Harnessing fragment-based drug discovery at CRT

In the past decade, fragment-based drug discovery (FBDD) has gained increasing popularity in the pharmaceutical industry as a powerful alternative and complement to traditional high-throughput screening (HTS) as a source of preliminary hits for drug discovery. In theory, FBDD represents a highly efficient method for rapidly identifying starting points for structure-based drug design. Furthermore, FBDD is generally unbiased by preconceptions gleaned from computer modelling or existing structural data. This technique is aimed at evolving (growing) or merging new tightly binding molecules in a step by step approach and differs from HTS where larger compounds are screened in a single step procedure. Sensitive biophysical methods are essential to identify the typically weak interactions between fragments and the target protein. When combined with x-ray crystallography, FBDD allows the complementarity between a protein active site and fragments to be rapidly sampled and has led to the discovery of drug-like leads against previously intractable targets. In this review, we discuss FBDD at Cancer Research Technology Ltd (CRT) and its impact on the screening rationale within the company.

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Throughout the past two to three decades, HTS has played a pivotal role in drug discovery in the pharmaceutical sector. However, in recent years FBDD has developed into a significant alternative for identifying preliminary hits. FBDD facilitates the efficient development of novel leads because it is a design-driven process. FBDD is an approach in which much lower molecular weight (MW) compounds are screened compared to HTS campaigns. Fragment hits are typically weak inhibitors with affinities in the µM to mM range and need to be screened at high concentrations using sensitive techniques such as nuclear magnetic resonance (NMR), x-ray crystallography, surface plasmon resonance (SPR), thermal shift assay (TSA) and high concentration screening (HCS) in order to be reliably detected.

FBDD and CRT: screening novel targets

CRT is a specialist commercialisation and development company which aims to develop new discoveries in cancer research for the benefit of patients and is wholly-owned by Cancer Research UK. CRT's Discovery Laboratories bridge the gap between academia and industry, building on exploratory research to create attractive commercial opportunities through collaboration with research institutes worldwide. The Discovery Laboratories' focus is on novel targets emerging from Cancer Research UK-funded and other academic basic research, and we see FBDD as an exciting approach which is complementary to our HTS capabilities for the generation of novel chemical matter to interact with previously 'undrugged' targets.

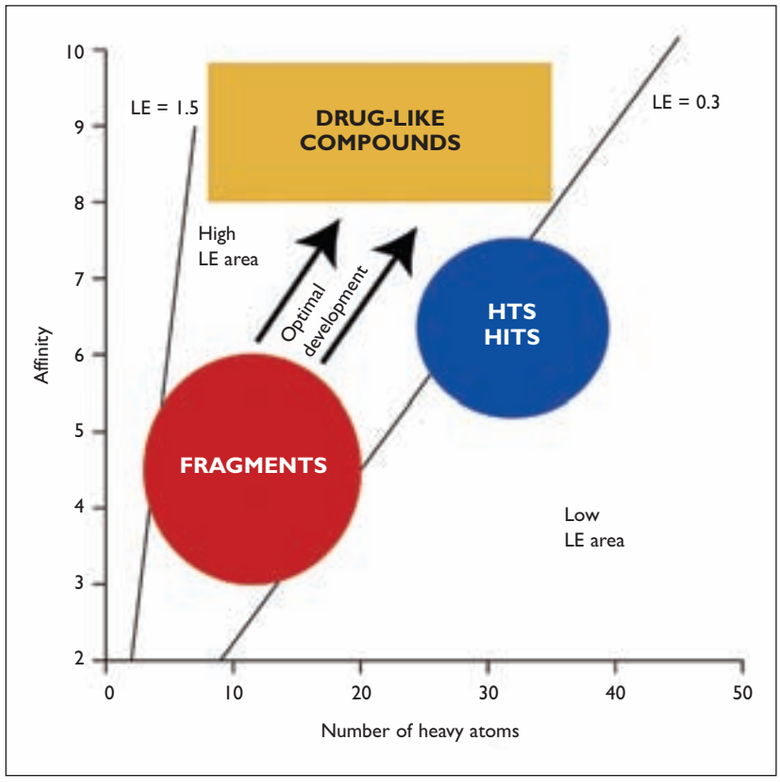

Figure 1
Graph of ligand efficiency

What is a fragment?

A fragment is a low MW compound, typically less than 300 Daltons (Da) in size. In contrast, a drug like compound usually has a MW of up to around 500 Da. Because of their small size, fragments have a number of advantages compared to HTS compounds.

First, the hit rate for fragment screening is usually much higher than HTS as there is an inverse relationship between molecular complexity and the probability of a compound matching a target protein-binding site. There are approximately 10^7 synthetically feasible small molecule fragments with up to 12 heavy atoms compared to in excess of 10^{60} small drug like molecules with up to 30 heavy atoms. Therefore, a fragment library covers a wider range of chemical diversity space for low MW compounds than a large library of drug-sized molecules does for higher MW compounds. Large pharmaceutical companies routinely screen targets against HTS libraries exceeding one million compounds in size. In contrast, fragment libraries can be much smaller (typically between 100 and a few thousand fragments) in order to identify hits for the same target. There are many advantages associated with the construction, storage and screening of fragment libraries compared to HTS libraries.

Second, in HTS, it is often difficult to find compounds of the required shape and diversity. Fragments, on the other hand, can find small niches within the active site of a protein and subsequently its core or functional groups can provide starting points for evolving hits into lead compounds through growing (evolving) and linking.

Fragments are weak hits with low binding affinities (typically in the high µM to low mM range) compared to drug-like molecules (nm to low µM). A key metric for ranking a set of active fragments is ligand efficiency (LE) not the absolute affinity. LE is defined as the affinity of a fragment divided by the number of heavy atoms (ie non-hydrogen atoms) in its structure (**Figure 1**). The maximum theoretically possible increase in LE is approximately 1.5 kcal/heavy atom whereas, in practice, LEs of around 0.3 kcal/heavy atom are usually achievable. LE increases linearly with increasing size up to about 15 atoms beyond which there is very little increase. Fragments are likely to have higher LEs than classical HTS hits and should therefore be capable of being developed into more efficient drugs with better pharmaceutical properties including lower MW, better pharmacokinetics, lower toxicity, etc. In order to evolve fragments, the x-ray structure is essential to generate structure-activity-relationships (SAR) to drive medicinal chemistry and to track the LE.

Fragments usually follow Astex Therapeutics' 'rule of three' concept: compounds are limited to a MW of 300 Da, three or less hydrogen bond donors and acceptors and a calculated logP (hydrophobicity) of ≤3. Furthermore, fragments must be chemically tractable with unwanted functionalities removed. Fragments with 'synthetic handles' are often selected to maximise synthetic accessibility. For example, SGX Pharmaceuticals Inc championed the use of fragments containing a bromine atom, a feature which enhances synthetic elaboration and aids in the detection and validation of crystallographic screening data.

A fragment library can either be generic or target-focused. Focused sets may be derived for a particular class of targets, for a specific target or from the structures of molecules with desirable biological activity. For example, Boehm and colleagues at Hoffmann-La Roche Ltd used molecular docking studies to identify a focused fragment set with favourable binding characteristics to DNA gyrase. Finally, the throughput of the technique being used to screen the library will influence the size of library that can be screened.

CRT fragment library design

A number of suppliers' collections were evaluated to identify a CRT fragment library for screening. Filtering was primarily carried out based on

calculated physicochemical property fit to 'rule of three' guidelines and immediate availability. Compounds bearing undesirable functionality, such as reactive groups and known toxicophores, were also removed, and scaffold analysis was carried out to ensure maximum structural diversity. As championed by scientists at SGX to facilitate library evolution, the presence of a single bromine atom was tolerated with the molecular weight ceiling raised to 350 where appropriate to accommodate this. After final manual review of shortlists of compound structures, a 2,000-member fragment library was selected and purchased from the Maybridge Fragment Range (Thermo Fisher Scientific, Tintagel) to form the foundation of our new fragment screening collection.

Fragment screening

The major issue facing fragment screening is that conventional biochemical assays normally fail to detect compounds with very low affinities. Therefore, more sensitive biophysical screening approaches including NMR, x-ray crystallography, SPR and TSA are generally required to identify fragment hits. NMR provides very high quality binding site and affinity information, x-ray crystallography delivers detailed atomic resolution ligand-binding information, SPR is capable of generating high quality selectivity and affinity data and TSA ranks binding strengths. However, these techniques do not identify whether a bound fragment modulates activity and it is common to perform HCS to provide functionally relevant activity data. Each technique has its advantages and disadvantages and it is generally beneficial to run two or more orthogonal screening approaches in parallel to validate hits. The choice of techniques employed usually depends on what is already available within the organisation. The major FBDD screening techniques – NMR, x-ray crystallography, SPR, TSA and HCS will be discussed in turn.

NMR: NMR-based fragment screening was first pioneered by Fesik's group at Abbott Laboratories

Figure 2
Biacore T100

over a decade ago and now dominates FBDD due to its high sensitivity and its unique binding site resolution. Its major strength is the ability to use changes in one or more NMR parameters to detect ligand binding events, including chemical shifts, cross-relaxation in the protein–fragment complex (STD-NMR) or cross-relaxation between the fragment and protein-bound water molecules (water-LOGSY). For example, Vernalis uses NMR screening in its strategy for FBDD called SeeDs (Selection of Experimentally Exploitable Drug start points) which identifies fragments that bind competitively to a specific site of the protein. Additionally, cocktails of between 10 and 12 fragments can be screened. Compounds in the original Vertex 'SHAPES' library all have simple ^{1}H NMR spectra and contain at least two protons within 5 Å to facilitate screening mixtures of fragments by NMR. Recently, a new technique called target immobilised NMR screening (TINS) has been developed by Siegal and co-workers at Leiden University that, in principle, can be applied to screening integral and membrane-associated proteins including G protein-coupled receptors (GPCRs) – a class of proteins which represents approximately 60% of all current pharmaceutical targets and which are notoriously difficult to develop drug candidates against.

X-ray crystallography: X-ray crystallography is a well established technique for studying protein structures and can provide the most complete picture of fragment binding to a target. Fragment-protein crystal structures show the binding orientation of the fragments in the active site, enabling the active site binding properties to be mapped to help guide efficient lead optimisation programmes. This technique is reliant on being able to identify robust crystallisation and fragment soaking conditions which is often not possible. However, recent advancements in methods of protein production and crystallisation, and an increased use of automation and robotics have seen this technique develop into a viable primary FBDD method. Several companies including Astex Therapeutics routinely use x-ray crystallography to identify fragments and screen their libraries in shape-diverse cocktails of between 5 to 10 compounds. However, certain classes of targets, such as membrane proteins, are simply not amenable to routine analysis by x-ray crystallography.

SPR: SPR can be used to directly detect fragment binding based upon mass changes at a sensor surface and is capable of generating high quality selectivity and affinity data to facilitate fragment selection. Also, most of the hits identified by the NMR screening techniques are also identified by SPR. Vernalis uses SPR to complement fragment screening by NMR to deliver affinity rankings of fragments from its SeeDs library. Furthermore, cross-competition experiments between fragments or

between a reference compound can indicate whether a fragment binds to the same or different site. SPR can also be used in the absence of a three-dimensional x-ray structure to drive SAR. In such cases, decisions about which fragments to progress can be based on thermodynamic and kinetic parameters such as slow dissociation rates (K_{off}) or enthalpy criteria for selection.

TSA: TSA, also known as differential scanning fluorimetry and ThermoFluor, is a high-throughput assay that measures T_m (the midpoint temperature of the protein unfolding transition) to rank binding strengths. Only relatively small T_m shifts are generally seen for fragments and those with ΔT_m greater than 0.5 C are considered to be positive hits.

HCS: The simplest approach to fragment screening is to perform biochemical assays at higher concentrations (typically 250µM to 5mM) than those used in conventional HTS (10 to 30µM). HCS is a relatively fast method which can exploit a wide variety of detection techniques. Assays with high reproducibility and stability are needed and the screening cascade has to be properly designed to have clear selection criteria for true positives since HCS is likely to lead to a comparatively high rate of false positives (due to compound aggregation) and false negatives (due to a lack of solubility). HCS can detect direct changes in the activity of a target upon fragment binding but still requires structural biology back up to define the precise mode of binding.

Fragment screening at CRT

At CRT, SPR (assessed using a Biacore T100 instrument, **Figure 2**) and HCS are employed in parallel in the FBDD screening cascade (**Figure 3**). We consider it much more efficient to employ x-ray crystallography after screening when the majority of fragments have been filtered out by applying various affinity, selectivity and concentration-response filters. Using two orthogonal screening techniques facilitates the cross-validation of hits and the selection of good starting points: compounds that are likely to have a high probability of success in soaking or co-crystallisation experiments with the target protein. HCS has been incorporated into the screening cascade by adapting the existing HTS platform at CRT as a method to directly detect functionally active fragments. Hits identified by SPR are ranked according to affinity (K_d) and compared to their potency in HCS prior to entering the crystallography pipeline. Further SPR validation can also be performed to assess whether a hit binds to a specific site on the target protein through competition assays with a reference compound (for example, an allosteric

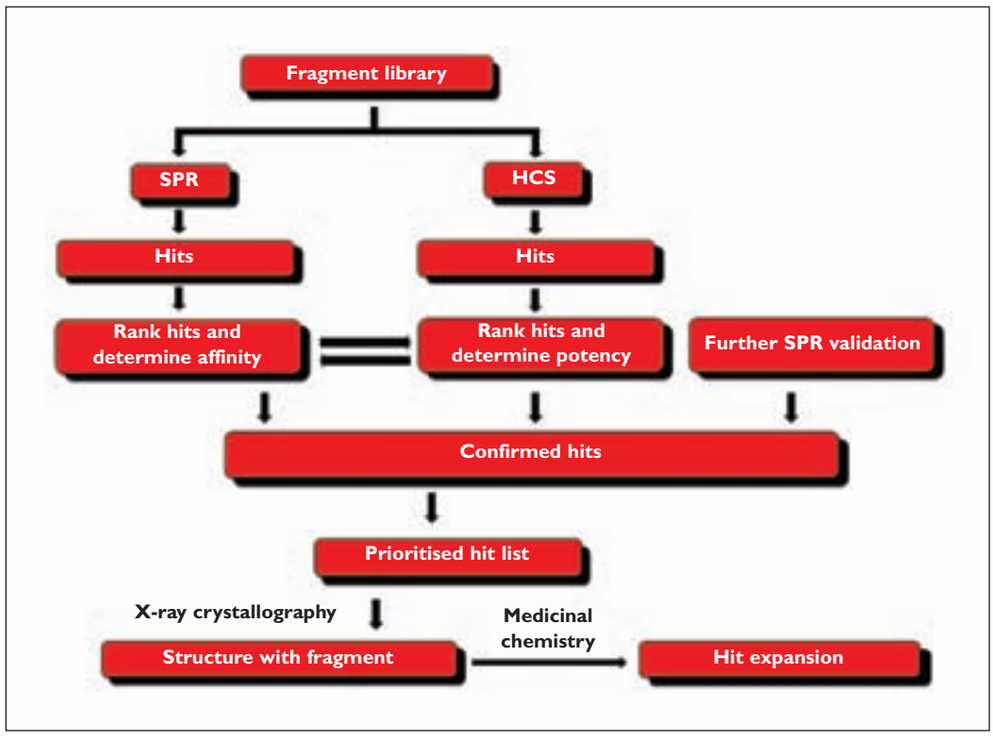

Figure 3
CRT fragment screening cascade

inhibitor). The structure information for the validated hits subsequently aids in the design of more potent compounds via hit expansion.

Other groups within the network of Cancer Research UK-funded drug discovery are also engaged in FBDD, employing complementary screening strategies. In the Drug Discovery Programme at the Beatson Institute for Cancer Research, Glasgow, UK, NMR-based screening will be employed with SPR as an orthogonal biophysical technique. In the Cancer Research UK Centre for Cancer Therapeutics at the Institute of Cancer Research, Sutton, UK, HCS, TSA and hit follow up by x-ray crystallography are chosen techniques.

Fragment optimisation

Structure-guided approaches can rapidly generate compounds with increased potency, however, advancing fragments to good quality lead molecules remains a significant hurdle. After an initial fragment has been identified it can be either evolved (grown) or merged to generate a hit compound. By selecting fragments with the highest LE, optimisation can concentrate on improving potency and other molecular properties by increasing molecular complexity. Additionally, target selectivity, compound patentability and ADMET liabilities (drug properties) can be considered at an early stage in the drug design process.

There are three complementary approaches that can be used for fragment optimisation: analogue-by-catalogue, fragment evolution and fragment linking.

The analogue-by-catalogue approach aims to enrich SAR around a fragment using chemoinformatic searches. Selected active fragments are used as substructure queries for large databases of commercially available compounds, generating a set of analogues which are subsequently docked into the target protein. Analogues which provide additional favourable interactions with the target protein are purchased and evaluated.

In the fragment evolution approach, the crystallographic binding mode of a fragment is used to identify new hot spots in the active site of the protein into which a fragment can be evolved (grown) to provide new points for interaction. *In silico* docking studies are used to predict the binding modes of compounds designed to exploit the hot spots and to prioritise compounds for synthesis.

Fragment linking relies on structural information for two active fragments that bind to different sites in the target protein. The challenge is to design a chemical linkage between the two fragments to produce a single molecule that adopts a low energy conformation and retains the binding mode of each individual fragment. However, in practice, it is extremely difficult to design and synthesise effective chemical linkages that conserve the binding mode of the individual fragments.

Summary and outlook

FBDD is most effective when applied to targets that are amenable to structure determination. At CRT, we consider FBDD to be a valuable alternative and complement to the traditional HTS platform to help identify novel chemical matter for projects where HTS hits have been elusive. The success of FBDD ultimately resides in the interactions between the biologist, structural biologist and medicinal chemist to generate SAR and guide the drug discovery process. We have also found that structural information significantly improves the efficiency of hit validation and hit progression. The focus is now on how best to use FBDD so it adds significant value to lead identification and drug discovery programmes. DDW

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