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Fragment-Based Approaches in Drug Discovery and Chemical Biology

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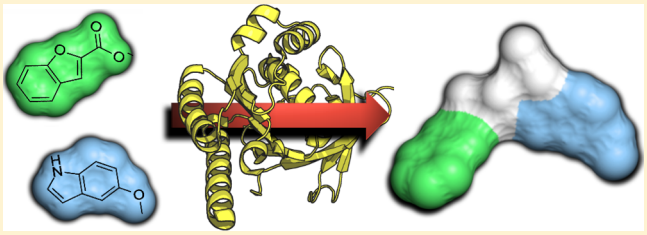
ABSTRACT: Fragment-based approaches to finding novel small molecules that bind to proteins are now firmly established in drug discovery and chemical biology. Initially developed primarily in a few centers in the biotech and pharma industry, this methodology has now been adopted widely in both the pharmaceutical industry and academia. After the initial success with kinase targets, the versatility of this approach has now expanded to a broad range of different protein classes. Herein we describe recent fragment-based approaches to a wide range of target types, including Hsp90, β-secretase, and allosteric sites in human immunodeficiency virus protease and fanesyl pyrophosphate synthase. The role of fragment-based approaches in an academic research environment is also examined with an emphasis on neglected diseases such as tuberculosis. The development of a fragment library, the fragment screening process, and the subsequent fragment hit elaboration will be discussed using examples from the literature.



The discovery of new molecules for modulating biological function is a key motivation in chemical biology and the primary focus in drug discovery programs. Over the past two decades, the pharmaceutical industry has become very reliant on high-throughput screening (HTS) approaches, to screen large libraries of compounds (up to 10^6), that they have assimilated over time. Maintaining the diversity and quality of these libraries is a major overhead and requires constant attention. Inevitably, the libraries contain molecules that are not very druglike; i.e., they are relatively lipophilic and have poor thermodynamic solubility. As a consequence, false positives arise, and even genuine hits may not be good starting points for drug development. It is also inevitable that these HTS libraries represent only a very tiny fraction of possible chemical space and so limit confidence in finding a really good starting point for subsequent development. Finally, even if the structure of a HTS hit binding to the target protein can be obtained, it may still not be clear which parts of the molecule contribute most to the binding energy, leading to ambiguity about how best to increase potency.

Fragment-based methods, which involve the elaboration of weakly binding small molecules with molecular masses of <300 Da, have emerged as a complementary and contrasting approach for early stage drug development in recent years. The approach is fundamentally different from HTS in almost every aspect: library size, screening method, value placed on each hit, dependence on structural methods, etc. Fragment-based drug discovery (FBDD) depends upon the close interaction of structural biology and synthetic chemistry. It is an attractive area to both practitioners of chemical biology, structural biology, and synthetic and computational chemistry, as it takes a structural and molecular view of biological targets, combined with a stepwise and logical medicinal chemistry strategy. The synergy of a structure-based approach and an iterative small-to-large-molecule chemistry strategy means that lead development is guided by combining structural and assay information generated at each step in the program. There is now a growing body of published work to support the power of this approach. An important landmark was passed in 2011 when Zelboraf (PLX4032) became the first FDA-approved drug developed using the fragment-based approach.[1,2] Despite this, there is still more progress that needs to be made in the implementation of this approach.

The origins of FBDD can be traced to a seminal paper published by Jencks in which it is proposed that small "fragments", although weakly binding, form high-quality interactions that can be optimized into highly potent larger molecules.[3] Another notable contribution to the early thinking in this field came from Verlinde who developed a method to link together low-molecular mass fragments to develop more potent compounds against trypanosomiasis.[4] The methodology became established though the pioneering studies at Abbott using SAR by NMR[5] and Astex,[6,7] where the use of X-ray crystallography was, and still is, the main screening platform. FBDD was initially successful when it was employed principally against kinase targets that proved to be highly druggable and well-suited to the approach. Subsequently, it has become clear that the approach is generic and can be applied successfully to any protein family for which structural information is readily available, and indeed, there are examples of success with fragment-based methods against targets that have proven to be very difficult with traditional HTS approaches.[8,9] Fragment-based methods are now being used against targets as diverse as

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protein−protein interactions, transcription factors, protein chaperones, and RNA.[10−17]

FRAGMENT-BASED DRUG DISCOVERY

The fragment-based approach involves the construction of potent small-molecule ligands from low-molecular mass fragment molecules (Figure 1). Fragments typically conform

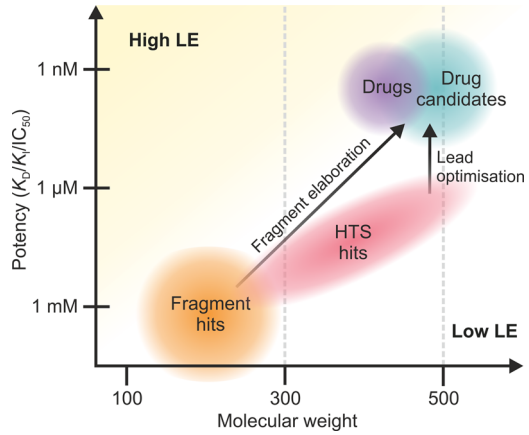

Figure 1. Comparison of molecular mass vs potency of leads developed from conventional HTS and FBDD approaches. The dashed lines show Lipinski's Rule of Five 500 molecular weight cutoff and the Rule of Three 300 molecular weight limit.

to the Rule of Three:[18] molecular mass of <300 Da, up to three hydrogen bond donors, up to three hydrogen bond acceptors, and calculated logP (clogP) of ≤3. In the general fragment-based process, there are three key stages: (1) fragment library design, during which a library of fragment molecules is assembled, (2) fragment screening, during which the library of fragments is screened in vitro using an array of biophysical techniques to detect noncovalent binding to a target biomolecule, and (3) fragment elaboration, during which the validated fragment hits are synthetically elaborated into lead compounds by iterative cycles of synthesis, guided by a combination of structural information, in silico design, and bioaffinity data (Figure 2).

There are two primary advantages to the methodology. First, it is estimated that there are 10^{60-200} possible druglike

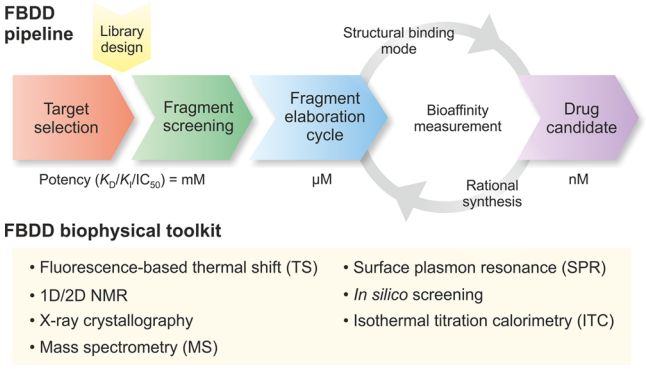

Figure 2. General workflow of the fragment-based approach, indicating the process of taking validated fragments through to a lead series. The iterative cycle of design, binding mode determination, and affinity measurement to incrementally improve potency and biophysical properties is shown.

compounds of HTS size (300−500 Da)[19,20] but only 10^7 possible molecules composed of up to 11 atoms of C, N, O, and F that follow the Rule of Three.[21] Thus, a significantly larger proportion of chemical space can be sampled with a fragment library (usually ~10^3 fragments) than with the ~10^5−10^6 larger molecules typical for an HTS campaign. Second, although fragment hits are weakly binding, they must make high-quality interactions with the target to bind with sufficient affinity for detection (Figure 3). Because of these optimal

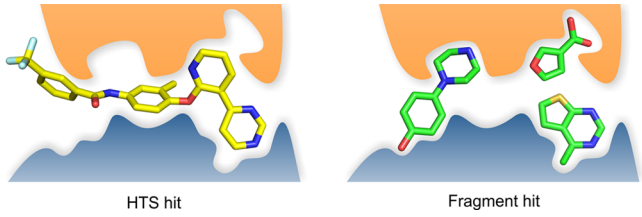

Figure 3. HTS hits may bind by virtue of numerous suboptimal interactions. By contrast, fragment hits are more ligand efficient and involve fewer but more optimized interactions.

interactions, fragments are very "atom efficient" binders, demonstrated by high ligand efficiency (LE), defined as $-\Delta G$ in kilocalories per mole divided by the number of heavy (non-hydrogen) atoms.[22]

FRAGMENT LIBRARY DESIGN

With the emergence of more challenging targets, there is now an incentive to reassess the composition of fragment libraries.[23] A reasonable starting point is to include primarily compounds that are Rule of Three compliant and include functionalities that facilitate future fragment elaboration, while avoiding reactive, unstable, or toxic scaffolds such as alkylating or acylating groups.[24] For example, Gibbs et al.[25] report that they exclude more than 45 functional groups and substructures as part of a primary library filter. Interestingly, some groups such as arylamines or arylnitros remain well-represented in marketed oral drugs despite being flags for carcinogenicity.[26,27] It may be beneficial therefore to leave such moieties in a fragment library but aim to replace them with bioisosteres if any developed leads show undesirable properties.[28] While individual compounds in many commercial fragment libraries are Rule of Three compliant, the library as a whole can be somewhat limited with regard to chemotype and shape diversity. A number of pharmaceutical companies and academic groups have begun to examine the composition of fragment libraries in detail to address these issues, using in silico methods to analyze fragment screening data. Hubbard et al. used the results from fragment screens against 12 diverse targets to guide the development of their fragment library.[29] For the 12 targets examined in this study, all the fragment hits for a given target possessed similar physicochemical properties. For example, fragments that were hits against protein−protein interaction targets tended to be more hydrophobic and heavier than other fragment hits from other programs. They also note a lower hit rate for protein−protein interactions as compared to more "druggable" target classes such as kinases. Of the fragment hits against the kinases screened, 11% of hits were common to all three kinases and at least 52% were unique to each kinase. Stewart et al. have developed a "Fragments of Life" library of 1329 compounds using fragment-like naturally occurring metabolites, derivatives, and biaryl mimetics of protein α-, β-,

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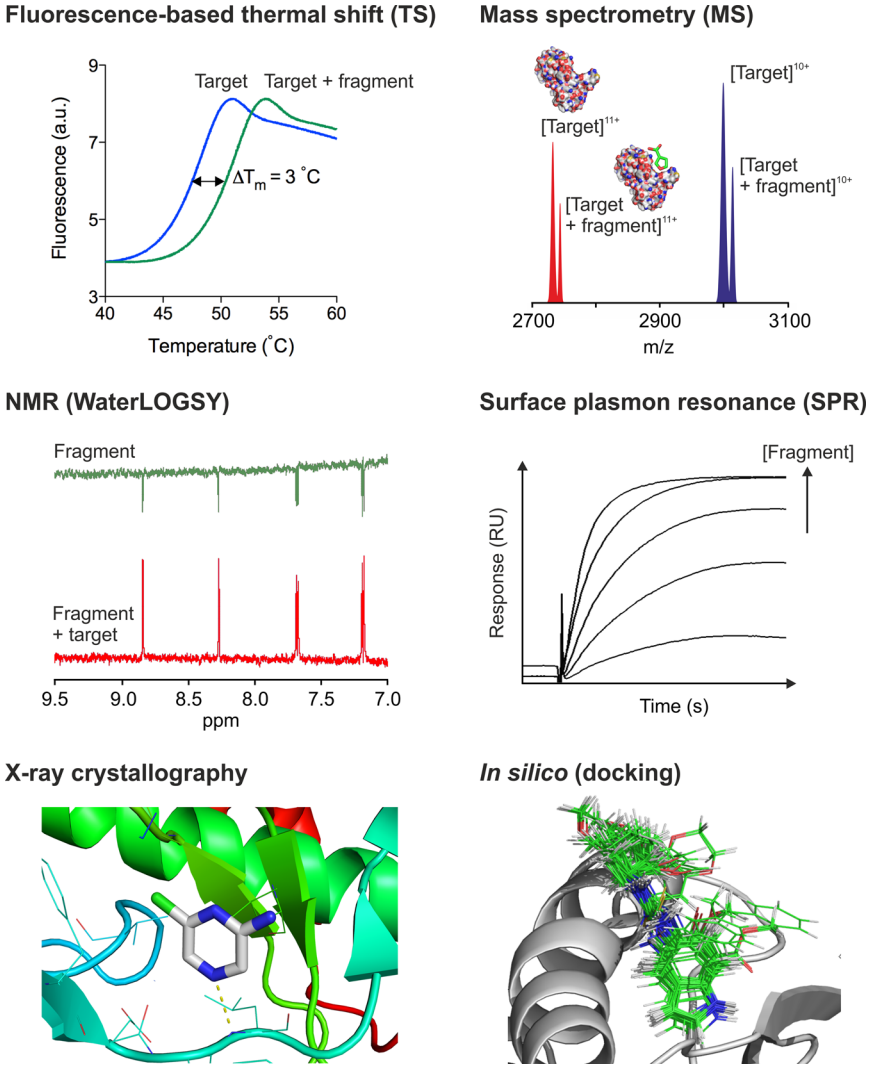

Figure 4. Examples of representative data from fragment-based screening techniques. Fluorescence-based thermal shift has been used to measure the induced increase in the melting temperature of a protein in the presence of a fragment. Mass spectrometry has been used to detect a protein:fragment complex directly. NMR (WaterLOGSY) spectra which show unbound ligand signals (green spectrum) and inversion in the presence of target (red spectrum). Surface plasmon resonance (SPR) sensorgrams showing a fragment titration, with each response line representing a different concentration of fragment. X-ray crystallography is used for unambigious determination of fragment binding mode, essential for fragment development. In silico (docking) produces an ensemble of ranked, docked conformations.

or γ -turns, with the goal of interrogating enzyme active sites, allosteric pockets, and protein−protein interactions.[30] This library was screened against leukotriene A4 hydrolase, which yielded 12 fragments that bound with affinities in the range of 40 μM to 5 mM, also identifying a fragment that bound in a shallow surface pocket (IC_{50} = 1 mM). Makara used an in silico approach to examine the success rates of screening fragment-like libraries and has suggested that a small number of readily available chemotypes can yield potent activities against a large number of targets.[31] Nunez et al. at Abbott have used molecular graph theory to design a more fragment efficient library. They performed an in silico screen of their library against human trypsin and identified virtual fragments that were highly chemically similar to known trypsin inhibitors.[32] Similar approaches using in silico methods have also been applied to the structure-guided expansion of kinase fragment libraries.[33] Hung et al. at the Broad Institute have recently reported the synthesis of a set of Rule of Three compliant fragments that contained non-aromatic sp^3-rich skeletons, using a diversity-oriented approach, although they have not reported screening of this library.[34]

■ FRAGMENT SCREENING

Fragment hits make a small number of high-quality interactions with the target, and typically, their binding affinities are in the range of 0.1−10 mM. To detect binding, sensitive biophysical screening methods must be used. Compared to those in typical HTS bioactivity assays, higher concentrations of fragments are required. However, as fragments are chosen because of their good aqueous solubility, fragment screening should lead to fewer false positives from aggregation (often identified as compounds that cannot be displaced by a known binder), a common problem in HTS programs.

Although X-ray crystallography is potentially the most powerful primary screening technique, it depends upon an infrastructure of automation, powerful in-house X-ray detectors, and good access to synchrotrons. More typically, fragment screening involves a cascade of biophysical screens that provide

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increasing confidence that the fragment does bind to the target and may additionally provide some information about binding location through competition studies. Using this strategy, the initial screen is likely to be by thermal shift (TS) or surface plasmon resonance (SPR), although other approaches are being adopted, e.g., mass spectrometry. Subsequent screening by NMR spectroscopy can provide conclusive evidence of binding and often precedes structural studies by X-ray crystallography to determine the exact mode of fragment binding (Figure 4).

Fluorescence-Based Thermal Shift (TS). Perhaps the quickest method for fragment screening is fluorescence-based thermal shift (TS).[35,36] This technique detects compounds that increase the unfolding temperature of a target protein (ΔT_m) by binding to and stabilizing the folded state. The thermal unfolding process is typically monitored in a plate-based format via an exogenous environmentally sensitive fluorescent dye. Because fragment binding is weak, the expected shifts are small. A hit is often classified as a compound that gives a ΔT_m that is twice the standard deviation of the measured melting point, typically 1 °C. The results are not always highly reproducible, and the process should be seen as an enrichment exercise, ahead of, for example, an NMR screen. Fersht et al. have reported the use of fluorescence-based TS to screen for fragments binding to the mutational cavity of the oncogenic Y220C p53 tumor suppressor protein.[14] Approximately 1900 fragments were screened, leading to the identification of 47 fragments with a ΔT_m greater than 0.5 °C, with a maximal ΔT_m of 1.8 °C.

NMR Spectroscopy. Ligand-detected one-dimensional (1D) ^{1}H NMR experiments [i.e., Carr−Purcell−Meiboom−Gill (CPMG), saturation transfer difference (STD), or water-ligand observed via gradient spectroscopy (WaterLOGSY)] provide the most simple and rapid methods of NMR screening.[14,17,37−39] In CPMG experiments, a reduction in the intensity of the fragment proton signals due to binding to the slow tumbling protein is observed.[40,41] In the STD and WaterLOGSY experiments, an irradiation pulse is applied at either the resonance frequency of bulk water (WaterLOGSY) or the biomolecule directly (STD), resulting in the transfer of magnetization to the fragment via the target−fragment complex.[42,43] In each experiment, the resonance signals of binding versus non-binding fragments become easily distinguishable. The acquisition of 1D spectra is relatively quick (∼15 min), and higher throughput can be achieved by screening two or more fragments at the same time in "cocktails". Information about the fragment binding site and its affinity can also be gained by adding a displacer ligand with a known binding mode. Brough et al.[17] utilized this method to find fragments binding to the ATP pocket of the Hsp90. Cocktail screening of 10−12 fragments was employed in the presence and absence of the previously known ATP-competitive ligand PU3.[44] Of the 1351 fragments screened, 82 displaceable hits were found and a series of novel ATP-competitive Hsp90 inhibitors were developed.

Fragments can be screened and validated simultaneously by more complex two-dimensional (2D) target-detected ^{15}N−^{1}H HSQC experiments, which monitor fragment-induced chemical shifts in the ^{15}N−^{1}H cross-peaks of the ^{15}N-labeled target protein.[37,45−47] Although the method consumes much time and material, it provides information about the localization and characterization of fragment binding. For example, following an initial WaterLOGSY NMR and TS screen, Fersht et al. used ^{15}N−^{1}H HSQC to identify fragment hits that bound to the mutational surface pocket of the Y220C p53 mutant.[14]

X-ray Crystallography. X-ray crystallographic screening provides both validated hits and structural binding information in one step.[25,48−54] Crystals of the target biomolecule are soaked with either high-concentration cocktails of fragments or individual fragments (up to 50 mM per compound is not unusual). In the case of cocktail screening, it may be appropriate to rescreen the cocktail components as singletons for unambiguous electron density assignment. The protein crystal must be robust, diffracting to generally better than 2.5 Å resolution, and have a solvent-exposed and/or unhindered binding site in the crystal lattice. For more difficult crystal systems, for example, in cases where ligand binding induces a large conformational shift that cracks the crystal, cocrystallization with cocktails of fragments may be possible.

Astex Pharmaceuticals has pioneered the approach of high-throughput X-ray fragment screening. It is a key component of their proprietary Pyramid platform. In one example, Astex conducted a screen of 500 fragments against cyclin-dependent kinase 2 (CDK2) by soaking apo CDK2 crystals in cocktails of four.[53] CDK2 is a key regulatory kinase for mammalian cell cycle progression and thus an attractive anticancer target. The X-ray screen yielded more than 30 cocrystal structures, showing all fragments binding in the kinase ATP binding site. The structure-guided optimization of an indazole hit, along with optimization of its cellular activity and pharmacokinetic properties, led to the discovery of AT7519. The lead is currently in phase I/II clinical trials for controlling tumor growth of a range of hematologic carcinomas.

Mass Spectrometry (MS). Recent improvements in mass spectrometry techniques have led the use of native mass spectrometry as a complementary fragment screening method.[51,55,56] In its simplest form, fragment/target mixtures are ionized by soft electrospray ionization (ESI), and fragment binding is observed by an increase in the mass of the biomolecular ion corresponding to that of a bound fragment. In principle, fragments can be analyzed in large cocktails and the gas phase abundance of fragment−target pairs can be used as a crude measure of relative binding affinity. However, because the soft ionization techniques necessary to see noncovalent complexes lead to a significant loss of spectral resolution, it is often not possible to detect fragment binding directly.

Surface Plasmon Resonance (SPR). In direct SPR fragment screening, the target biomolecule is covalently linked to the gold surface of an SPR biosensor chip and solutions of single fragments are sequentially passed over it.[52,57−61] As fragments bind to the immobilized target, the increase in the surface mass is detected in real time. From the time-dependent fragment association−dissociation response, the binding kinetics can be measured and the binding affinity calculated. Recent advancements in SPR biosensors and instrumentation have allowed the technology to detect binding of small molecules with molecular masses down to 100 Da, with significantly reduced numbers of false positives from non-specific binders. The experiments can also be performed in a competition mode, in which a known ligand is tethered to the surface and the protein is allowed to form a complex with it. When a competing fragment is introduced, the decomplexation of the protein from the surface is detected as a large signal change. Rapid screening of entire fragment libraries by SPR is now possible within a few weeks, requiring as few as 25−50 μg of protein.[57] The power of this technique for screening against

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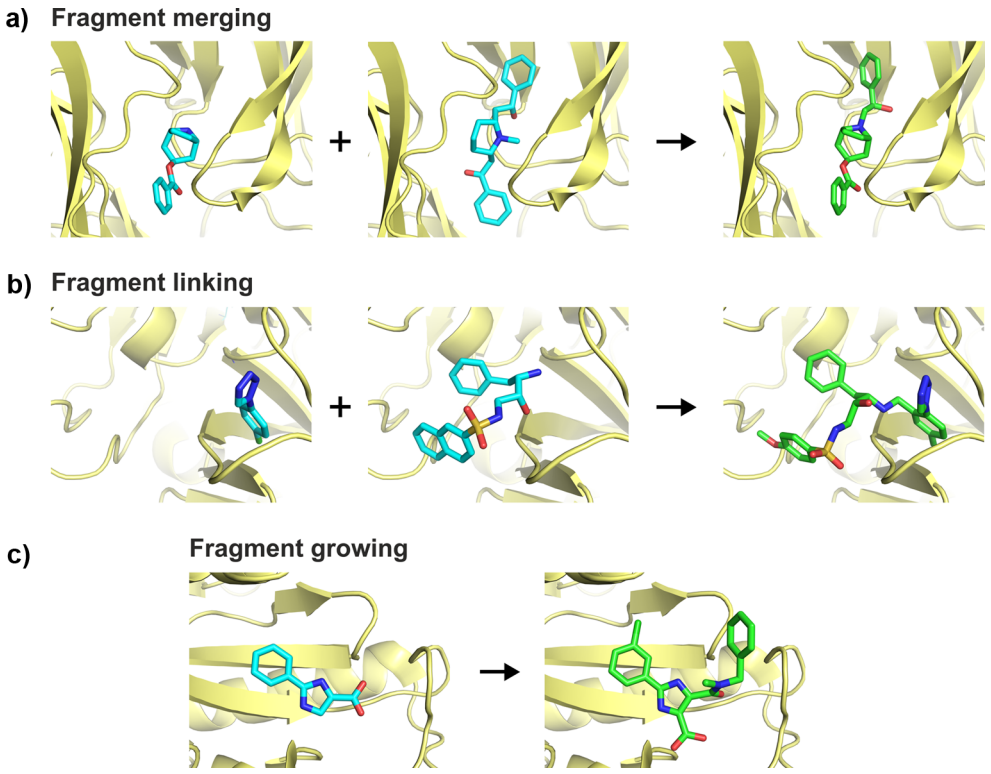

Figure 5. (a) Fragment merging strategy with acetylcholine binding protein A. The natural alkaloid lobeline (K_D = 32 nM) was merged with an identified benzoate-substituted nortropine fragment (K_D = 20 μM) to produce a compound with a K_D of 320 nM.[68] (b) Fragment linking of *p*-chlorophenyltetrazole (IC_{50} = 330 μM) and an amino alcohol (IC_{50} = 100 μM) to give a compound with an IC_{50} of 1.4 nM against thrombin.[75] (c) Fragment growing of a phenyl imidazole fragment (IC_{50} = 180 μM) by addition of a chlorine atom and a benzylic amide group to improve potency to 2.0 μM against Pin1.[78]

difficult targets such as G-protein-coupled transmembrane receptors (GPCRs) is described in further detail below.

Virtual Screening. In recent years, in silico techniques have become more sophisticated and widely available. Molecular docking has been used in two ways: to produce the binding mode for a biophysically validated hit in the absence of structural information and as part of an in silico screening effort. To assess the former application, Verdonk et al. systematically examined a range of scoring functions and docking methodologies for fragments and druglike molecules against a range of targets.[62] The X-ray crystal structure of the molecule in complex with protein was available for each molecule docked. Given the molecule had already been validated as a binder, how well could the docking recapitulate the binding pose in a useful manner? In broad terms, they found that approximately half the time both fragments and druglike molecules were correctly docked. Interestingly, an analysis of the failed dockings revealed that fragments and druglike molecules primarily failed for different reasons. In "failed cases", fragment poses were correctly produced but not scored highly enough, and for druglike molecules, undersampling of the conformational space failed to produce the correct pose.

For molecular docking to be useful for screening, it must not only produce the correct binding mode but also evaluate the free energy change of a ligand in solvent binding to the protein. For such a process, conformational entropy penalties of the ligand, desolvation terms, water interactions, and protein conformational changes need to be considered. With these additional complications and given the failure rates even for known binders, the use of docking as a screening technique seems to be less viable. To argue that in silico screening enrichs a pool of compounds, it is necessary to demonstrate that a randomized sample from the library produces significantly fewer experimental hits. While in silico screens may be justified in HTS campaigns, the many fewer compounds typically screened and the throughput of biophysical screens for FBDD make the use of in silico screening to filter a fragment library less useful. Certainly, in silico techniques find application at a later stage, particularly in fragment and lead elaboration, where comparisons to existing structural information can be made.[63]

Clearly, there is an opportunity to improve docking algorithms and especially scoring functions. Favia et al. have recently compiled a publicly available database, SERAPhiC (Selected Fragment Protein Complexes), of high-quality protein−ligand complexes, providing a resource against which new computational tools can be assessed.[64]

■ FRAGMENT ELABORATION CYCLE

Validated fragment hits are structurally elaborated to improve potency in an iterative process of rational design and synthesis, guided by a combination of structural binding information and quantitative affinity data (Figure 2). Knowledge of a compound's binding mode is paramount in any FBDD campaign and is derived primarily by X-ray crystallography. Protein-based 2D and three-dimensional (3D) NMR spectroscopic methods can also be used to determine compound binding modes.[45,46] Additional structural information about fragments that bind simultaneously in adjacent sites may be obtained by observing interligand nuclear Overhauser effects

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(ILOEs).[65,66] If structural data are unavailable, there may be some merit to in silico docking. However, the in silico approaches may not provide sufficient confidence in the predicted binding poses for a significant investment in subsequent medicinal chemistry.

Quantitative binding data can be collected by several methods. Isothermal titration calorimetry (ITC) is commonly used to measure the K_D of a fragment or elaborated molecule. The technique works by measuring the heat released by binding during a series of titrations of ligand into a solution of the biomolecule.[51] It is generally applicable, has high sensitivity, yields full thermodynamic characterization, and can be used to measure a large dynamic range of K_D values. One disadvantage is that each experiment requires a significant amount of the target protein. Affinity measurements can also be obtained with NMR[14,45,46] or SPR.[38,52,57,60] Nonspecific aggregation and the typically low potency of fragments mitigate against them being used in kinetic assays. These should ideally be introduced only when more elaborated and potent molecules have been generated with improved physical properties. Thereafter, quantitative measurement of enzyme inhibition becomes an important parameter in subsequent compound optimization.

Fragment Merging. There are three main approaches to increasing the potency of compounds derived from fragments: fragment merging, linking, and growing (Figure 5). Fragment merging is the incorporation of structural portions of overlapping molecules into a fragment, using structural information of other fragments, substrates, and known ligands in complex with the protein.[67,68] Trends in potency from different series can be used to identify important binding motifs and interactions, and this information can be used to produce a hybrid series. Edink et al. applied such a fragment merging strategy to target acetylcholine binding protein (Figure 5a). From an overlay of the crystal structures of the natural alkaloid lobeline (SPR at 32 nM) and an identified benzoate-substituted nortropine fragment (SPR at 20 μM), a merged compound was produced with a K_D of 320 nM.[68] Similarly, in an FBDD program against Hsp90, Brough et al. have reported the merging of a triazine fragment (IC_{50} = 350 μM) with a molecule identified through an in silico screen (IC_{50} = 0.9 μM) to produce a merged molecule with an IC_{50} of 58 nM.[17] Other applications of fragment merging have also been reported with Jun N-terminal kinase 3 (JNK3),[69] where overlapping fragments were merged to improve potency, and with thymidylate synthase, where the glutamate tail of the natural cofactor mTHF was appended to a fragment with a K_I of 1.1 mM to produce a 45-fold increase in potency.[70] Hughes et al. reported a fragment merging strategy against PI3γ kinase.[71] A series of imidazopyridine fragments that were found by X-ray crystallography to overlay with an inhibitor from the literature were identified. Incorporating a thiazolidinedione moiety from the previously reported inhibitor produced a novel compound (K_D = 34 nM) that displayed a high degree of selectivity against a panel of 43 kinases.

Fragment Linking. Fragment linking is conceptually the most appealing strategy for fragment elaboration. It requires the efficient joining of two fragments that are known to bind at non-overlapping sites. In theory, a compound derived from linking fragments with an ideal linker is expected to have a Gibbs free energy of binding that is better than the sum of the individual fragment binding energies.[3] This is because the rigid body entropy barrier that has to be overcome by the fragments is largely independent of molecular weight, so the linked compound has only a single rigid body penalty term, not the sum of two terms.[72] Consequently, it is highly desirable to be able to identify fragments in adjacent pockets.

When presented with two fragments binding in different pockets on a target and considering how to best link them, we must make various compromises. The fragments have revealed their preferred orientation to the protein, to optimize their interaction; hence, it is assumed that any attempt to link them should allow the linked fragments to adopt and recapitulate the conformation of the individual fragments. An ideal linker not only will allow this but also may make additional favorable interactions with the protein and add little additional entropic penalty. Reducing the degrees of freedom of a linked compound in solution by appropriately rigidifying the linker will reduce the entropic cost upon binding the linked compound, as compared to a more flexible linker. It is also desirable to reduce the number of rotatable bonds as this has been empirically linked to improved oral bioavailability.[73]

A highly flexible linker may allow a small molecule to undergo a degree of "hydrophobic collapse" in solution, i.e., the intramolecular burial of hydrophobic surfaces. If the "folded up" conformation in solution then does not resemble the bound conformation, there will be an energetic barrier to adopting the correct conformation for binding. In other cases, however, hydrophobic collapse of the solution ground state of a molecule that is similar to its bound conformation may help, as observed in an interesting cis configuration of a urea moiety in AT9283, an Aurora A inhibitor.[74]

Howard et al. linked *p*-chlorophenyltetrazole (IC_{50} = 330 μM) found to bind in S1 of thrombin to an amino alcohol (IC_{50} = 100 μM) bound in an adjacent pocket to produce a series of compounds, the best of which bound with an IC_{50} of 1.4 nM (Figure 5b).[75] Although there are impressive examples of fragment linking, these tend to be the exceptions. Often, it is difficult to find an ideal linker that has the correct attributes of length and rigidity while allowing the joined molecule to adopt the optimal conformation. In a comparative study of fragment linking and growing, Hung et al. noted that the limitations imposed by fragment linking gave fewer opportunities for optimization than incremental fragment growing.[76] Therefore, although impressive when it works, fragment linking is much less common than fragment growing.

Fragment Growing. More frequently, a set of fragments that bind at a single site are discovered and are "grown" through chemical synthesis to explore further interactions. Before investing time and resources into growing a fragment, we should optimize the potency of the fragment. The optimal fragment may have been identified in the original screen or could found by testing related fragments acquired either by minor chemical elaboration or by sourcing available analogues. The choice of which fragments to pursue, however, will be influenced not only by potency but also by ligand efficiency, synthetic tractability, and more general medicinal chemistry considerations.

Structural information is a key aspect for successfully growing a fragment. The interactions the target makes either with known substrates or with existing drug molecules can be instructive with regard to which interactions may be advantageous to make during fragment elaboration. At each stage of design, synthesis, and testing, an evaluation of the degree of success can be made by examining metrics such as the ligand efficiency (LE). Monitoring the LE from fragment to lead compound is a convenient way to objectively assess whether

the extra molecular mass was efficiently added to a fragment. Ideally, this is strictly adhered to throughout the fragment growing exercise, as an LE of worse than 0.3 for a final compound is likely to produce an overly large molecule, with impaired pharmacokinetics. A further development of the concept of LE is a group efficiency (GE) analysis, where the contribution from individual chemical groups within a molecule is assessed.[77]

Incorporating lipophilic groups into lead molecules can be a quick way to improve potency by virtue of the hydrophobic effect. However, this can lead to insoluble, aggregating compounds with likely nonspecific binding and poor bioavailability. Compound handling also becomes much more difficult and SAR more dubious in low-solubility regimes. Increasing potency this way and then incorporating polarity into the molecule at a late stage to make compounds Rule of Five compliant is consequently a poor strategy. Rather, a general strategy for growing fragments into potent leads should be to elaborate a fragment initially by adding polar functionality, to establish hydrogen bonds to the protein, and then to add hydrophobic groups in subsequent growing steps. Although the introduced polarity may not initially produce great increases in potency, solubility, specificity, and pharmacokinetic properties will be improved in the final molecule. Potter et al. elaborated identified fragments through a fragment growing strategy. An initial phenyl imidazole was identified (IC_{50} = 180 μM), and the potency was improved by the addition of a chlorine atom to the phenyl ring and the addition of a benzylic amide group, to give a compound with an IC_{50} of 2.0 μM (Figure 5c).[78]

APPLICATIONS OF FBDD

The majority of biomolecules targeted by FBDD to date have been soluble proteins, but membrane proteins[60] and nucleic acid targets, such as structured mRNA riboswitches,[10] are also amenable. Various recent, novel, and interesting examples are discussed below.

Comparison of Approaches with Hsp90 and BACE-1. Fragment-based approaches have been applied to a wide range of targets, including Hsp90 and BACE-1, which have both been a focus for a number of pharmaceutical companies. Hsp90 is a molecular chaperone that plays a critical role in the activation and stabilization of several proteins.[44,79] It has been shown to facilitate cancer cell survival and growth. One of the first inhibitors of this protein was the antibiotic geldanamycin. This macrocyclic benzoquinone binds to the ATP binding site of the N-terminal domain with a K_D of 1.2 μM.[80] However, geldanamycin has been found to cause severe hepatotoxicity and is metabolically unstable. Consequently, a number of groups have used fragment-based approaches against this target to find novel inhibitors with improved absorption distribution metabolism excretion toxicity (ADMET) properties (Figure 6A).[15−17,81,82] One of the first such studies was described by researchers at Abbott.[81] An NMR-based fragment screen (2D-HSQC) of a library of 11520 fragments was performed on the N-terminal domain of Hsp90. Three fragments that bound with affinities of 10−250 μM were identified. Subsequent fragment elaboration guided by a combination of NMR and X-ray crystallography resulted in the discovery of three series of compounds, one of which bound to the N-terminal domain with a K_I of 60 nM. More recently, both Astex and Vernalis have conducted fragment-based screens against Hsp90.[15−17] Vernalis performed both an NMR and an in silico screen and

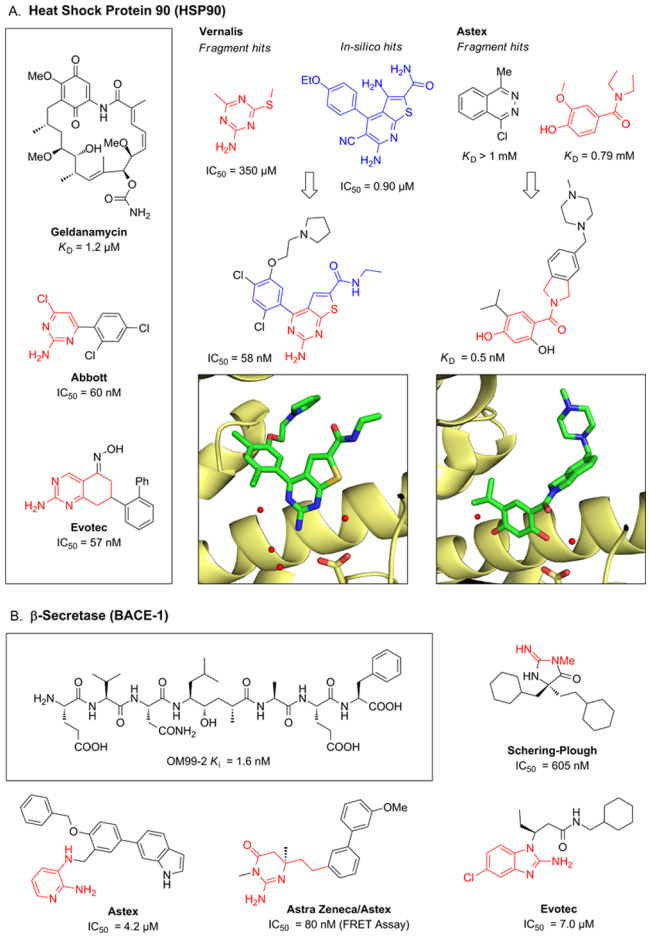

Figure 6. (A) Fragment-based approaches to target Hsp90. The development of fragments into a lead series from Vernalis and Astex is shown [Protein Data Bank (PDB) entries 2WI7 and 2XJX]. The inset shows the natural product geldanamycin and lead molecules derived from fragment hits from Abbott[81] and Evotec.[82] (B) Fragment-based approaches to target β-secretase. Representative molecules for demonstrating the variety of lead series derived from various FBDD programs are shown.[54,83−88] The inset shows the pseudopeptide mimetic OM99-2. Red parts of structures represent original fragments.

obtained a number of fragment hits that bind in the N-terminal domain. The fragment hits were elaborated to give a final compound which had an IC_{50} of 58 nM. Researchers at Astex have also identified a number of fragments against Hsp90 using a combination of NMR screening and high-throughput X-ray crystallography. One of the fragments identified in the screen was the known drug Ethamivan (K_D = 0.79 mM), which bound in the ATP binding site. Subsequent synthetic elaboration of this fragment improved the affinity 1 million-fold with the addition of only six heavy atoms.

Another target that has been of interest to a number of research groups has been the aspartyl protease enzyme β-secretase (BACE-1) (Figure 6B). This enzyme is implicated in Alzheimer's disease, and targeting this enzyme is considered likely to help slow the progression of this disease. Although pseudopeptide mimetics such as OM99-2 are potent against BACE-1, they possess poor pharmacokinetic properties. Researchers at Astex identified a selection of fragment hits using a combination of X-ray crystallography and NMR screening. The hits bound with millimolar affinity and had good ligand efficiency. An in silico screen was then conducted

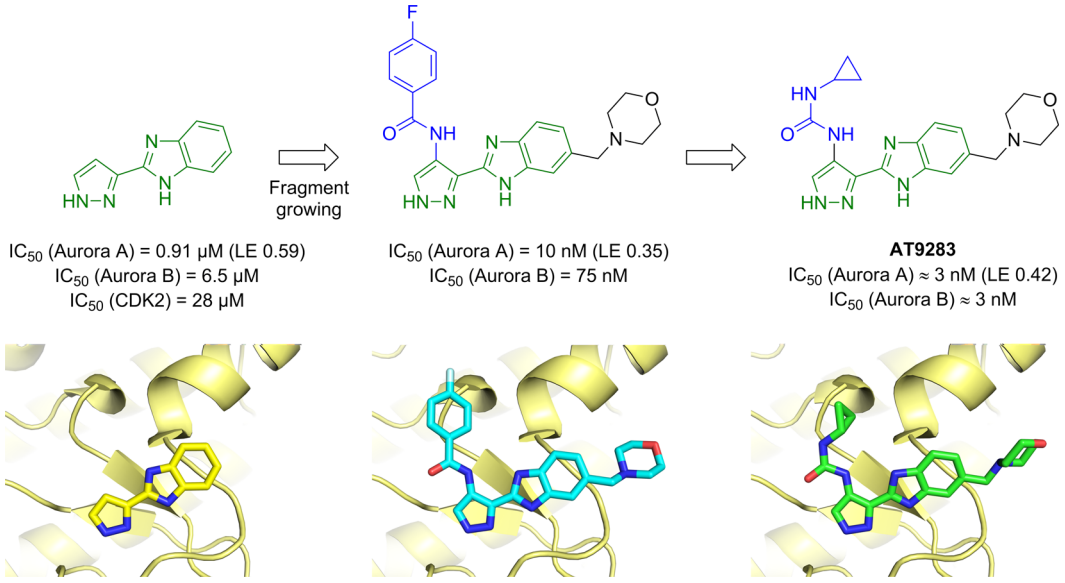

Figure 7. Fragment growing leading to the development of AT9283, an ATP site binding inhibitor of the Aurora A and B kinases (PDB entries 2W1D, 2W1C, and 2W1G).[74]

with analogues of these fragment hits, and more potent fragments that were developed into low-micromolar inhibitors were identified.[54,83,84]

Researchers at Astra Zeneca in collaboration with Astex used a fragment-based approach to target BACE-1 using a combination of NMR, SPR, X-ray crystallography, and a FRET assay. This led to a series of developed inhibitors, the best having an IC_{50} of 80 nM (FRET) and a cellular activity of 470 nM.[85] Evotec has also examined BACE-1 as a target using a fragment-based approach and discovered novel 2-amino-benzimidazole inhibitors that bound with an IC_{50} of 7.0 μM. However, this series of compounds was eventually discontinued because of hERG activity.[86] Researchers at Schering-Plough found a novel amidine-containing heterocycle from an NMR fragment-based screen. From the initial fragment hits, small molecules based upon an iminohydantoin were developed. The most potent of these had an IC_{50} of 605 nM against BACE-1.[87,88]

These fragment-based studies on Hsp90 and BACE-1 have resulted in lead compounds with very different structures and affinities. This illustrates that results from a fragment-based approach are dependent upon a number of factors such as the fragment library used, the screening techniques, and the ingenuity of the medicinal chemists in developing different lead series.

Aurora A and Aurora B Kinase. Aurora A and Aurora B are serine/threonine kinases that play key roles in the regulation of mitosis, making them attractive targets for the treatment of cancer. Howard et al. have reported the discovery of AT9283, a selective inhibitor of Aurora A based upon a pyrazole–benzimidazole fragment that was previously identified in a CDK2 program (Figure 7).[74] The fragment formed new interactions in Aurora A that have no counterpart in CDK2. Examination of the binding mode showed possible vectors of growth from position 4 of pyrazole or position 6 of benzimidazole. Introduction of a *p*-fluorobenzamide and a weakly basic *N*-methylene morpholine motif gave excellent affinity for both Aurora A and B but poor pharmacokinetic properties. Optimization of the *p*-fluorophenyl group to a urea-linked cyclopropyl group AT9283 gave the best combination of in vitro affinity, druglike physicochemical properties, a mouse pharmacokinetic profile, and anti-HCT116 carcinoma cell activity. AT9283 has progressed into clinical trials for the treatment of metastatic solid tumors and hematological malignancies.[89]

FBDD: An Academic Approach. While fragment-based drug discovery has been primarily developed in the pharmaceutical industry, a number of research groups in academia have now adapted this approach as a method for the development of lead compounds and chemical tools. Unlike high-throughput screening libraries, fragment libraries are commercially available and are within the budget of academic research groups. In academia, there is the possibility of using fragment-based approaches to study targets that are deemed difficult or not of primary interest to the mainstream pharmaceutical industry.

Abell et al. have explored a number of tuberculosis targets using the fragment-based approach. In a study of *Mycobacterium tuberculosis* pantothenate synthetase (PS), a fragment screen was conducted using a combination of thermal shift, NMR, and X-ray crystallography.[76] A number of fragments were found to bind in the ATP pocket of PS (Figure 8A). Two fragments, benzofuran-2-carboxylic acid and 5-methoxyindole, were found to bind in non-overlapping pockets of the enzyme simultaneously, each with a K_D of ~1.0 mM. These two fragments were linked with an acylsulfonamide linker to give a compound with a K_D of 2 μM. An overlay of the X-ray crystal structures of the fragments with the linked compound revealed an excellent alignment of the original fragment binding modes (Figure 8A).

Drinkwater and co-workers used a fragment-based approach against phenylethanolamine *N*-methyltransferase (PNMT), the enzyme that catalyzes the final step of the biosynthesis of adrenaline (Figure 8B).[51] Fragment screening was conducted using X-ray crystallography in the presence of the product *S*-adenosyl-L-homocysteine (AdoHcy). The screening was performed in cocktails of four fragments, and 12 fragments were found to bind in the noradrenaline binding site. The binding of these fragment hits was further validated by ITC. Mass spectrometry (ESI-MS) was conducted on 12 of the 384

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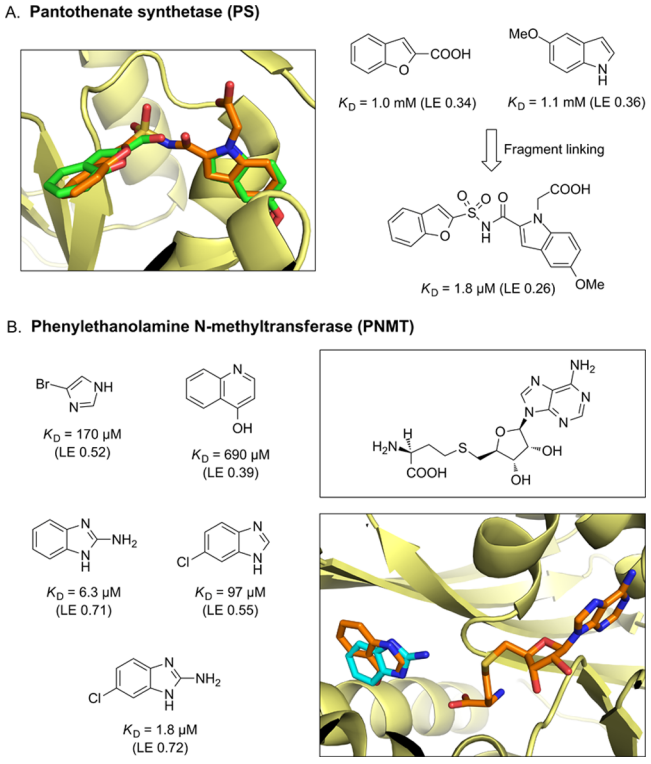

Figure 8. (A) Fragments binding to the ATP binding pocket of *M. tuberculosis* pantothenate synthetase shown as green sticks (PDB entry 3IGM).[76] The synthetically elaborated compound (orange) is derived from the two fragments and linked with an acyl sulfonamide (PDB entry 3IVX). (B) Fragment binding to PNMT with the cofactor AdoHcy (orange) (PDB entries 3KQM, 3KPU, and 3KQS).[51] A selection of the fragments found using X-ray crystallography and mass spectrometry is also shown.

fragments from the library. Of these, four were observed to bind to PNMT.

Other academic groups have used fragment-based approaches in novel ways to enhance the fragment hit rate and apply this methodology to new and unusual targets. Cohen et al. examined a focused library of fragments that were designed to bind metals to target metalloenzymes. Screening against five matrix metalloprotease classes, they found a high hit rate of 30−40%, demonstrating that using a focused library can significantly enhance the hit rate.[90]

Fragment-based approaches have also been reported on oligonucleotide targets. For example, equilibrium dialysis was used as a primary screen to identify fragments that bound to the *Escherichia coli* thiM riboswitch. Riboswitches are regions on mRNA that directly bind metabolites leading to alteration of gene expression and are found in many prokaryotic and some eukaryotic organisms. As they control essential metabolic pathways, they are potential candidates for modulation by small molecules. The *E. coli* thiM riboswitch is involved in thiamine pyrophosphate biosynthesis. A fragment library of 1300 fragments was screened by equilibrium dialysis in cocktails of five using radiolabeled [^{3}H]thiamine as a reporter ligand. After the hits had been deconvoluted, 20 fragments were identified, and these were confirmed using WaterLOGSY NMR. Seventeen fragments were shown by ITC to have a K_D in the range of 22−280 μM.[10,11]

NEW AND FUTURE TARGETS

Fragment-based approaches have become an accepted part of early stage drug discovery in industry and academia. There is a desire to push the methodology, e.g., to find allosteric inhibitors, or to use it on systems where structural information is less available, e.g., GPCRs and ion channels.

Jahnke et al. performed a fragment screen against farnesyl pyrophosphate synthase (FPPS) (Figure 9A).[91] Nitrogen-

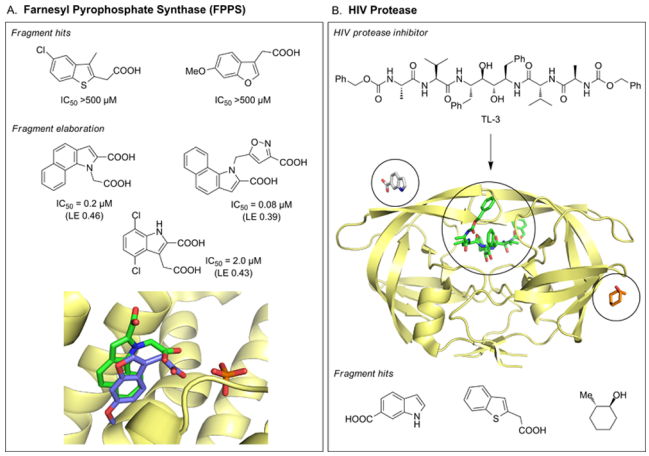

Figure 9. (A) Fragment screening against farnesyl pyrophosphate synthase (FPSS) showing fragment hits and subsequent fragment elaboration.[91] Overlay of X-ray crystal structures showing representative fragments bound (PDB entries 3N3L and 3N6K). (B) HIV protease dimer with TL-3 bound in the active site and 2-methylcyclohexanol in the exosite. Indole-6-carboxylic acid and benzothiopheneacetic acid bind in another surface pocket (PDB entries 3KFN and 3KFR).[93]

containing bisphosphonates inhibit this enzyme and block excessive bone resorption. These bisphosphonates are potent inhibitors of FPPS and used in the treatment of osteoporosis, Paget's disease, and tumor-induced osteolysis. The fragment library was screened by WaterLOGSY NMR experiments. From a library of 400 fragments, four hits were identified. X-ray crystallography revealed that the four fragments bound outside the active site, in an allosteric pocket near the C-terminus. To investigate this further, they screened a focused small library of 40 fragment analogues. From sequential NMR displacement experiments, they then identified fragments of increasing potency with IC_{50} values in the low micromolar region (2−12 μM). Synthetic elaboration of these fragments led to a compound with an IC_{50} of 80 nM. This is the first reported non-bisphosphonate inhibitor of FPPS and also binds in a previously unknown allosteric binding site. This allosteric pocket has the potential to be utilized in a new generation of drugs that have potential antitumor effects in soft tissue.

The pharmaceutical industry has had good success in developing small molecule drugs to target HIV.[92] However, the emergence of resistant strains means that new therapeutics are still needed. Stout et al. have reported a fragment-based approach against wild-type (NL4-3) HIV protease (Figure 9B).[93] Using a commercially available fragment library, 96 cocktails of four fragments were screened by X-ray crystallography in the presence of the known HIV protease inhibitor TL-3, to find novel sites and to allosterically trap the inactive protein conformation. Three of the identified fragments were found to bind to the HIV protease binding site and induce a

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distinct change in the conformation in the presence of TL-3. The fragment 2-methylcyclohexanol bound in an exosite that picks up a hydrogen bond to Gly17 of the protein backbone and hydrophobic interactions with Lys15 and Leu63. Two other fragments, indole-6-carboxylic acid and 2-benzothiopheneacetic acid, bound at a different site on the "outside/top of the flap" of the HIV protease.

A different approach involved comparative fragment binding studies against a target enzyme and important mutants. Danielson et al. used a fragment-based screening approach for HIV-1 reverse transcriptase using SPR as the primary screening method.[94] Ten fragments were identified and their affinities measured against the wild type and three drug resistant enzyme variants (K103N, Y181C, and L100I). One fragment, a bromoindanone, exhibited submillimolar (<300 μM) K_D and IC_{50} values with activity against all four proteins. An examination of current non-nucleoside reverse transcriptase inhibitors (NNRTI) showed that the fragment structure was novel. The bromoindanone fragment bound competitively with the NNRTI drug Nevirapine, suggesting a common binding site, whereas the remaining fragments did not compete with Nevirapine.

Forty percent of all marketed drugs target G-protein-coupled receptors (GPCRs).[95,96] Because of the inherent hydrophobicity, low natural abundance, and conformational flexibility of these transmembrane signaling proteins, it has proven to be extremely difficult to express and isolate soluble GPCRs in stable, functionally folded forms.[97] However, technological advances in the field have recently led to the generation of stable, detergent-soluble mutants giving high-resolution crystal structures in complex with agonists or antagonists to increase conformational homogeneity.[97−103] The majority of these mutants involve a combination of point mutations and deletions at highly flexible regions or fusion of the receptor with T4 bacteriophage lysozyme at the position of its third intracellular loop. In a first proof-of-concept study, Congreve et al.[60] successfully performed fragment screening on human adenosine A_{2A} and avian β_1-adrenergic stabilized receptors, which carry point mutations to stabilize the receptor into a single antagonist-binding conformation. A small fragment library was screened by SPR and target-immobilized NMR screening (TINS), and in both cases, the immobilized receptors remained active and reusable throughout the assay. A control screen was also run in parallel with an alternative membrane protein, to remove false positives caused by nonspecific binding. Several fragment hits were identified for each GPCR, with K_D values ranging from 10 μM to 5 mM for A_{2A} and IC_{50} values ranging from 5 μM to 5 mM for the avian β_1-adrenergic stabilized receptor. Efforts to obtain fragment-bound crystal structures are now ongoing, and it is envisaged that fragment screening methods will allow discovery of new binding sites that could be missed by traditional radioligand displacement assays. This study is an important step toward the development of potent and selective GPCR agonists and antagonists using FBDD.

LIMITATIONS OF FBDD

FBDD is not a "one size fits all" solution for the problems of drug discovery against any given target. The low potency of fragments means they are unsuitable for whole cell screening and also for kinetic assays, where a millimolar potency would necessitate high fragment concentrations and result in false positives. In our laboratory, some targets have proven to be less amenable to the FBDD methodology than others, with some fragment programs yielding no hits. It is interesting to speculate briefly, on a case-by-case basis, why some targets seem resistant to fragment binding. For example, we were unable to produce any fully validated fragment hits against the potential antibacterial target shikimate kinase from *M. tuberculosis* (K. Bromfield, D. Osborne, S. Surade, T. L. Blundell, and C. Abell, unpublished work). The enzyme binds ATP and shikimate in a shallow groove-like binding site and catalyzes the phosphorylation of the substrate to produce ADP and shikimate 3-phosphate. Most fragments that seemed to bind were negatively charged, but none were displaced in control experiments with the natural ligands. Interestingly, the protein possesses a distal surface from the active site, which is comprised of 11 positively charged residues.[104] It is possible that the anionic fragment hits might be binding nonspecifically to this positively charged surface, masking any well-behaved displacement from the active site. Regardless of the nature of the ligand binding site, the presence of a highly charged nonspecific patch on proteins may frustrate the ability to biophysically validate fragments. In other work, a fragment screening program against the Polo Box Domain (PBD) of anticancer target Plk1 failed to find fragment hits (P. Sledz, C. Stubbs, S. Lang, Y.-Q. Yang, and C. Abell, unpublished work). The PBD recognizes a phosphoserine or phosphothreonine motif, and it was this interaction that was anticipated to be a binding "hot spot". However, phosphate mimetics are known to offer challenges of their own in drug discovery, and this binding site in PBD is also characterized by an extensive network of waters that may form an ice-like barrier and prevent binding of the fragment to the protein.[105] At the other end of the spectrum, traditionally very challenging targets such as oligonucleotides and protein−protein interactions can be targeted to give fragments from which chemical elaboration can begin.[10,45]

CONCLUSIONS

The approval of the first drug to be discovered by the fragment-based approach, Zelboraf (PLX4032), has been an important milestone for this methodology.[1,2] Currently, there are a large number of drugs in clinical development that were discovered using fragment-based approaches. These approaches are evolving, being used with increasing frequency by both academic and industrial groups. Some of the most significant areas where research is focused are fragment library design, expanded target selection, and higher-throughput biophysical methods of screening.

In the past decade, there have been major leaps in methodology for fragment screening using X-ray crystallography and NMR spectroscopy. More recently, surface plasmon resonance has become more widely used for fragment screening. However, the field would still benefit from the development of more high-throughput methods, where the screening of libraries could be conducted in hours as opposed to days or weeks. Improvements could also be made in the composition of fragment libraries. Until recently, libraries have generally been either devised in-house or purchased from commercial suppliers. The structural makeup of these has been biased toward small flat heterocyclic molecules.[106] However, there is now a concerted effort to broaden the structural diversity of fragment libraries, e.g., by including more three-dimensional fragments.[23,29,30,32,34] It is hoped that these libraries may prove to be more successful for screening difficult targets such as protein−protein interactions.

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The initial focus of fragment-based drug discovery was kinases. In recent years, however, the target range has diversified to include *inter alia* metalloproteins and ion channels. There is the potential to expand to new targets in areas such as epigenetics,[107,108] multiprotein complexes, and DNA−RNA complexes, which may provide new starting points for the next generation of therapeutics and tools for chemical biology.

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Notes

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