 13. The highest selectivity was 5 kJ/mol in terms of $\Delta G$ that had been achieved by the most enthalpic compound ($\Delta H$=-40.4 kJ/mol) and again, this was not the highest affinity compound considering the MMP12 target. The linear correlation coefficient (r) between the $\Delta G_{MMP12}$ and $\Delta\Delta G_{MMP12\text{-}MMP13}$ values was found to be -0.15 (p=0.85) while the linear correlation coefficient between the $\Delta H_{MMP12}$ and $\Delta\Delta G_{MMP12\text{-}MMP13}$ is higher: -0.68 (p=0.32). Observations on this limited congeneric ligand set further strengthens that not the affinity, but the binding enthalpy has higher contribution to off-target selectivity.

*Aldose reductase*

Aldose reductase (ALR2) is a promising therapeutic target to prevent late complications of diabetes. An optimal drug candidate should possess a high level of selectivity for ALR2 over the related aldehyde reductase (ALR1) [17]. In order to obtain a comprehensive overview of the binding event, X-ray crystallography and thermodynamics measurements were carried out for ALR2. Based on human X-ray structures published, the six studied ligands can be grouped into those penetrating to the specificity pocket and others leaving the specificity pocket closed. According to the X-ray structures, the favourable enthalpy of IDD393 might be the result of specific polar contacts between the nitro moiety of the ligand and the Ser302 residue. This was further strengthened by the Ser302Arg mutation, where the binding enthalpy of IDD393 dropped by 19.4 kJ/mol. Similarly, comparing the structures of Sorbinil and Fidarestat revealed that Sorbinil possess a strong H-bond with the backbone NH of Leu300, not present in the case of Fidarestat. This observation has been further supported by the decreased enthalpic contribution of Sorbinil binding by 8.2 kJ/mol in the Leu300Pro mutant protein. In both cases the increased selectivity against ALR1 might be due to the specific enthalpic contact with the primary target that is less than optimal in the case of the antitarget ALR1.

On the other hand, we did not find correlation between selectivity and binding enthalpy for the whole dataset. This might be explained by the different physicochemical profiles of the two ligand classes. Ligands occupying the selectivity pocket are carboxylic acids, and share similar pharmacophoric features, while those bound outside the

pocket (Sorbinil and Fidarestat) are small, weak NH acids, with less pharmacophore elements compared to compounds in the first group.

*Thrombin*

Medicinal chemistry optimization of triazole- and tetrazole-containing sulphonamide type thrombin inhibitors was published by Siles et al. [18]. The most promising compound had 828 times selectivity against trypsin. Evaluation of tryptase and chymase selectivity revealed favourable selectivity profile, since 0.95 and 0.98 residual enzyme activities were determined in the presence of 10 μM inhibitor. Thermodynamic profiles of thrombin binding, the primary target, were measured by isothermal titration calorimetry. The highly selective lead compound was found to bind thrombin with favourable -38.1 kJ/mol enthalpy contribution and unfavourable 3.3 kJ/mol entropy. Due to the significant selectivity against other related human serine proteases and the encouraging thermodynamic profile, this lead compound serves as high quality starting point for further optimization. Although, in this study only the top ranked compound was thermodynamically characterized, the high selectivity and the corresponding enthalpy-driven binding fits into the proposed relationship of these properties.

*Cannabinoid receptors*

Binding thermodynamics of agonists and antagonists of cannabinoid CB1 and CB2 receptors were determined by van't Hoff analysis [19] a methodology that is generally considered to be less reliable than ITC measurements, and highly influenced by the heat capacity change of the system. In this case, similarly to many GPCR targets, binding thermodynamics separates the ligands by their functional activity. Binding of the five agonist compounds was entirely entropy driven, while binding of the three antagonists was mainly realized by favourable enthalpy contribution. The relationship between thermodynamics signatures and functional activities is a topic of a high number of studies [20] and thus out of the scope of the present paper. Among the two investigated CB receptors, the highest affinity target was evaluated as primary target and the lowest affinity target as secondary. Selectivity was defined as the difference in binding free energy between the primary and the secondary target. The enthalpy contribution calculated for the primary target was compared to the selectivity observed resulting in linear correlation coefficient (r) of -0.81 (p=0.016) for all the eight ligands. Accordingly, ligands possessing more favourable binding enthalpy contribution had higher selectivity considering the two CB receptors. Among the eight ligands, ACEA, 2-Fl-AEA and CP-55,940 had different physicochemical profiles and pharmacophore sets. ACEA and 2-Fl-AEA are arachidonic acid derivatives, while CP-55,940 is an octane derivative. Therefore their binding mode might be significantly different from that of the remaining five ligands that might explain their distinct thermodynamic profiles. Leaving these ligands out resulted in higher linear correlation coefficient (r=-0.95, p=0.012) between the $\Delta\Delta G$ and $\Delta H$ values. According to these observations, the relationship between selectivity and enthalpy contribution seems to be valid on G-protein coupled receptors with thermodynamic profiles obtained from van't Hoff analysis for compounds having similar binding mode.

*Nucleic acid binding*

A specific DNA aptamer that recognized L-argininamide was discovered by Sytematic Evaluation of Ligands by EXponential enrichment system (SELEX) approach [1]. This construct had approximately 100-fold selectivity for L-argininamide over several other arginine analogues and amino acids. The highly selective binding of L-argininamide to the DNA was found to be relatively weak, -21.3 kJ/mol only. However, the binding was accompanied by a large, favourable enthalpy, in range of -36 to -38 kJ/mol, and unfavourable binding entropy, in range of 15 to 17 kJ/mol. Based on this observation, it was concluded that high specificity can be achieved without high affinity, if the binding is mediated by large enthalpy contribution.

Thermodynamics signatures of Amiloride binding to an abasic (AP site) site in RNA and DNA was reported recently [21]. In spite of the typically promiscuous binding of aminoglycoside antibiotics to various RNA targets, Amiloride was found to bind strongly and selectively to an AP site of RNA duplex. The thermodynamics measurements on AP-RNA revealed that the -45.2 kJ/mol binding free energy is composed of favourable -69.0 kJ/mol enthalpy and unfavourable 23.8 kJ/mol entropy contributions. Interestingly, the Amiloride affinity to AP-DNA was 78 times lower, -34.3 kJ/mol. Such remarkable preference of Amiloride binding to RNA relative to DNA is quite characteristic compared to typical small DNA-binding ligands [21]. Analysis of the binding thermodynamics data measured for RNA and DNA revealed that the unique selectivity is associated with a large 10.9 kJ/mol enthalpy difference, while the entropy contribution was almost equivalent. As a consequence, selectivity between the two targets was explicitly the result of the enthalpy change.

*Ligand-binding protein*

Anti-digoxigenin antibodies are administered to remove overdosed Digoxin, which has narrow therapeutic window. A very interesting approach was published recently in which protein binding sites were computationally designed to bind Digoxigenin (DIG) [22]. The binding pocket was in silico engineered to have specific, energetically favourable hydrogen-bonds and van der Waals interactions along with high overall shape complementarity. The best construct obtained was able to bind DIG with extremely high affinity (541 pM), similar to those of anti-digoxin antibodies. Isothermal titration calorimetry and X-ray crystallography revealed that the best host site is able to form three specific hydrogen-bonds with the ligand that is associated with favourable ~-45.3 kJ/mol enthalpy and ~-7.5 kJ/mol entropy. The designed protein has 29, 372 and 3216 fold DIG preference against structurally highly similar compounds: digitoxigenin, progesterone and β-oestradiol, respectively. In order to assess

the role of specific hydrogen bonds with DIG ligand on selectivity Tyr101, H-bond donor interacting with DIG C-ring oxygen, was mutated to Phe. This mutation resulted in lower affinity for DIG ($K_i$: 39 nM) and reduced selectivity: ~0.1, 0.8 and 43 fold for digitoxigenin, progesterone and β-oestradiol, respectively. Mutation of the other important H-bonding partner, Tyr43 to Phe also decreased the affinity for DIG (59 nM) and the selectivity against the three investigated compounds (DIG had 12, 1.3 and 254 fold selectivity against digitoxigenin, progesterone and β-oestradiol, respectively). These experiments confirmed that the selectivity of the artificial ligand-binding protein for DIG was conferred through the designed hydrogen-bonding interactions. Therefore this study is a plausible example for the role of highly oriented specific interactions that results in favourable binding enthalpy and high specificity. Considering medicinal chemistry aspects, designing ligands that are able to form optimal, enthalpically favourable hydrogen bonds with the target binding site might similarly result in high specificity against different binding environments having lower complementarity and suboptimal hydrogen-bonding pattern.

**Thermodynamic profile of marketed drugs**

Retrospective analysis of thermodynamic signatures of marketed drugs was published for series of statins binding to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, small-molecule inhibitors of HIV protease [10, 23] and bisphosphonate inhibitors for farnesyl pyrophosphate synthase (FPPS) [24]. Investigating the compounds in terms of their market entry time revealed a clear trend regarding the enthalpy contribution of the binding free energy (Figure 2).

Binding to their primary target first in class drugs were typically entropy driven, with positive or negligible enthalpy contribution in all the three cases. During the time course of drug evolution, as novel drugs with the same mechanism of action are introduced to the market, they must show advantages over the predecessors. Those drugs that were better than the previous compounds in the class can be characterized with sequentially increasing binding enthalpy contribution. In the case of statins, for the first in class Fluvastatin the binding enthalpy is zero, and the binding entropy is -37.6 kJ/mol, while for Rosuvastatin, -the last entry in this analysis- the corresponding values are -38,9 kJ/mol and -12.5 kJ/mol, for enthalpy and entropy, respectively. Considering the protease inhibitors, Indinavir binding is realized by -59,4 kJ/mol favorable entropy term and the enthalpy contribution is 7.6 kJ/mol. In contrast, Darunavir binding is mainly enthalpy driven (-53,1 kJ/mol), and the entropy contribution is -9.6 kJ/mol only. The tendency is also obvious for bisphosphonate FPPS inhibitors. Etidronate binding is entirely entropy driven, with -36,4 kJ/mol value, that is associated with unfavorable, 7.1 kJ/mol enthalpy. In contrast, Minodronate binding is enthalpy driven (-37.7 kJ/mol) and the entropy contribution is -3.3 kJ/mol only. The underlying reasons for the success of the more enthalpic compounds may include better physicochemical parameters, such as lipophilicity and solubility arising from the more polar moieties that are necessary for favorable enthalpic binding. However we should emphasize that these drugs were optimized without controlling their binding thermodynamics and obviously there are a vast amount of other parameters influencing the success of the given drug. Although we are aware to the limitations of thermodynamics signatures, increasing enthalpy contribution to binding free energy during drug evolution in a given class is significant.

The most common adverse effect associated with statins is myalgia that is thought to be linked to myocyte HMG-CoA reductase inhibition. One possibility to avoid this side effect is the hepatocyte selective distribution of statins that can be achieved by decreasing passive cell permeability by increasing the hydrophilicity. In this respect the most enthalpic Rosuvastatin has the highest hydrophilicity and hepatoselectivity. Myocyte $IC_{50}$ is 926 times higher compared to the hepatocyte value [25]. Hepatoselectivity of less enthalpic binders such as Pravastatin, Atorvastatin and Cerivastatin is somewhat lower: these drugs show 444, 144 and 4.1 fold selectivity, respectively. Therefore monitoring binding thermodynamics become integrated part of statin optimizations [26]. Optimizing the Atorvastatin scaffold a promising lead compound was derived possessing -74 kJ/mol enthalpy contribution, and higher than 1000 fold hepatic selectivity.

Selectivity against off-targets can be another reason for the success of the best in class compounds. In the case of HIV protease inhibitors it was shown that more enthalpic compounds possess higher adaptability to drug-resistant mutants along with more enhanced selectivity to the off target cathepsin D [27, 28]. Interestingly, no correlation was observed between adaptability and binding affinity, however a reproducible correlation was found between the logarithm of the corresponding $K_d$ ratio and the proportion of binding enthalpy contribution to the binding affinity in the wild-type protease [27]. The benefit of enthalpic optimization was also shown during the evaluation of Indinavir, Nelfinavir, Saquinavir, Ritonavir, KNI-764 and KNI-272 in terms of the inhibition of resistant mutant V82F/I84V. In this case compounds forming optimized H-bonds and thus realized more enthalpic binding to the wild type HIV protease show higher residual inhibition against the resistant mutant that of the entropy driven binders. Here specific and enthalpically favorable contacts provided more desirable adaptability [29].

**Selectivity profile analysis of marketed drugs**

Our evaluation on the link between selectivity and binding thermodynamics profiles indicated that enthalpy driven compounds show typically higher selectivity than entropy driven analogues with similar binding mode to primary targets. In order to expand the domain of the selectivity-thermodynamics relationship, our intention was to challenge this hypothesis on broad selectivity profiles. Therefore we have collected examples possessing binding

thermodynamics data on the primary target and were subjected to broad off-target assay profiling. The thermodynamics data were collected from literature including BindingDB, PDBCal and Scorpio databases [30]. In vitro profiling data were collected from the coherent DrugMatrix database available through ChEMBL [https://ebi.ac.uk/chembldb] and the CerepBioprint (Cerep) profile [31]. We have selected these databases to ensure that the compounds were tested on the same number of assays within the same laboratory conditions in order to minimize the noise originated from the inter-laboratory differences. On the other hand, the thermodynamics data pooled from various sources might include inter-laboratory errors of thermodynamics measurements.

Nineteen compounds acting on six targets fulfilled our criteria (Table 4). Five compounds are HIV-1 protease inhibitors, five are dopamine D2 receptor ligands, four are HMG-CoA reductase inhibitors, two act on histamine H1 receptors, two compounds are beta-blockers and finally we included a DNS-gyrase inhibitor. HIV-1 protease and HMG-CoA reductase inhibitors were evaluated in terms of their thermodynamics profile [10, 23]. It was proposed that favourable interplay between enthalpy and entropy is reflected in their progress to the market. It was suggested that enthalpically more favourable drugs tend to be the best in class compounds, while the entropy-driven binders represent pioneer, first-in-class drugs. In accordance, evaluating the enthalpic efficiency as the measure of ligand-protein complementarity has also been discussed in the literature [10, 32].

First, promiscuity and thermodynamics relationships were evaluated within target groups. Table 4 shows (more details are given as Supplementary Table 1) that binding of three HIV-1 protease inhibitors Nelfinavir, Indinavir and Saquinavir are entropy driven. Ritonavir binding is also entropy driven, but the enthalpy contribution is more favorable than that for the first group. Amprenavir binding is characterized by balanced entropy-enthalpy contributions. The change from entropy driven binding to more balanced thermodynamic profile is also reflected in the selectivity profile. Amprenavir hits only 2 targets out of ~134 involved in the DrugMatrix panel and 3 out of 185 on Cerep. In contrary, Saquinavir hits 11 targets on the DrugMatrix assay panel and 18 on the Cerep panel.

Enthalpy contribution of the five drugs acting on dopamine D2 receptor possess significant, -0.92 ($p=0.026$) linear correlation coefficient (r) with the number of hit targets on the Cerep profile. The entropy-driven binding of Flupenthixol is translated into high promiscuity, hitting 52 targets (Table 4). In contrast, the enthalpy-driven binding of Sulpiride highlights the enhanced complementarity to the target binding site, and results in significantly reduced promiscuity. Ligands of the dopamine D2 target show univocal tendencies on the DrugMatrix and the Cerep profile.

In case of HMG-CoA reductase inhibitors Fluvastatin binding is entropy driven, while Cerivastatin, Pravastatin and Atorvastatin binding have increased enthalpy contribution. Accordingly, Fluvastatin, Cerivastatin and Pravastatin hits 5, 4 and 1 target on the Cerep assay panel, respectively. The increasing selectivity is in line with the entropy-promiscuity relationships, since the decreasing binding entropy results in lower promiscuity.

Binding of the histamine H1 ligands is entropy driven. Accordingly, Clozapine and Diphenhydramine are highly promiscuous compounds hitting 26 and 11 targets on DrugMatrix, 44 and 29 targets on Cerep profile, respectively.

Thermodynamics signatures of beta blockers revealed that Pindolol binding is balanced in terms of enthalpy and entropy contributions, while Isoproterenol binding is entirely enthalpy-driven. Thermodynamic profiles are in line with their medium and low promiscuity, respectively.

Our last example is Novobiocin, a selective compound characterized by enthalpy-driven binding and correspondingly no off-target activity on the Cerep panel. This compound is specific, with no promiscuity issue reported.

Next we investigated the whole dataset that represents broad chemical diversity and spans six targets. In order to assess the relationship between thermodynamics profiles and observed hit rates linear and rank correlation coefficients were calculated. Sum of ranking differences (SRD) were also calculated (results are presented as Supplementary Tables 2-3 [36]). Although differences in binding site characteristics and measurement conditions might impact the results of this analysis we found that compounds hitting higher number of targets have more remarkable entropy and typically less favorable enthalpy contributions. It is worth to mention that higher affinity achieved by entropy-driven optimization do not necessary results in high selectivity (significant negative correlation coefficients), in contrast to those with lower affinity but higher enthalpy contributions. Lopinavir, Atazanavir and Amprenavir clearly exemplify this statement. Results acquired for this limited dataset revealed that binding enthalpy and entropy tends to correlate with the number of off-targets (Table 4 and Supplementary Figure 1). In the case of SRD calculations the entropy-based ranking was found to be significantly different form a random distribution, with p value of 0.017 and 0.016 for DrugMatrix and Cerep datasets. More favorable binding enthalpy and less favorable binding entropy might result in higher specificity and lower promiscuity. Enthalpy and entropy correlation was found to be -0.99 ($p<0.001$), in accordance with the phenomenon of entropy-enthalpy compensation. Although correlations were found to be statistically significant, it is worth to be mentioned that they are generally weak and were obtained on a limited dataset preventing its over-interpretation. Leaving one compound out generally does not change the correlation coefficients (±0.02) except for Clozapine. Linear correlation between enthalpy and promiscuity without Clozapine drops to 0.32 and 0.39 for DrugMatrix and Cerep data, respectively.

In order to compare continuous variables we calculated the sum of binding energies on off-targets using the activity values presented in DrugMatrix database (Table 4). In contrast to the simple sum of targets hit the summed affinity avoids biases due to high hit rates with limited affinities compared to low hit rates with high affinity. Since the DrugMatrix database contains equivalent number of assay results for each ligand, binding energies can be summed. This analysis resulted in similar tendencies to that of the hit target type assessment.

The quality of optimized compounds is usually quantified by changes in the physicochemical profile. In terms of these parameters enthalpy driven optimization is generally preferred over entropy driven optimization [10, 15, 30, 32, 37, 38]. Ligand promiscuity, as well as thermodynamic signature of binding [39] are also interrelated to physicochemical properties. It was shown that three physicochemical features, as basic character, molecular weight and lipophilicity (logP/logD) have highest influence on promiscuity [40]. It is interesting to note that lipophilicity (AlogP) generally showed higher correlations with the off-target occurrences compared to molecular size descriptors, such as heavy atom count (Nh) and molecular weight (Mw) (Table 4). However, all of these correlations were statistically non-significant on this dataset (Table 4). The general correlation between size and potency is well established [41, 42], in our case the correlation between binding energy and Mw was found to be also remarkable (r= -0.45, p=0.052). Linear correlation (r) between entropy and lipophilicity (AlogP) was found to be more pronounced r=-0.63 (p=0.004).

The correlation between binding enthalpy and lipophilic ligand efficiency (LLE=pAct-logP) has been recently investigated by Shultz [43]. Therefore we have collected all the cases collected in Tables 1-4, to evaluate the enthalpy-LLE correlation. The linear correlation coefficient (r) was found to be -0.501 (p=0.001), resulting in significant correlation for the investigated 37 cases. However correlation using the off-target assay profiling data collected in Table 4 did not resulted in significant correlation with DrugMatrix (r=-0.003) and Cerep (-0.257) sets.

Regarding the application domain of the concept of increasing selectivity by thermodynamics optimization it is important to be emphasized that the key point is the rational optimization of the interaction pattern of the protein-ligand complex with specific contacts. The experimentally determined entropy and enthalpy values measure the sum of the changes related to the complex formation, including solvation terms, protein and ligand conformational changes, and protein-ligand enthalpic contacts. The flexibility of the protein target and the nature of the binding site have also crucial impact, as exemplified by the entropy-enthalpy transduction theory [44]. Decoupling the protein-ligand entropy and enthalpy contribution is therefore a difficult task that has not been solved entirely to date. Accordingly, it is highly recommended to synergistically deploy both experimental and computational approaches to understand the biophysical background of the free energy changes.

**Conclusions**

The pioneer hypothesis of Kawasaki and Freire [15] has been evaluated here regarding the interplay between selectivity and thermodynamics profiles. According to their study, ligand selectivity can be achieved by favorable binding enthalpy, since it is a straightforward measure of protein-ligand complementarity [10, 15]. Highly oriented interactions accomplished by enthalpic interactions result in higher bias towards the primary target and making compounds less promiscuous. We showed that structurally diverse ligands of several validated drug targets (HIV-protease, HMG-CoA reductase, D2, beta adrenergic and histamine H1 receptors) possessing broad selectivity profile data support this concept. The limited number of cases collected and discussed here strengthens the link between selectivity and thermodynamics and facilitates the generation of more thermodynamics data on compounds with wide range of selectivity assay data. The objective of the present review is to stimulate further debate supporting or challenging this hypothesis by publishing further experimental data. From thermodynamics point of view drugs are acting in an open-system, therefore emphasizing that the recent observations were made under equilibrium conditions is crucial. Under physiological conditions the binding kinetics might also influence the selectivity profile realized in vivo [45-47].

From practical point of view, monitoring binding thermodynamics at project milestones can facilitate the selection of the higher quality compounds. Desirable hit and lead compounds having the highest enthalpy among the chemical series might be in line with the greater complementarity with the primary binding site and therefore impacts selectivity profile. Due to the complex nature of the binding event and the difficulties of understanding the thermodynamics background of the SAR contributes to the obstacles of successful optimization towards higher enthalpy. Structure-based approaches, both experimental and computational have crucial role to rationalize these modifications. Based on the current state of our understanding this hypothesis can be exploited during medicinal chemistry programs optimizing affinity and selectivity in parallel.

Figure 1. Correlation between binding free energy difference and binding enthalpy for HIV-1 protease inhibitors.

Figure 2. Thermodynamic profile of marketed drugs.

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Table 1. Binding thermodynamics data of MMP12 ligands.

| Compound | $\Delta G_{MMP12}$ (kJ/mol) | $\Delta H_{MMP12}$ (kJ/mol) | $-T\Delta S_{MMP12}$ (kJ/mol) | $\Delta\Delta G_{MMP13-MMP12}$ (kJ/mol) |
|---|---|---|---|---|
| 1 (1)[a] | -41.1 | -38.0 | -3.1 | 1.0 |
| 2 (3)[a] | -43.9 | -35.6 | -8.3 | 2.3 |
| 3 (4)[a] | -49.2 | -37.0 | -12.2 | 1.0 |
| 4 (6)[a] | -46.2 | -40.4 | -5.9 | 5.0 |

[a]Compound identifier in the original article [16].

Table 2. Binding thermodynamics and selectivity data of ALR2 ligands.

| Compound | $\Delta G_{ALR2}$ (kJ/mol) | $\Delta H_{ALR2}$ (kJ/mol) | $-T\Delta S_{ALR2}$ (kJ/mol) | $\Delta\Delta G_{ALR1-ALR2}$ (kJ/mol) |
|---|---|---|---|---|
| Selectivity pocket occupant | | | | |
| Zopolrestat | -46.0 | -58.1 | 12.1 | 17.7 |
| Compound 2[a] | -42.5 | -48.5 | 6.0 | >23.8 (rat) |
| IDD 388 | -42.7 | -59.0 | 16.3 | 14.9 |
| IDD 393 | -42.2 | -81.2 | 39.0 | 21.0 (fluoro derivative) |
| Non-occupant | | | | |
| Sorbinil | -37.9 | -54.7 | 16.8 | 2.4 |
| Fidarestat | -46.7 | -79.5 | 32.8 | 11.9 |

[a]Compound identifier in the original article [17].

Table 3. Binding thermodynamics data of CB ligands.

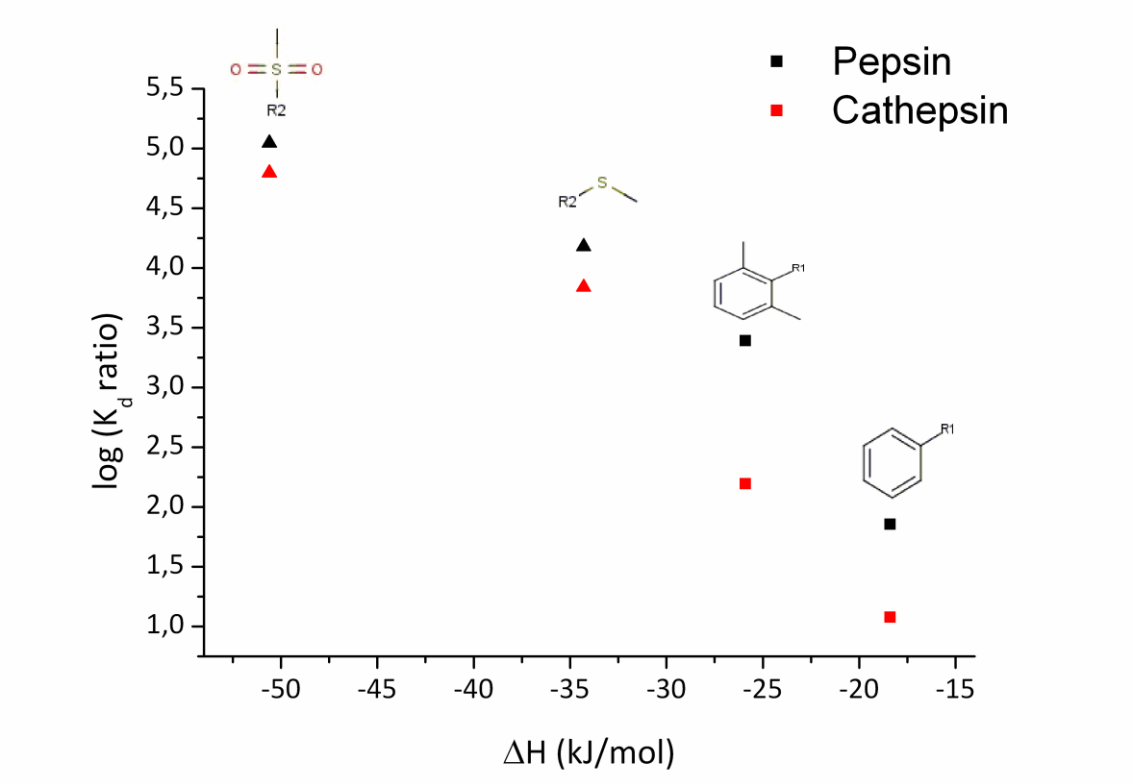
| Compound | $\Delta G_{(primary)}$ (kJ/mol) | $\Delta H_{(primary)}$ (kJ/mol) | $-T\Delta S_{(primary)}$ (kJ/mol) | $\Delta\Delta G_{(primary-secondary)}$ (kJ/mol) |
|---|---|---|---|---|
| WIN 55212 | -46.8 (CB2) | 27 | -73.8 | 2.6 |
| JWH-015 | -41.4 (CB2) | 48 | -89.4 | 2.6 |
| ACEA | -47.8 (CB1) | 59 | -106.8 | 8.2 |
| 2-Fl-AEA | -46.1 (CB1) | 17 | -29.1 | 6.2 |
| CP-55,940 | -51.2 (CB1) | 56 | -107.2 | 3.3 |
| AM630 | -41.2 (CB2) | -19 | -22.2 | 8.0 |
| AM281 | -45.9 (CB1) | -35 | -10.9 | 13.7 |
| AM251 | -48.8 (CB1) | -52 | 3.2 | 13.3 |

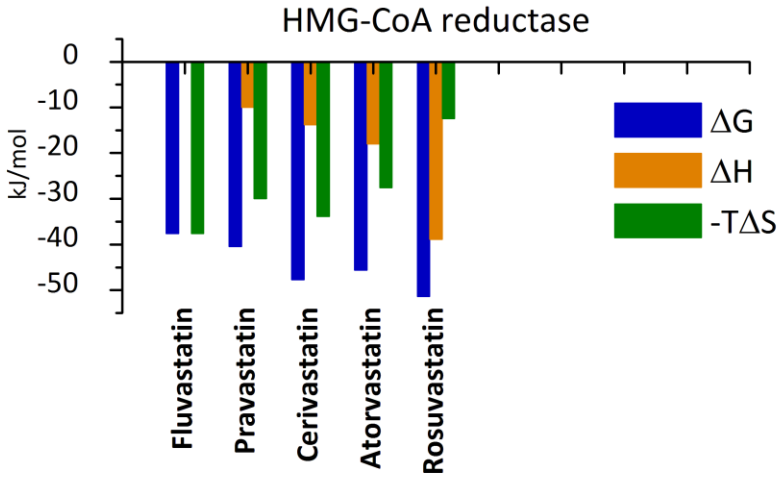
Table 4. Compounds with thermodynamic and broad specificity assay profiles. Calculated octanol-water partition coefficient (AlogP), heavy atom count (Nh) and molecular weight (Mw) is indicated.

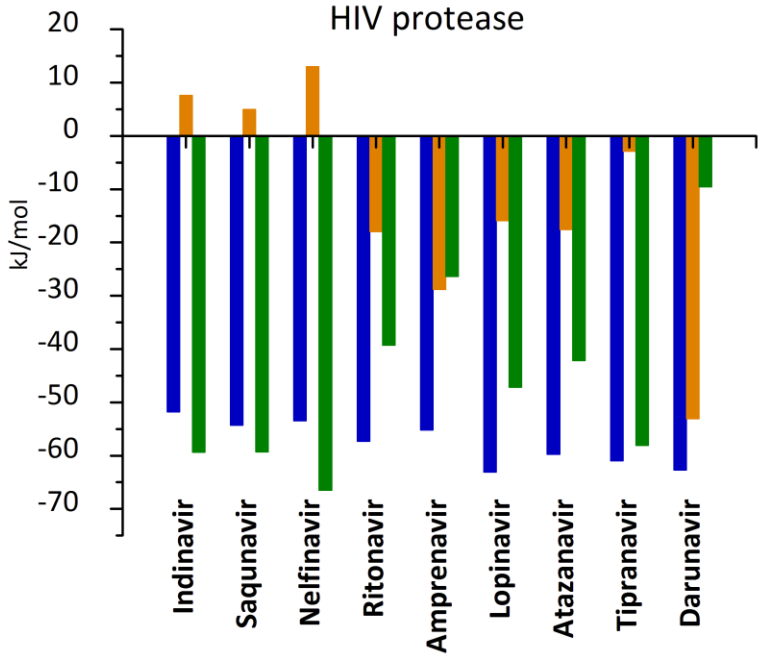
| # | **Drug** | **Target** | **Drug Matrix** | **Drug Matrix $\Sigma\Delta G_{off}$** | **Cerep** | **$\Delta G$[a]** | **$\Delta H$[a]** | **$-T\Delta S$[a]** | **AlogP** | **Nh** | **Mw** | **Ref.** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Nelfinavir | HIV-1 protease | 7 | -204 | | -53.5 | 13.0 | -66.5 | 5.3 | 40 | 567.8 | [10] |
| 2 | Indinavir | HIV-1 protease | 3 | -102 | 7 | -51.8 | 7.6 | -59.4 | 3.1 | 45 | 613.8 | [10] |
| 3 | Saquinavir | HIV-1 protease | 11 | -338 | 18 | -54.3 | 5.0 | -59.3 | 3.7 | 49 | 669.9 | [10] |
| 4 | Ritonavir | HIV-1 protease | 8 | -277 | 15 | -57.3 | -18.0 | -39.3 | 5.0 | 50 | 720.9 | [10] |
| 5 | Amprenavir | HIV-1 protease | 2 | -64 | 3 | -55.2 | -28.8 | -26.4 | 2.4 | 35 | 506.6 | [10] |
| 6 | Flupenthixol | Dopamine D2 | | | 52 | -47.7 | 15.2 | -62.9 | 4.82 | 30 | 434.52 | [33] |
| 7 | Haloperidol | Dopamine D2 | 18 | -682 | 27 | -53.2 | -12.8 | -40.4 | 3.76 | 26 | 375.9 | [33] |
| 8 | Alizapride | Dopamine D2 | | | 13 | -42.3 | -50.8 | 8.6 | 1.87 | 23 | 315.4 | [33] |
| 9 | Metoclopramide | Dopamine D2 | 6 | -214 | 19 | -41.4 | -54.8 | 13.4 | 1.78 | 20 | 299.8 | [33] |
| 10 | Sulpiride | Dopamine D2 | 2 | -76 | 10 | -41.9 | -88.6 | 46.7 | 0.7 | 23 | 341.4 | [33] |
| 11 | Fluvastatin | HMG-CoA reductase | 1 | -36 | 5 | -37.6 | 0.0 | -37.6 | 4.2 | 30 | 411.5 | [10] |
| 12 | Cerivastatin | HMG-CoA reductase | 0 | 0 | 4 | -47.7 | -13.8 | -33.9 | 4.2 | 33 | 459.6 | [10] |

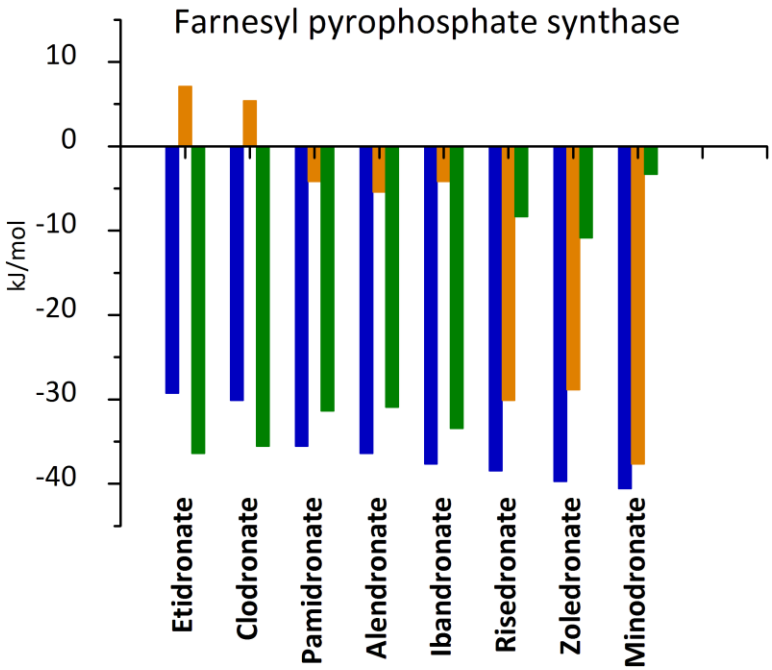
| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 13 | Pravastatin | HMG-CoA reductase | 0 | 0 | 1 | -40.5 | -10.5 | -30.0 | 2.2 | 30 | 424.5 | [10] |
| 14 | Atorvastatin | HMG-CoA reductase | 0 | 0 | | -45.6 | -18.0 | -27.6 | 5.6 | 41 | 557.6 | [10] |
| 15 | Clozapine | Histamine H1 | 26 | -1100 | 44 | -47.9 | 72.0 | -119.9 | 3.42 | 23 | 326.8 | [34] |
| 16 | Diphenhydramine | Histamine H1 | 11 | -398 | 29 | -43.6 | 22.6 | -66.2 | 3.38 | 19 | 255.4 | [34] |
| 17 | Pindolol | Beta-blocker | 1 | -43 | 9 | -49.6 | -21.3 | -28.3 | 1.93 | 18 | 248.3 | [35] |
| 18 | Isoproterenol | Beta-blocker | 1 | -33 | 5 | -50.2 | -143.2 | 92.9 | 1.1 | 15 | 211.6 | [35] |
| 19 | Novobiocin | DNA gyrase | | | 0 | -42.7 | -51.8 | 9.2 | 3.45 | 44 | 612.6 | [30] |
| Linear correlation | | DrugMatrix (N=16) | 1.00 | -0.99 (<0.001)[b] | 0.95 | -0.26 | 0.55 (0.026) | -0.58 (0.020) | 0.19 | -0.07 | -0.06 | |
| | | DrugMatrix ΣΔG (N=16) | -0.99 (<0.001) | 1.00 | -0.95 (<0.001) | 0.21 | -0.55 (0.027) | 0.57 (0.022) | -0.16 | 0.13 | 0.12 | |
| | | Cerep (N=17) | 0.95 (<0.001) | -0.95 (<0.001) | 1.00 | -0.12 | 0.54 (0.025) | -0.54 (0.023) | 0.38 | -0.20 | -0.19 | |
| Spearman rank correlation | | DrugMatrix (N=16) | 1.00 | -0.99 (<0.001) | 0.92 (<0.001) | -0.42 | 0.47 | -0.63 (0.009) | 0.10 | 0.04 | 0.05 | |
| | | DrugMatrix ΣΔG (N=16) | -0.99 (<0.001) | 1.00 | -0.92 (<0.001) | 0.37 | -0.45 | 0.61 (0.013) | -0.06 | 0.00 | 0.00 | |
| | | Cerep (N=17) | 0.92 (<0.001) | -0.92 (<0.001) | 1.00 | -0.15 | 0.46 | -0.57 (0.019) | 0.20 | -0.26 | -0.24 | |

[a]Thermodynamic data are presented in kJ/mol units. Statistically significant correlations are marked with red color. [b] P-values are indicated in parentheses.


Pepsin
Cathepsin
log (K_d ratio)
5,5
5,0
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
-50
-45
-40
-35
-30
-25
-20
-15
ΔH (kJ/mol)
R2
S
R1


HMG-CoA reductase
kJ/mol
0
-10
-20
-30
-40
-50
ΔG
ΔH
-TΔS
Fluvastatin
Pravastatin
Cerivastatin
Atorvastatin
Rosuvastatin


HIV protease
kJ/mol
20
10
0
-10
-20
-30
-40
-50
-60
-70
Indinavir
Saqunavir
Nelfinavir
Ritonavir
Amprenavir
Lopinavir
Atazanavir
Tipranavir
Darunavir


Farnesyl pyrophosphate synthase
kJ/mol
10
0
-10
-20
-30
-40
Etidronate
Clodronate
Pamidronate
Alendronate
Ibandronate
Risedronate
Zoledronate
Minodronate