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Application Review

Fragment Based Drug Discovery:

Studying low affinity ligands by ITC

Dissecting a ligand into smaller fragments can provide a strategy for analyzing the role of key functional groups in a protein-ligand interaction. Conversely, low affinity ligand fragments that are able to occupy a receptor binding site simultaneously, can often be linked together to form high affinity inhibitors. More recently, fragments have been used as starting points for 'fragment growth' lead optimization programs. One of the key challenges to these design strategies is in accurately measuring the K_d of low affinity interactions. This application review demonstrates how ITC can be used to address this issue.

Introduction

When two or more low affinity fragments can be accommodated simultaneously in a receptor binding site, it is possible to connect them to form a ligand with an affinity considerably higher than the sum of the component parts (Figure 1). Once one half of a bivalent ligand has bound to its receptor, there is a very high local concentration of the second recognition element in the vicinity of the binding site, thus increasing the probability that the second interaction will take place.

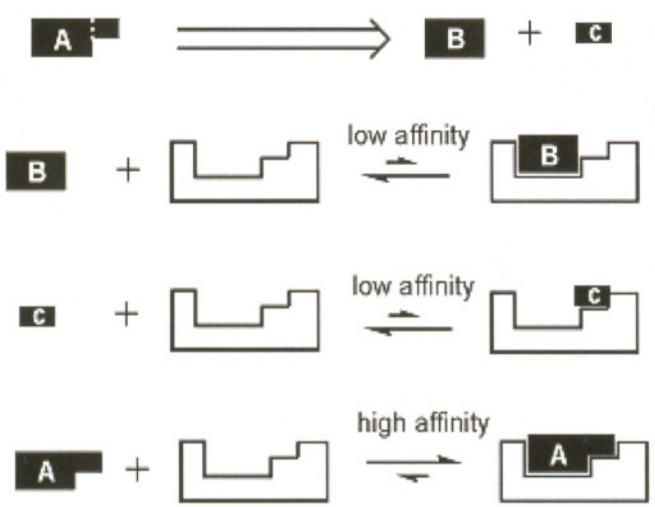

Figure 1: Low affinity "fragments" can be linked together to form high affinity ligands.

The "fragment-based" strategy for ligand design is now well established in the pharmaceutical industry, in particular in situations where high throughput screening of existing compound libraries has failed to identify a suitable lead compound for a given target protein. Typically, NMR and X-ray crystallographic based screening methods are employed to identify pairs of small molecule "fragments" (<250 Da), and to provide structural data on their orientations in the receptor binding site. However, additional information can be provided by Isothermal Titration Calorimetry (ITC.)

ITC is a universally applicable, complementary method that provides highly quantitative affinity data, as well as mechanistic information about the specific, non-covalent forces that are involved in the binding. This technique directly measures the heat of interaction without the need for immobilization, chemical modification or assay design. Measurement of this heat enables accurate determination of binding constants, reaction stoichiometry, enthalpy and entropy. Selecting fragments that display the most favourable enthalpy changes can prove a useful strategy for maximising binding selectivity for the final ligands.

The ability to study low affinity interactions is central to the fragment based approach to drug discovery. This review describes a case study that uses ITC in a straightforward manner for such an application.

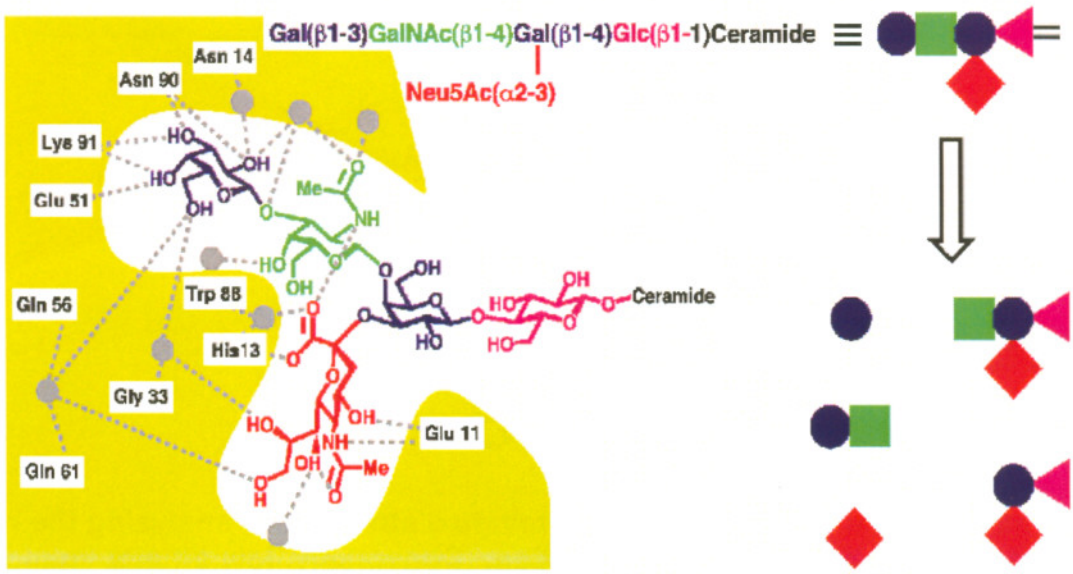

Figure 2. Cartoon representation of the CTB-GM1 interaction. The pentasaccharide was dissected into the fragments (bottom right) for subsequent binding studies.

Case Study: Dissection of the cholera toxin-GM1 oligosaccharide interaction

CTB and its complexes with GM1 oligosaccharide (GM1os), have been studied extensively by X-ray crystallography. The crystal structure reveals that the branched GM1os binds to the protein in a "two fingered grip" (Figure 2), with extensive hydrogen bonding to the three sugar residues at its non-reducing terminus. The remaining two sugar residues point away from the protein surface, and form the point of attachment to the ceramide lipid, which anchors GM1 in the cell membrane, *in vivo*.

ITC Binding Experiments for High and Low Affinity Ligands

The GM1os pentasaccharide was dissected into fragments from mono- to tetrasaccharides to assess the contributions made by each of the three terminal sugar residues to the overall binding energy. ITC was used to measure the K_d of the parent ligand and the smaller fragments.

The GM1os pentasaccharide was found to bind very tightly (K_d = 43 nM), and gave a well defined sigmoidal curve with an excellent level of signal-to-noise (Figure 3a). All other fragments bound very weakly and were analyzed using a 'low c value' (Figure 3b) and/or the 'displacement' method (Figure 3c). These approaches allow the use of ITC for measuring weak affinities while minimizing the amount of protein required. The former is a direct method requiring an assumption about the stoichiometry of the interaction whereas the latter involves competition with a higher affinity ligand.

Good agreement was found for those systems that were measured using both weak affinity methods. However the displacement method uses lower protein concentrations than normally associated with weak affinity assays.

The SAR are conveniently assessed by comparing the free energies of binding (ΔG). These can be simply calculated from the K_d ($\Delta G = RT\ln K_d$). The data for the pentasaccharide and all the fragments is tabulated below.

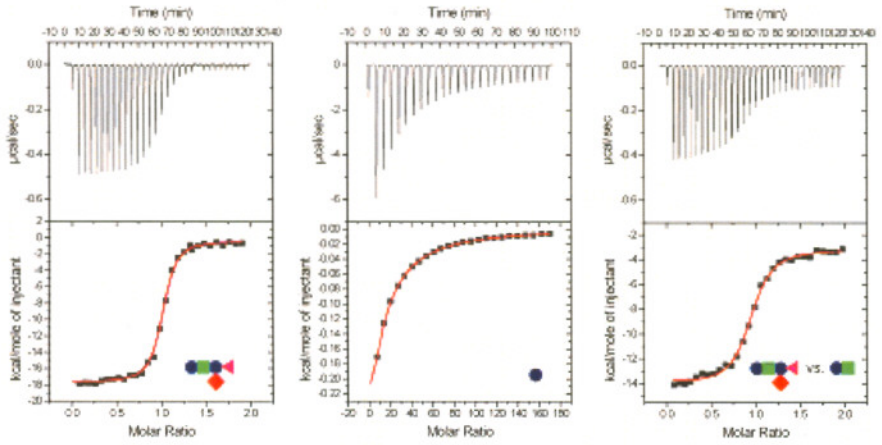

Figure 3. Direct and displacement titrations of CTB with various oligosaccharides. Left: 10 µM CTB / 110 µM GM1os (direct); Center: 145 µM CTB / 100 mM GalbOMe (direct); Right: 10 µM CTB / 25 mM GalGalNAc / 110 µM GM1os (displacement).

The potential gains in affinity from using the fragment based approach are demonstrated here. If we add the ΔG of GalβOMe to that of GM2os we obtain -6.17 kcal mol^{-1}, which is considerably less than the -10.04 kcal mol^{-1} we find for the full ligand even though all the component saccharides are considered. The same is true if we consider adding the contributions from GalGalNAc and Neu5AcαOme (-3.81 kcal mol^{-1}) even though these saccharides are responsible for all the direct contacts made by the full ligand to the CTB in the crystal structure (see Figure 2). Using the same logic, but in reverse, a small, weakly binding fragment can be made to bind with higher affinity by adding relatively few additional functional groups.

Ligand	K_d	ΔG kcal mol^{-1}
GM1os	43.3 ± 1.4 nM	-10.04 ± 0.02
GalβOMe	14.8 ± 1.6 mM	-2.50 ± 0.07
GM2os	2.0 ± 0.2 mM	-3.67 ± 0.09
GalGalNAc	7.6 ± 0.8 mM	-2.89 ± 0.08
Neu5AcαOMe	210 ± 100 mM	-0.92 ± 0.28

Table 1. Summary of K_ds and free energies (ΔG) for GM1os and fragment ligands

Summary/Conclusions

Fragment based drug discovery is gaining impetus in the pharmaceutical industry, particularly in those cases where HTS campaigns have been unsuccessful and in the biotech sector which cannot afford libraries of 2-5 million compounds typically available to large pharmaceutical companies. Even with such large libraries companies are only searching within a tiny fraction of 'chemical space' for their drug candidates. High resolution structural data of the protein targets is making this search for hits far more efficient and one such outcome of this development is the fragment based approach.

Small, focused libraries containing compounds with the appropriate reactivity to specific centers within the protein target can be tested for their ability to modulate activity. These compounds are small (~200 Daltons) giving the medicinal chemists the opportunity to improve the binding efficiency (affinity/mass) by adding new moieties to the fragment.

This approach depends on the ability to measure weak affinity interactions in a quantitative way and the ability to increase the affinity/mass ratio significantly in the lead optimization process. Both of these questions have been addressed. It is clear that ITC can be used to measure weak affinity interactions with high accuracy and, with improved methodology, considerably lower concentrations than previously thought. In addition the question of improved binding efficiency has been demonstrated powerfully in this example. The tight binding 'parent' ligand has a 63% improved binding efficiency over the sum of that obtained for the GalβOMe and GM2os and a 264% improvement of that obtained for GalGalNAc and Neu5AcαOme.

It is clear that ITC is an important and universally applicable tool in fragment based drug discovery and is perfect complement to the structural data available for many of the drug targets today.

These methods have been described fully in the application note 'Divided We Fall? Studying low affinity fragments of ligands by ITC' written by Dr. W. Bruce Turnbull. Please contact MicroCal to obtain a copy.

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