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Fragment-based approaches to enzyme inhibition

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Abstract

Fragment-based approaches have provided a new paradigm for small-molecule drug discovery. The methodology is complementary to high-throughput screening approaches, starting from fragments of low molecular complexity and high ligand efficiency, and building up to more potent inhibitors. The approach, which depends heavily on a number of biophysical techniques, is now being taken up by more groups in both industry and academia. This article describes key aspects of the process and highlights recent developments and applications.

Introduction

The search for new enzyme inhibitors and, more generally, for new ligands for proteins is central to chemical biology and a key focus in the early stages of drug discovery. Fragment-based approaches enable one to identify weak-affinity small molecules, termed ‘fragments’, and use them as suitable anchors for the elaboration of more potent inhibitors. Several compounds discovered using a fragment-based approach over the last decade have now entered clinical trials or are in pre-clinical development. The efficacy and successes of fragment-based lead discovery have now led to this being adopted in more industrial and academic organisations. Several reviews [1-5] and a book [6**] on this area have recently appeared. This review will focus on practical aspects and issues associated with fragment-based inhibitor discovery. The literature cited is drawn primarily from that published over the last two years. Earlier studies have been reviewed in this journal by Erlanson in November 2006 [7**].

Smaller is better

The rational design of enzyme inhibitors often involves modifying the structure of substrates and cofactors, or mimicking intermediates or transition states in the postulated reaction mechanism. When this does not provide an appropriate starting point, inhibitors are often identified from screening libraries of compounds (often tens of thousands of compounds) [8]. There is increasing interest in academia in accessing compound libraries, and national and international initiatives to establish suitable collections are underway. Fragment libraries are much smaller. The low molecular weight of fragments (typically <250 Da) and their correspondingly reduced functionality mean they will generally only bind to a target protein with weak affinity ($K_d > 0.1$ mM), if at all. Despite their weak affinities, fragments possess

high intrinsic binding energy to overcome a large entropic barrier upon binding [9] and often exhibit high ligand efficiency (G of binding per heavy atoms) [10]. Ligand efficiency is a useful yardstick, widely used in industry to assess the success of elaboration of a hit to a lead. Therefore, fragments are suitable starting points for elaboration into larger, more potent ligands.

A fragment-based approach to inhibitor design can be divided into four phases, as shown in Figure 1: (a) design and assembly of fragment libraries; (b) screening of fragments against a suitable form of the target enzyme; (c) validation of the hits; (d) elaboration of fragments into more potent inhibitors.

Starting with fragments: design and assembly of fragment libraries

The choice of fragments for the screening library is a crucial first step. On a similar framework to the 'rule of five' [11] for drug-like compounds ($MW < 500$ Da, $clog P \leq 5$, hydrogen bond donors ≤ 5 and hydrogen bond acceptors ≤ 10), a 'rule of three' ($MW < 300$ Da, $clog P \leq 3$, hydrogen bond donors and acceptors each ≤ 3) has been proposed for fragments [12]. Fragmenting the structures of known inhibitors, natural product compounds or of marketed oral drugs can provide useful information for library design [13]. In this regard, it has been shown that 50% of existing drugs share only a few molecular frameworks [14]. While maintaining diversity, libraries can be successfully biased to specific enzyme classes, for example kinases or proteases.

The quality of a fragment library is of paramount importance. The chemical and physical properties of fragments have received specific attention [15]. Compound solubility is a crucial issue [16]. Fragments need to be soluble at least $\sim 10^{-2}$ M in buffers from concentrated (10^{-1} – 10^0 M) stocks in dimethyl sulfoxide, to allow detection of their inherently weak affinities. By exhibiting high solubility and low $clog P$, fragments should be less prone to aggregation and non-specific effects in screening [17].

Amongst other criteria to be considered when assembling a fragment library are its size, diversity and chemical tractability. Fragments have lower molecular complexity than drug-like compounds, and so have a correspondingly higher probability of binding to a target [18]. Hit rates from screening fragments are higher than from screening lead-like compounds, so libraries of 10^2 – 10^3 fragments are usually sufficient to find hits. Several computational tools using molecular fingerprints and descriptors are available to estimate similarity and diversity to aid library design [19]. Synthetic tractability is an important consideration. Fragments should include functionality that facilitates chemical expansion and optimisation; however compounds with reactive groups should be excluded to avoid unwanted covalent modification of the target protein. Finally, the curation, quality control and maintenance of the compound libraries is a significant undertaking. Fragment collections are now available from several commercial sources, providing good starting points for expanding and building more target-focused libraries.

Fragment screening and identification

Screening small molecules that bind to a target protein is often considered in terms of sampling chemical space [20]. It has been estimated that there are only ~13 million molecules of up to 11 heavy atoms which are also 'rule of 3' compliant [21], compared to $\sim 10^{63}$ molecules of molecular weight less than 500 Da [22]. The molecular simplicity of fragments means few interactions are formed with the target enzyme, so binding affinities are typically low and outside the detection range of routine bioassays. There has been a move to develop high concentration bioassays for fragment screening [23]. However, this strategy can be problematic, leading to a high rate of false positives and artefacts from the high concentrations used.

These problems are largely avoided if fragment screening is performed using biophysical methods. Although initial studies focused on X-ray crystallography and NMR spectroscopy [24], there is an increasing interest in extending the repertoire of tools to other techniques, for example isothermal titration calorimetry (ITC), thermal shift, mass spectrometry and surface plasmon resonance [25]. The application of these biophysical techniques to fragment-based approaches has been reviewed [26], and their advantages and disadvantages contrasted [2,4]. Here we highlight key aspects of three methods: fluorescence-based thermal shift, ligand-based NMR spectroscopic experiments and X-ray crystallography.

Biophysical methods for fragment screening

In thermal shift experiments, compounds are added at 1–10 mM concentration to 1–10 μ M protein, in the presence of Sypro Orange, a fluorescent dye that is sensitive to the chemical environment, which binds preferentially to the unfolded state of a protein [27]. The samples are warmed in a thermal cycler at ~ 0.5 °C/min, and the fluorescence is monitored continuously. Compounds can be readily screened in a 96-well plate format in a relatively high-throughput manner. Hits are identified as compounds that stabilize the folded state of the target protein by at least 0.5 °C. Only relatively small shifts are generally seen from fragments, but the value of the technique is enhanced because a correlation between T_M and binding G is often observed.

NMR spectroscopy is well suited for fragment screening. In different experiments, it can be used to rapidly confirm whether a small molecule binds to a protein, or to determine the location of the binding site. For a comprehensive review on applications of NMR spectroscopy for screening, see Ref. [28]. One of the most useful techniques for ligand screening is ^1H WaterLOGSY. This works by magnetization transfer from bulk water to fragments, via stably bound water molecules in the protein-fragment complex [29]. A related 1D technique is saturation transfer difference (STD), which exploits a magnetization transfer process from the methyl resonances of the protein to detect binders [30]. These NMR techniques monitor the resonances of the small molecules directly, so are often referred to as ligand-based methods. Hits can be rapidly identified from cocktails, ideally of 3–4 fragments to minimize the overlap of signals, with no need to deconvolute the mixture. They have the general advantage of working with low amounts (~ 10 μ M) of proteins of any size (MW > 10 kDa), and requiring no knowledge of the protein NMR spectrum. They are

most powerful when performed as competition experiments with known ligands to determine if the hits bind in the active site and to eliminate interference from non-specific binding, a common caveat of highly sensitive NMR detection. NMR-based competition studies were used to identify novel fragments binding at the pY pocket of the SH2 domain of the Src protein tyrosine kinase [31*].

X-ray crystallography is arguably the most powerful tool for fragment screening, as it provides direct evidence of where and how the fragment binds to the enzyme [32]. Fragments are soaked as cocktails at high concentration (up to 50 mM per compound) into the enzyme crystal. After data collection and processing, difference electron density maps in the active site are analysed to detect fragment binding (Figure 2a). Depending on the quality of the data it may be necessary to deconvolute the cocktail by repeating the soaking experiment with single compounds to confirm the identity of the binder. Ideally the hit rate should be less than one compound per cocktail, to avoid multiple partial occupancies of electron densities, which make fragment identification difficult. Protocols for automated fitting of electron density obtained from soaking cocktails of fragments have been developed, such as the AutoSolve program developed by Astex Therapeutics [33].

Examples of fragment cocktail screening using X-ray crystallography include studies on the enzymes p38 MAP kinase, CDK2, ribonuclease A, and PTP1B [34], thrombin [34,35*], and more recently β -secretase [36] and protein kinase B (PKB) [37**]. Fragment crystallographic screening has also been reported on *Trypanosoma brucei* nucleoside 2-deoxyribosyltransferase [38]. Soaking times used in these experiments vary from ~10 s [38] to up to 24 h [34]. In rare cases longer incubation times were required [39*].

Key issues of fragment screening

Traditional bioassay-based approaches require only low quantities of *active* enzyme for kinetic assays. By contrast, fragment-based approaches require significant quantities of pure, soluble recombinant protein. It is important that the binding site of interest is free from endogenous ligands and thus able to bind fragments. Inactive forms of the enzyme can be targeted. To allow screening using X-ray crystallography, high diffraction-quality crystals of the unliganded enzyme have to be obtained, and should be robust enough to be soaked in the presence of fragments. Fragments can induce conformational changes in the protein upon binding [34]. However, enzymes that undergo major conformational changes such as domain movements may be problematic as this may prevent fragments from soaking into crystals.

The nature of the binding site of the target enzyme is also important to the success of fragment-based approaches. Shallow grooves are more difficult to target compared to deep and well-defined pockets. When several sub-pockets of a large active site are available, for example with proteases or dehydrogenases, fragments are more likely to bind to 'hot spots', that is regions that contribute most to the binding affinity of natural substrates or cofactors [40**]. In this regard, fragment screening may be a way of assessing the druggability of a target [41].

Computational methods have been developed to identify and characterize hot spots for fragment binding [42]. However, computation of free energies and binding modes by docking experiments are not yet sufficiently accurate to make an impact on our ability to find fragments or to correctly predict early structure-activity relationships (SAR) around weak-affinity fragments.

Hit validation

Once hits are identified their binding needs to be confirmed. The three-dimensional structure of the protein-fragment complex should be determined, ideally by X-ray crystallography, before a hit is progressed into chemical optimisation. This can be surprisingly problematic and often constitutes a bottle-neck step [39]. Specific issues arise because of the high concentration of fragment required and limitations because of solubility. It is clear that using data from a range of biophysical methods can increase the success in obtaining liganded crystal structures [43].

In addition to X-ray crystallography, structural information can be obtained using NMR spectroscopy by monitoring perturbations of HN resonances in two-dimensional ^1H - ^{15}N HSQC protein spectra. However, these techniques are primarily limited to small (<30 kDa) and soluble proteins. The protein needs to be labelled with at least ^{15}N and all the ^1H - ^{15}N cross-peaks assigned. In the absence of crystallographic or NMR spectroscopic data, the binding site can be located by performing competition experiments with a known ligand, or by using site-directed mutants to report on fragment binding in the active site [40, 44].

As part of validating a fragment hit, its binding affinity should be determined – allowing the ligand efficiency to be calculated. The gold-standard technique for this purpose is isothermal titration calorimetry (ITC). ITC can provide information on the enthalpic and entropic contribution to the binding free energy. The utility of considering thermodynamic parameters for drug design has been discussed [31, 45], for example monitoring ΔH and ΔS by ITC may reveal changes in binding modes [44, 45]. On the other hand, it is difficult to rationalize enthalpic and entropic contributions to ΔG using classical SAR analysis. Their compensating nature means correlation with other structural or physico-chemical parameters of organic molecules is rarely observed.

Elaboration of fragments

Once a fragment hit is confirmed, the aim is to find analogues with improved binding affinity, guided by structural information. Before committing to synthetic chemistry, compounds that are structurally related to the hit should be tested to establish that the best fragment has been found. As fragment binding is expected to be especially sensitive to even minor structural modifications, it is very important to establish SAR around an initial hit, early on, ideally using ITC.

Once a course of action has been decided, a larger molecule is synthesised that incorporates the original fragment(s), and the structure and affinity of the molecule bound to the enzyme are determined. Compared to traditional medicinal chemistry approaches, iterative cycles of synthetic chemistry are needed earlier on to incrementally build up the potency of

compounds by optimally exploiting interactions at the active site. Availability of a few suitable starting fragments is desirable to generate more than one chemical series. Once compounds of sufficient potency have been synthesised the activity of the inhibitor series can be tracked using kinetic assays.

The chemistry ideally involves high yielding reactions such as amide coupling, palladium-catalysed alkylations, reductive aminations, and Click chemistry. Reversible imine and disulphide chemistry can be exploited for fragment self-assembly in a dynamic combinatorial fashion to amplify high-affinity inhibitors. A related approach, dynamic combinatorial X-ray crystallography, was used to identify potent inhibitors of CDK2 in the presence of a single protein crystal [46].

Growing versus linking fragments

Two different strategies can be employed to construct larger inhibitors from one or more starting points: fragment growing and fragment linking. Arguably, the former is likely to be more straightforward and less problematic, since only one starting point is required. The fragment binding orientation is used to decide how to grow the fragment to pick additional interactions (Figure 2b), often aided by *in silico* docking and knowledge of the binding pocket. Depending on their binding mode and synthetic tractability, it may be difficult to use some fragments as starting points for design [36]. By contrast, before a fragment-linking approach can be initiated, a second fragment needs to be identified that binds in close proximity to the first fragment. Strategies using NMR spectroscopy have been reported, to screen for a second-site fragment in the presence of a known compound [47], or to identify a pair of ligands that bind simultaneously in a single cocktail [48]. An example of a successful fragment-linking approach is the study to discover inhibitors of thrombin [35^{*}] (Figure 3). Reductive amination of the S2–S4 binder (Figure 3a) with the relevant benzaldehyde derivative of the S1 fragment (Figure 3b) was used to generate nanomolar inhibitors (Figure 3c) [35^{*}].

Extending fragments is an iterative process during which the starting elements should maintain their binding geometry as they are incorporated into bigger compounds. An example showing how little the initial fragment moves during a growing process has been recently described [37^{**}]. The linking strategy is very sensitive to designing an ideal linker, which should not perturb the binding mode of the fragments. This is difficult to achieve in practise, so fragments are more prone to movements during the linking process [5]. More flexible binding pockets would be expected to accommodate these movements more easily. However, significant changes in binding modes should raise suspicion, as they can result from changes in crystallization conditions such as pH, solution components and additives [44,49].

Deconstructing binding modes and ligand efficiency

It is remarkable how fragments can maintain their binding mode during the elaboration process. A crystallographic study has addressed the question of whether the inverse logic also applies [50^{*}]. Can fragments recapitulate the binding mode exhibited in a larger molecule? Fragments of an inhibitor of β -lactamase (K_i 1 μ M, MW 340 Da and ligand

efficiency = 0.38) were found to explore a new pocket as well as a novel binding mode within the known pocket. Only when a certain degree of complexity was restored was it possible to recapitulate the original binding mode. This experiment could be perceived to undermine the fragment-based approach, but only if the starting inhibitor was optimal. There is no convincing evidence this was the case. There are other examples in which binding modes have been maintained during the deconstruction of ligands into fragments, notably early studies on thymidylate synthase [51]. More recently, the binding of fragments of the cofactor NADPH to ketopantoate reductase was studied to investigate the structural determinants of its affinity [40**]. The different parts of the ligand were classified according to positive or negative contributions to ligand efficiency, leading to identification of two hot spots that contributed to significant increases in affinity. Further, using ITC and site-directed mutants, the binding of the fragments at these sites was shown to recapitulate that of the cofactor [40**].

Ligand efficiency is a remarkably useful criterion for evaluating the quality of the starting fragment hits and for assessing intermediate inhibitors en route to the final lead compound [52*]. Dissecting the contribution of each group modification to the ligand efficiency during the fragment optimisation process is very helpful to understand which regions in the binding site are more likely to improve affinity [40**]. This helps to prioritize which compounds to make. The development of a nM inhibitor of PKB is an example of maintaining a high ligand efficiency (above 0.4) during the process of growing a fragment [37**] (Figure 4).

Concluding remarks

Fragment-based inhibitor discovery is a logical and powerful approach to rapidly generate small molecules that interfere with biological processes. A clear challenge is the highly multi-disciplinary platform that requires strong collaborations between crystallographers, biologists, biophysical screeners and medicinal chemists. However, worldwide access to synchrotron radiation sources and sophisticated biophysical instrumentation are making these approaches more tractable.

The intellectual process is strongly structurally and hypothesis driven, on the basis of our current understanding of non-covalent protein–ligand interactions and of structure–activity relationships. The utilization of fragments to improve our understanding of molecular recognition is starting to expand into new areas. Fragments are now being used to address increasingly challenging targets, such as protein–protein interactions [53] and RNA [54]. All these areas are likely to generate intense interest in the near future. It could not be a more exciting time to start with fragments.

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Figure 1.

The multistage strategy adopted for fragment screening, verification and elaboration into enzyme inhibitors. The process involves qualitatively identifying fragments that interact with the target protein, confirming and characterizing this interaction to precisely locate the fragment and define its binding mode, and finally the iterative elaboration of the fragment into a larger potent inhibitor.

Figure 2. X-ray crystallographic analysis of fragment binding. **(a)** Electron density showing multiple fragment-binding in an enzyme active site. **(b)** Core fragment chosen for synthetic elaboration showing possible growth vectors to two adjacent binding sites.

Figure 3.

An example of fragment linking, adapted from Ref. [35*]. X-ray crystal structure of fragments binding at (a) S2–S4 sites ($IC_{50} = 12 \mu M$) and (b) S1 site ($IC_{50} = 330 \mu M$) of thrombin. (c) Structure of the final inhibitor ($IC_{50} = 3.7 nM$) generated by linking the fragments in (a) and (b).

Figure 4.

Evolution of inhibitors of PKB, adapted from Ref. [37**]. The initial fragment **1** was elaborated successively to inhibitors **2** and then **3**. The high ligand efficiency and the binding mode of fragment **1** were maintained through the synthetic elaboration.