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Fragment-Based Lead Discovery and Design

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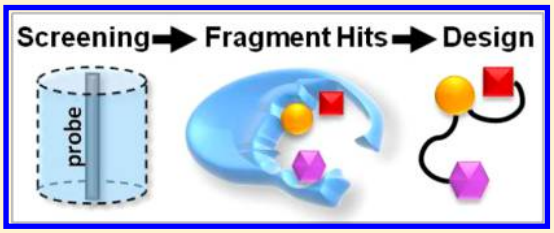
ABSTRACT: Fragment-based lead discovery and design has and continues to show increasing promise in drug discovery. In this article, the current state of the art in terms of hot-spot characterization, fragment screening techniques, and fragment-based design is discussed. Three overall fragment-based lead generation strategies are explored and involve the chemical biology characterization of biological targets via fragment screening, fragment screening as a complementary approach to high-throughput screening of drug-like compounds, and direct fragment-based drug discovery, respectively. The evolution and development of fragment libraries is described. With an emphasis on computational approaches and the strategies applied at AstraZeneca, the review illustrates how integration of data from one regime can inform the design of experiments in the other, ultimately leading to the discovery of high quality chemical matter.



Fragment-based lead generation (FBLG) encompasses a broad range of methodologies and strategies for discovering small, organic fragments with desired bioactivities. While there is no standard FBLG approach, the characteristics and properties of small organic fragments serve to define the field to some extent. Fragments possess a number of attractive features from the perspective of drug discovery. They have low molecular weights and are selected to be more polar and soluble than larger "drug-like" compounds; these characteristics are often thought to translate into compounds with favorable physical properties, pharmacokinetics/bioavailability, and ADME/toxicology properties. From a cheminformatics perspective, they tend to be of lower chemical complexity than larger compounds, which can simplify the interpretation of structure−activity relationship (SAR) data and consequently accelerate optimization. The principal disadvantage of fragments is that they most often bind with low affinity to a protein target, requiring physical screening approaches to use high concentrations and large amounts of material.

The low-affinity binding of a fragment to a protein target involves fewer protein−ligand interactions than the binding of a larger, drug-like compound, and consequently, most common FBLG strategies employ significant biophysical and structural characterization of fragment binding at the very early stages of the lead discovery process. Generally, FBLG yields higher content data at a much lower throughput than traditional high-throughput biochemical screening of larger, drug-like compounds. The low throughput/high information content nature of FBLG presents challenges to effective integration of FBLG into drug discovery programs. The key to successfully utilizing FBLG in drug discovery is understanding how to use data obtained from biophysical, biostructural, and biochemical approaches in lead identification and optimization. Computational tools and approaches can play a particularly important role in building models that integrate data from these sources to guide decision-making.

In this article, we review the common methodologies used in fragment screening and present them in the context of the application of FBLG strategies to drug discovery programs. We emphasize the critical component of computational approaches in designing and interpreting FBLG experiments.

FRAGMENT BINDING: REVEALING PROTEIN HOT-SPOTS, WHICH DRIVE HIGH-AFFINITY LIGAND BINDING

Protein−ligand binding is comprised of widely varying contributions from different atoms and functional groups on the ligand; often, certain atoms or groups contribute much more toward the free energy of binding than others. This phenomenon—described as an "activity cliff"—often confounds SAR analysis and has motivated significant study and method development.[1] Studies of protein−protein interfaces also reveal highly variable contributions to the free energy of binding from different residues, often with a relatively small number of "hot-spot" residues responsible for the vast majority of the binding free energy.[2,3] Taken together, these findings imply that ligands which bind with high affinity to their protein target are optimally exploiting interactions with protein hot-spots by properly orienting key functional groups in the right regions of the binding site. Activity cliffs result from changes in the ligand that incorporate or eliminate a hot-spot interaction, thus leading to a large change in the free energy of binding.

FBLG approaches are intrinsically well-matched to the hot-spot-driven model of protein−ligand binding, as fragments often exploit strong interactions with hot-spots that overcome the limitations of low molecular weight. Many of the perceived advantages of FBLG approaches—fragment leads with increased polarity and better physical properties than traditional HTS hits—derive from the high ligand efficiency (binding free energy per ligand heavy atom) demonstrated by fragments that

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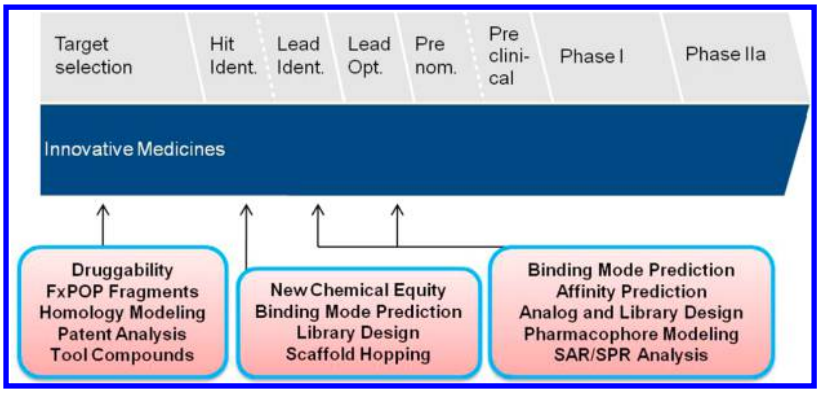

Figure 1. Potential impact of virtual screening across the drug discovery value chain.

bind to hot-spots. A major challenge for the field has been developing methods to exploit this information about fragment–hot-spot binding to generate drug-like leads without losing too much ligand efficiency and favorable physical properties. As the field has matured, a diverse range of FBLG strategies have emerged that utilize a variety of types of fragment binding data. Here, we describe common experimental and computational methods and the FBLG strategies that employ them.

COMPUTATIONAL PREDICTION OF FRAGMENT BINDING

Tools for Predicting Fragment Binding. Computational fragment positioning methods actually predated the experimental approaches with GRID in 1985[4] followed by MCSS,[5,6] LUDI,[7] Sprout,[8] Superstar,[9] and MUSIC[10] in the early 1990s. Most of these methods are force field based approaches that rely on molecular mechanics models of protein–ligand interactions to predict bound fragment positions. In contrast, Superstar is a knowledge-based computational approach for identifying hot spots in a protein structure, which uses a database of frequently occurring intermolecular interactions observed in X-ray crystal structures to predict which functional groups have high propensity to bind in a protein pocket.[9] As this area of computational drug design has evolved over the last twenty years, enhanced fragment positioning methods have emerged that employ more sophisticated force fields. A recent example of this is the 3D-RISM approach,[11] which generates the most probable binding modes for ligands and involves the use of the 3D reference interaction site model theory of solvation. Another example is the FTMap method that generates consensus sites or regions of the protein surface where clusters of different fragment types overlap and uses a fast Fourier transform correlation approach and the analytical continuum electrostatic (ACE) model for solvation.[12] The FTMap approach has recently been employed to detect hot spots for DJ-1, a Parkinson disease target.[13]

Virtual Fragment Screening. Virtual screening, in general, is typically employed in the earlier stages of a drug discovery project to identify tool compounds and potential leads and in the later stages of a project for lead optimization and scaffold hopping (see Figure 1).

For large-scale virtual fragment screening, standard molecular docking techniques are often used to screen large molecular databases to identify those fragments likely to bind to the target.[14] Ligand-based screening or a combination of structure- and ligand-based screening can also be used if there is a known ligand or series of ligands for the target. Most molecular docking programs use a hierarchical scoring scheme, whereby a simple shape-based scoring function is applied to the entire database as a fast filter to rule out those compounds that are sterically incompatible with the binding site. Progressively more rigorous and time-consuming scoring functions are then applied to the filtered hits to account for effects such as solvation and entropy,[15,16] thereby producing the final list of (hopefully) more accurately ranked compounds. In this manner, databases of millions of molecules can be screened rapidly.

Most docking methods that are currently utilized for fragment screening have been optimized for screening larger, lead-like, small molecules, raising the concern that the force fields and scoring functions they rely upon are mismatched to the problem of fragment docking. In addition, fragment hits are expected to have low initial specificity, as was demonstrated for CTX-M β-lactamase.[17] To address these concerns, there have been several studies investigating how well these approaches perform for virtually screening fragment libraries.[18,19] For fragment docking, as for docking in general, accounting for solvation can be important but difficult to do, protein flexibility can play a role, and docking accuracy generally decreases for weaker binding ligands. In spite of these limitations, studies have shown that the enrichment rates for docking fragment compounds can be similar to those generally observed for docking lead-like compounds, where, to evaluate the success of a VS approach, the enrichment of actives found in the set of experimentally tested compounds is compared to that for random screening (i.e., HTS). Therefore, these studies indicate that VS of fragment libraries can identify leads, though those leads may likely be of lower binding affinity than HTS-derived leads. When structural information on a target is used for VS, the enriched actives are expected to be those capable of binding to the conformation represented by that structure used. While, if known ligands are used for VS, the results will be necessarily be more biased toward compounds with chemical similarity. A recent example discusses the use of structure-based docking in combination with specific known ligand information to identify fragment hits for a histamine H1 receptor,[20] a G protein-coupled receptor (GPCR), a particularly difficult but important class of membrane-bound targets.

EXPERIMENTAL CHARACTERIZATION OF FRAGMENT BINDING

NMR Approaches for Fragment Screening. The results of a virtual fragment screen are sometimes funneled into a

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nuclear magnetic resonance spectroscopy (NMR) screen for experimental testing alongside a generic library. NMR has become a preferred method for identifying fragments that specifically bind to a protein or nucleic acid target of interest.[21] At AstraZeneca, we utilize NMR both for fragment screening as well as for the confirmation of fragment hits identified through high concentration screening (HCS). Either 1D ligand-observe or two-dimensional (2D) protein-observe NMR experiments can be employed for fragment screening. The advantages of using a 1D NMR approach are that the protein does not need to be labeled, a relatively small amount of protein is required, and library samples do not need to be in deuterated DMSO. A competing compound is needed to demonstrate that the observed fragment binding is specific as well as to determine subsequently ligand binding constants. The advantage of 2D NMR is that ligand binding constants can be directly obtained for fragments. This method, however, requires much more protein, the protein must be isotopically labeled, and some proteins do not yield a useful 2D spectrum. As a result, we generally employ 1D NMR when possible.

An automated NMR system is required for fragment screening. In a typical setup, a superconducting magnet encloses a cryogenic probe in its center. Although higher strength magnets will increase sensitivity and reduce experiment time,[22,23] an economic and highly efficient screening system can be built with a 600 MHz magnet. A fully automated system allows continuous preparation and analysis of typically 96 samples, which can contain up to 10 compounds per sample. Depending on the experiment time (typically 3−15 min), up to 1000 fragments can be tested in 24 h. Although automated spectral processing is available, data analysis and interpretation still remains the limiting step in using NMR for FBLG.

1D NMR. Ligand-observed NMR experiments are governed by chemical exchange.[22,23] In general, to observe a change in the spectrum upon ligand binding, the exchange rate must be much faster than the relaxation rate of the observed nucleus in the bound ligand, and for weak binders such as fragments, this is generally true. Common 1D NMR techniques used for fragment screening include relaxation edited 1D NMR,[24] water-ligand observed via gradient spectroscopy (WaterLOGSY),[25] and saturation transfer difference (STD) spectroscopy.[25,26] In general, ligand binding to a macromolecule translates into shortened relaxation properties of the complexed relative to the free ligand. In relaxation-edited 1D NMR, this effect is exploited by applying a $T_{1\rho}$ (longitudinal relaxation) or T_2 (transverse relaxation) filter in the pulse sequence. For fragments which bind to the target, there is a loss in signal intensity in the NMR spectrum, while for fragments which do not, there is no change in signal intensities (Figure 2A). Prior to running a screen, the spectrum of a weak competitor compound is observed with and without the protein present. The screen is then run in the presence of the competitor compound, and if a sample fragment competes with the binding and thus displaces the competitor, the peaks for the competitor compound reappear. Other techniques exploit "cross-relaxation" effects where, upon ligand binding, the change of polarization of one partner affects the polarization of the other. The WaterLOGSY technique utilizes bulk water magnetization that is transferred to the ligand. The cross-relaxation rate of dipole−dipole interaction between water and ligand is positive for free ligand and negative for bound ligands that are in proximity of water molecules associated with the

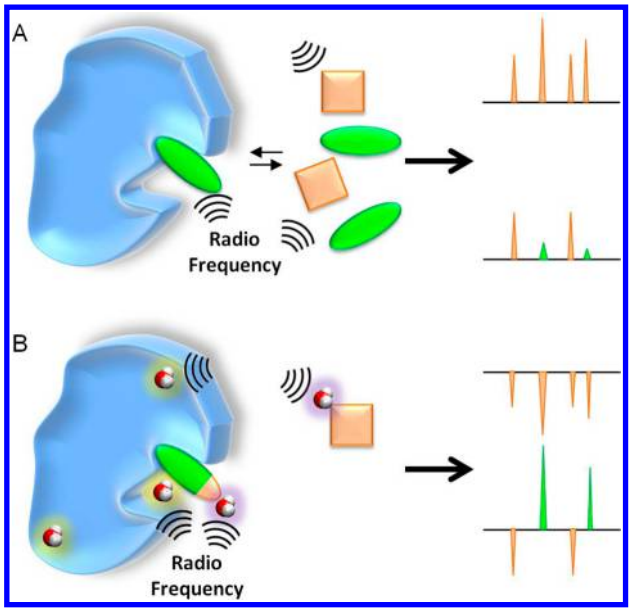

Figure 2. Schematic of typical 1D ^{1}H NMR experiments used to detect ligand binding: (A) relaxation edited NMR such as $T_{1\rho}$ or T_2 experiments and (B) WaterLOGSY experiments. Orange peaks represent free ligand signals, while green peaks are derived from binders. In each case, the top spectrum is for the ligand sample in the absence of the target, and the bottom spectrum illustrates a possible outcome in the presence of the target. Waters depicted in a purple cloud are fast tumbling while those in a yellow cloud are slow tumbling.

macromolecule. This sign difference of cross-relaxation rate translates into a phase difference in the WaterLOGSY NMR spectrum for free and bound ligands (Figure 2B). In STD experiments, the macromolecular target is continuously and selectively irradiated by radio frequency pulses. This irradiation saturates the magnetization of the whole target. This saturation is then transferred through space to the closely bound ligand; whereas, the free ligand is not affected. The final "difference spectrum" is obtained by subtracting the spectrum obtained with the magnetization saturated target from the spectrum obtained with the nonirradiated target.

While relaxation edited 1D NMR experiments exhibit the highest sensitivity, WaterLOGSY experiments are more reliable for monitoring ligand binding.[25] As such, when we carry out an NMR fragment screen for a given target, we generally apply both techniques to screen the library. The results of the two experiments combine to minimize false negative hits and allow good sensitivity for detection of weak binders. STD is the least sensitive of the described methods, and therefore, we do not routinely use this approach for fragment screening. A deuterated buffer is required for optimal sensitivity with STD, which prohibits its application in screening compound libraries, because the automation system uses H_2O for washing steps. Similarly to WaterLOGSY, however, STD can differentiate between the parts of a ligand that are in proximity to residues of the target versus those that are solvent exposed.[27]

Protein-Observed NMR. 2D NMR methods detect protein signal changes upon ligand binding. To overcome the signal overlap and line width broadening in proteins, a second dimension is achieved by ^{15}N or ^{13}C labeling of the protein.[28,29] Widely used 2D protein NMR experiments are the ^{1}H−^{15}N heteronuclear single quantum coherence spectroscopy (HSQC) and the more sensitive ^{1}H−^{13}C heteronuclear

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multiple quantum coherence spectroscopy (HMQC).[22] The HSQC pulse sequence has been further optimized to detect signals in larger proteins and is called TROSY.[30] These types of 2D NMR experiments provide a "fingerprint" of the target protein and resonances are very sensitive to changes in pH, temperature, salt concentration, or ligand binding.

Use of 1D NMR at AstraZeneca. Like enzyme assays, NMR experiments can probe fragment binding to all relevant conformations of a target protein accommodated during catalysis. 1D-NMR methods, however, allow detection of weak binders at low fragment concentrations of 50−200 μM, compared to the millimolar concentrations typical for enzyme assays, and therefore have a lower tendency to produce false positives or negative hits. When combined with competition experiments using a characterized binder, even the binding site can be confirmed and fragment affinities obtained. In cases where adequate assays cannot be established or where a certain enzyme form is targeted, NMR can become an efficient screening method to identify novel fragment hits.

At AstraZeneca in Infection, as mentioned above, we commonly use a combination of relaxation edited 1D NMR and WaterLOGSY experiments for efficient fragment screening. Libraries containing mixtures of 5−10 characterized compounds are screened. The mixtures are assembled so that each compound has at least one unique signal in the spectrum, and therefore deconvolution is not required to identify the binding fragments.

We employ 2D NMR approaches for fragment screening only for small proteins for which a gram or more can be readily labeled and purified. This requirement is satisfied for about 30% of the targets we study. 2D NMR, however, is often applied to confirm a smaller, focused set of hits from an HCS assay or from a 1D NMR screen instead of carrying out competition experiments. In particular, if a target has multiple binding sites, 2D NMR may be used to determine the precise pocket to which the fragment hit is binding.

Surface Plasmon Resonance and Other Biophysical Screening Methods. In addition to HCS, NMR, and X-ray crystallography, surface plasmon resonance (SPR), isothermal calorimetry (ITC), and mass spectroscopy (MS) have been applied successfully for FBLG.[31,32] Although SPR and MS can reach or even exceed the throughput achieved by 1D NMR, these technologies are more prone to artifacts since, in contrast to 1D NMR, they require high compound concentrations to detect weak binding. The SPR instrument with the highest throughput is the Corning EPIC OWG platform, which can observe binding simultaneously for 384 compounds in a few minutes. Therefore, this system is capable of screening tens of thousands of compounds in a reasonable time frame. With any SPR experiment, suitable conditions for either target or compound immobilization need to be determined. About 60% of the target proteins we have tested have been immobilized successfully on an SPR chip, such that they retained the ability to bind ligands. About 25% of target definition compounds (TDC) − ligands specially designed to be immobilized, we have tested, retain the ability to bind their receptors. When possible, the use of an immobilized TDC is preferable to the use of an immobilized receptor, in part due to the fact that the SPR signal generated by receptor binding to the surface is much larger than that generated by small molecule binding. In addition, lower compound concentrations are required to compete the receptor off the immobilized TDC, than are needed to measure binding of the compound to the immobilized receptor. Overall, SPR is a medium throughput method that yields binding affinities. Once immobilization protocols are validated, the method has high sensitivity with low protein and ligand consumption. However, developing these protocols can be time-consuming and may require substantial amounts of protein. An area where SPR may prove particularly useful in the future is in identifying and confirming fragment hits for membrane bound proteins such as GPCRs,[33] which represent a significant fraction of druggable targets.

X-Ray Crystallography and Automation. Fragment hits from virtual screening, HCS, and various biophysical screening approaches are eventually filtered through to X-ray crystallography to determine the 3D structure of any fragment hits bound to the target protein. In general, fragment hits are followed up on as leads and progressed into chemical optimization, if structural information is available to help with the design. In Infection at AstraZeneca, we have utilized a fragment-based crystallography proof of principle (fxPOP) in order to prosecute fragment hits.[117] The idea is that for a given target, substrate-like fragments are screened using the high concentration biochemical assay; any hits are then soaked into the crystal system and, in a successful fxPOP, the structure of at least one fragment bound to the target is solved. Virtual screening can be used to help select the fragment-like hits to increase the chances of success. Fragments of the substrate are used as queries for a ligand-based virtual screen. The top ranked hits from those searches, the ones most similar to the substrate fragments in terms of shape and pharmacophore features, are then docked into the target structure. The top hits from the structure-based screen are then selected for experimental testing. The most potent hits are tried first in crystallography soaking or cocrystallization experiments. A structure of one of the fragment hits bound to the target indicates that the target is likely to be amenable to a fragment-based approach.

Standard crystallography laboratories today use highly automated robots for mounting and retrieving crystals, allowing for rapid crystallography trials for active fragments. High-throughput X-ray crystallography involving a high degree of automation and robotics can also be used as a technique for fragment screening itself. Several years ago, Hartshorn et al. applied the approach to five targets and the results showed that different interaction types can drive fragment binding and that protein movement can be induced by fragments.[34] Typically mixtures of fragments are soaked into preformed crystals of the target. With the Astex Pyramid approach, described in detail in a recent review,[35] the time for acquiring protein−fragment complex structures is shortened, which is key to the use of crystallography as a screening technique, by implementing automated protocols and having a well-designed fragment library and informatics infrastructure. Astex typically screens mixtures of four fragments that are diverse in shape. The mixtures are generated by a computational method that minimizes chemical similarity. Others have described similar approaches for generating shape diverse mixtures[36,37] that allow for faster deconvolution. For any mixture of fragment hits, the electron density represents the average structure of the fragments in the mixture that bind to the target.

Alternatively, researchers at Johnson & Johnson have applied an orthogonal approach of screening mixtures of shape similar fragments.[38] They use the envelope of the electron density from each mixture that hits (has at least one fragment that binds) to then design a second library to screen. The second

library is intended to probe the boundaries of the envelope. It is estimated that with the current technology, they can screen 1000 fragments in mixtures of five in about 3 months and design, synthesize, and screen a second library of about 300 fragments in another 5 months, ultimately obtaining lead-like hits within about 13 months.

Obtaining Structural Information without Crystallography. Several emerging ligand-detected (1D) NMR techniques are being successfully used to obtain structural information for drug design in the absence of the availability of a full 3D structure of the protein or protein ligand-complex by crystallography or NMR. Examples include **S**tructural information, **O**verhauser effects, and **S**elective labeling STD (SOS-STD),[27] **in**terligand NOEs for **pharma**cophore mapping (INPHARMA),[39,40] and **i**nterligand **O**verhauser **e**ffect NMR (ILOE-NMR).[41,42] SOS-STD is a special STD method that employs perdeuterated targets with selectively protonated amino acid residues. As such, SOS-STD can provide some structural information for the complex and does not require protein resonance assignments and thus many large-sized proteins can be investigated. INPHARMA can be used to determine the pharmacophoric overlay of a pair of ligands that bind competitively and weakly and may prove to be particularly useful for GPCR targets. Researchers at the Max Planck Institute, EMBL, and Sanofi-Aventis together described the application of the method to GPR40, for the determination of bioactive conformations of ligands and cross-chemotype alignments.[43] The method is fast and requires only small amounts of crude membrane extracts containing the receptor. With the ILOE-NMR approach, small fragments that bind to the protein in the vicinity of a known binder are identified. The approach enables medicinal chemistry to be guided with directional information on how to expand the known ligand. ILOE-NMR has been applied to Bid, a proapoptotic member of the Bcl-2 family,[41] and pantothenate synthase (PtS).[42] For Bid, two millimolar fragments that bind in adjacent sites on the protein surface were identified and subsequently linked through the systematic synthesis of bidentate compounds to identify a low micromolar inhibitor (K_d 1.5 μM).

FBLG STRATEGIES

There are several ways in which FBLG is most often applied to drug discovery projects.

(I) Chemical biology exploration of biological targets: Fragment screening techniques have been applied to identify binding sites and to map out pharmacophores for receptor binding.

(II) Complementary to high-throughput biochemical screening of drug-like compounds: Fragment screening hits can be used to bias HTS libraries or to guide optimization of drug-like lead compounds.

(III) "Pure" FBLG for drug discovery: Fragment screening identifies leads which can be optimized (via linking, growing, etc.) to yield drug-like compounds.

(I) Chemical Biology Exploration of Targets. Both experimentally determined and calculated fragment positions can reveal hot-spot interactions and favorable chemotypes that can inform and guide hit discovery and lead optimization. For example, Ringe and Petsko showed that crystallography could be used successfully to map the binding modes of organic fragments and identify hot spots as those sites where bound fragment positions cluster.[44] Using their approach, called MSCS (multiple solvent crystal structures), protein crystals are cross-linked to increase their stability and solvent is then soaked into the crystals to obtain the structure of the protein with bound solvent molecules (such as acetonitrile, benzene, etc.) in sites not occupied by water molecules.[45] Fragment positions obtained by experimental methods such as MSCS, or through fragment docking, can be used to map out a target binding site to identify hot-spots and to define potential pharmacophores for the target. These pharmacophores can then be used to search a database to generate new leads for the target. Fragment positions can also be used more directly to help optimize an existing lead by merging or substituting the fragment onto the known scaffold for the target. Furthermore, fragment docking can be used to create a target-focused subset or library of fragments or small molecules for experimental testing.

In addition to these direct uses of fragment positions for inhibitor design, there are also a number of indirect or more "fuzzy" uses. One involves using the data from relatively small scale fragment screens to assess the druggability of a given target. Hajduk and co-workers have done pioneering work in this area.[46] Their published model including terms for polar and apolar surface area, surface complexity, and pocket dimensions is able to predict experimental screening hit rates. In addition, Gupta et al. at AstraZeneca in Bangalore, have developed QSAR models based on structural parameters to be used to prioritize targets for HTS screens.[47]

Another indirect use of fragment positions is to enable pharmacophore searches or to perform pharmacophore-based molecular docking. Previously, it has been shown that pharmacophore-based molecular docking leads to a greater retention of reasonable poses, or poses with better chemical matching to the site, and a greater enrichment of known actives in the hit list compared to docking without constraints.[48,49] Furthermore, hot-spots determined by fragment screening can be used to design combinatorial libraries of lead-like molecules. As an example, for HLA-DR4 computationally determined fragment positions were clustered, and the clusters were chosen to represent elements of a combinatorial library to develop the library design.[5] Then, the combinatorial chemistry determined actual allowed connections. In a sense, this approach is related to dynamic combinatorial library design with in situ chemistry and fragment screening by tethering.[50−52]

(II) Complementary Approach to HTS. Fragment-based lead generation (FBLG) can play an important role in the drug discovery process. Typically fragment-based methods, both experimental and computational, are applied in the early stages of a project when it is crucial to identify high-quality, novel leads for the target. Ultimately, the success of a drug discovery effort depends to a large extent on the quality of the one or two lead series taken forward into the lead optimization phase. Given the often desirable properties of fragment hits, it is not surprising that there are an increasing number of examples of successful fragment-based design leading to clinical candidates.[53,54] Fragment-based screening has been pursued by a number of technology-based companies since the mid 1990s.[55,56] Over the last 10 or so years, an increasing number of pharmaceutical companies have also invested in in-house fragment screening efforts and taken the general approach of combining a number of biophysical techniques resulting in increasing success rates.[57,58]

For structure-based projects in particular, fragment-based screening, including virtual fragment screening, can be used to

identify new leads for a given biological target. When coupled with virtual screening, the odds of doing so may be enhanced. In addition, fragment-based hits can sometimes be used to help optimize high-throughput screening (HTS) hits. If a lead series is identified through an HTS campaign and fragments that bind in the target binding-site are also identified, it may be possible to optimize the HTS series through addition, substitution, or merging of one of the fragment hits onto the HTS scaffold.

One of the main advantages of a fragment-based approach for lead generation is that it allows for a greater coverage of chemical space.[59−62] Another advantage is that fragment binders typically have high ligand efficiencies, where the ligand efficiency (LE) is commonly defined as the free energy of binding of the ligand divided by the number of heavy atoms in the ligand[63,64]

$$\mathrm{LE} = -\Delta G/N = -RT\ln(K_d)/N \approx -RT\ln(\mathrm{IC}_{50})/N$$

where ΔG is the free energy of ligand binding to the target, N is the number of heavy atoms in the ligand, K_d is the dissociation constant, and IC_{50} is the half maximal inhibitory concentration. One can think of the LE of a ligand as the average binding affinity per heavy atom. Fragments typically have higher ligand efficiencies relative to larger molecules even though their potencies may be relatively weak.[65] Ligands with high LE have the potential for a greater improvement in binding affinity through the addition of a heavy atom or small substituent.[66]

An extension of this idea is the lipophilic LE, called LLE or LiPE[67,68]

$$\mathrm{LLE} = \mathrm{pIC}_{50} - \log P$$

where pIC_{50} is $-\log(\mathrm{IC}_{50})$ and P is the partition coefficient for the un-ionized compound between octanol and water. Additional ligand efficiency indices have been described and used for lead identification.[69−71] Typically, as the potency of a lead is optimized, the molecular weight (MW) increases.[72] To have a drug candidate, at the end of the optimization process, the MW and other physical properties must be within an acceptable drug-like range.[73] Often Lipinski's "Rule of five" is applied to define that range.[74] HTS is very effective at identifying hits for most targets, and large pharmaceutical companies generally have state-of-the-art HTS capabilities. Sometimes, however, for a given target, HTS hits may end up having relatively low LE values. It can be difficult to take a low LE ligand and remove substituents during the optimization process so that the final compound ultimately has drug-like or acceptable properties. Furthermore, for certain therapeutic areas, such as infectious disease, HTS hit rates may not be as high as in other areas, such as oncology.[75] Fragment-based screening, like virtual HTS, is a complementary approach to HTS that increases the likelihood of identifying high quality chemical starting points for a given project.

Taking this idea of complementarity a step further, Crisman et al. at Novartis developed a "Virtual Fragment Linking" (VFL) approach to use fragment screening data to prioritize testing of lead-like compounds from a full HTS library.[76] In their tests, VFL was capable of identifying nanomolar potency compounds from micromolar fragment binding data in 57 of 75 target classes from the WOMBAT database. This approach has similarities to "SAR by catalog".[77,78] These examples highlight some of the benefits of having carefully and strategically designed fragment libraries, such that the results of relatively small-scale fragment screens can inform the experimental design of larger screens.

Development of Fragment Libraries. Commonly, fragments are defined as small molecules with MW ≤ 300 which obey more of a "Rule of three"[79] rather than Lipinski's "Rule of five".[74] The Congreve et al. "Rule of three" for fragments specifies MW < 300, hydrogen bond donors ≤ 3, hydrogen bond acceptors ≤ 3, and cLogP ≤ 3. Simultaneously with the development of fragment positioning methods, computational methods have been developed and applied for the design and analysis of fragment libraries.

Initial reports of designing more lead-like, as opposed to drug-like, libraries consisting of compounds with MW and lipophilicity lower than the limits prescribed by the "Rule of five" were described in the mid to late 1990s. Screens of lead-like libraries (MW < 350 and clogP between 1 and 3) generate hits that allow for further optimization of potency, metabolic, and pharmacokinetic properties.[80] In contrast, screening libraries of larger drug-like molecules often led to the identification of high MW hits that achieved micromolar affinities through many poor interactions making further optimization difficult. When screening more drug-like libraries, it has been estimated through probability analysis of HTS data that 50−100 analogs need to be screened for each series to be confident of identifying at least one active for each true active series for a given target.[81] The design of the "SHAPES library" for NMR screening, incorporated the finding that 32 different "molecular frameworks" or "shapes" described 50% of the then known drugs.[82,83] Similarly, Hann et al. observed that less complex molecules based on several properties (i.e., MW, clogP, number of aromatic rings, number of heavy atoms, calculated molecular refractivity, fingerprint bits set, interaction energy[84]) were more common starting points for the development of drugs.[85] They determined that the probability of finding matching ligand and pocket features decays exponentially as complexity increases. Baurin et al. subsequently showed that a relatively small fragment library, designed using three-point pharmacophoric fingerprints to measure molecular complexity and assess novelty, could be screened by NMR to identify hits that discriminate between related binding sites.[86−88] Recently Leach and Hann have reviewed the concept of molecular complexity and its relevance to fragment-based drug discovery.[89]

The AstraZeneca 20K fragment generic screening library, which is typically screened using high concentration biochemical assays, was designed using smarts-based substructural filters to restrict the molecular complexity.[90] Properties were calculated, but hard filters were not applied other than restricting the number of heavy atoms to less than or equal to 18. A diverse set of fragments was selected for inclusion in the library using fingerprint similarity measures and molecular shape indexing.[91] The goal was to design a fragment library that sampled many shapes with low complexity.

Recently, because of an increase in experimental capacity, we expanded an existing generic 1200 fragment NMR screening library, which was developed in a similar manner as the 20 000 fragment HCS library. In the process, we attempted to bias the extended library to contain compounds that would inhibit bacterial targets. The design of this new Infection fragment library is outlined in Figure 3. After all the fragments in our in-house collection were selected, we flagged fragments in the existing NMR library and added in flagged fragments from an "AstraZeneca infection friendly set". This Infection friendly fragment set was derived from (i) AstraZeneca Infection key transition compounds and compounds with MICs (minimum

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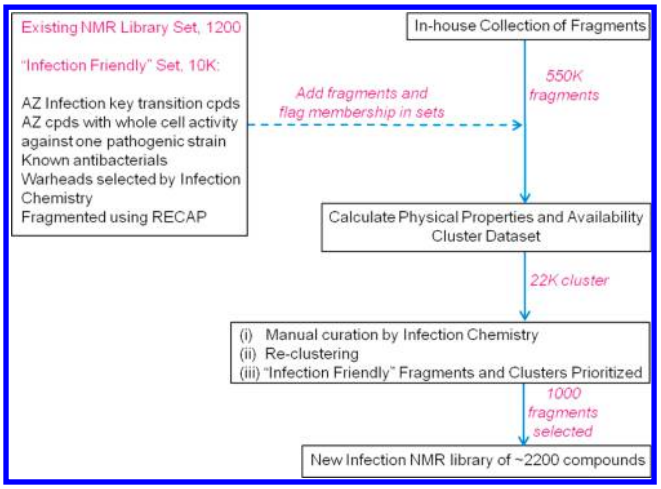

Figure 3. Infection specific NMR fragment library development scheme.

inhibitory concentration in a cell growth assay) against at least one pathogenic bacterial strain, (ii) known antibacterial compounds from external sources, and (iii) a small set of warheads selected by Infection Chemists. For the full fragment set of 550 000 compounds, physical properties were calculated and only compounds that were available in sufficient quantity for testing were considered. Clustering using Pipeline Pilot[119] FCFP_4 fingerprints and a Tanimoto of 0.5 yielded 22 000 clusters. After manual curation by Infection Chemists and reclustering, compounds were selected for inclusion in the new library, giving priority to "Infection friendly fragments" and "Infection friendly clusters". In the end, 1000 fragments were added to the existing NMR library to create an extended Infection NMR library of ~2200 fragments.[118] The library size is now matched to experimental capacity to maximize likelihood of obtaining hits. In the future, inclusion of fragments with greater three-dimensional (3D) character and diverse skeletal and stereochemical features, such as those created at the Broad Institute which using Diversity-Oriented Synthesis, may prove useful for infectious disease screening as well.[92]

Clearly a fragment library of approximately 2000 fragments is not fully representative of chemical space. Our analysis, described above, identified 22 000 distinct clusters of fragments based on fairly loose criteria and suggests that a library of 20 000 fragments is more likely to cover adequately chemical space. This analysis, based largely on examination of the AstraZeneca compound collection, is biased since the collection itself does not fully represent chemical space and therefore represents a lower bound on what is required. As such, practical considerations generally dictate the size of the library screened.

High Concentration Fragment Screening at AstraZeneca. At AstraZeneca, the fragment screening paradigm can involve a high concentration screen using a biochemical assay (HCS), virtual screens (VS), and/or a nuclear magnetic resonance spectroscopy (NMR) screen (Figure 4). Virtual screens are performed on fragment libraries of several hundred thousand in-house compounds as well as externally available fragments. Several hundred to a thousand compounds are then tested experimentally either in the HCS assay or by NMR. Virtual screening by design focuses on the target of interest, while HCS and NMR screens use generic fragment libraries. Any fragment hits, based on percent inhibition in a biochemical assay, are then confirmed by IC_{50} determination followed sometimes by NMR binding studies, and ultimately by X-ray crystallography.

In a one successful example, the program ROCS with color scoring[120] was used to carry out a shape and pharmacophore feature search of a large corporate collection of compounds. Substrate fragments and two other known fragment hits were used as the queries. Simultaneously, the program Glide with SP scoring[121] was used to perform structure-based docking into the target protein structure. A set of approximate 400 additional fragments was selected for experimental testing alongside our generic ~20 000 fragment library using a high concentration biochemical assay. In this particular case, the hit rate with for the fragments selected by virtual screening was ~17% compared to ~2% for screening the generic fragment library; while the number of hits from the generic HCS was still greater than from the virtual screening (454 vs 65), the diversity of hits was enhanced through the virtual screening. In addition, in this case, 5 of the 20 series that progressed to medicinal chemistry expansion were identified through the virtual screening. The virtual screening approach employed in this example was fairly typical, in that all available experimental information (about substrates, known and often weak ligands, structures, etc.) was taken into account.

Enzyme assays possess the highest throughput and provide a direct report on enzyme function. However, the use of high

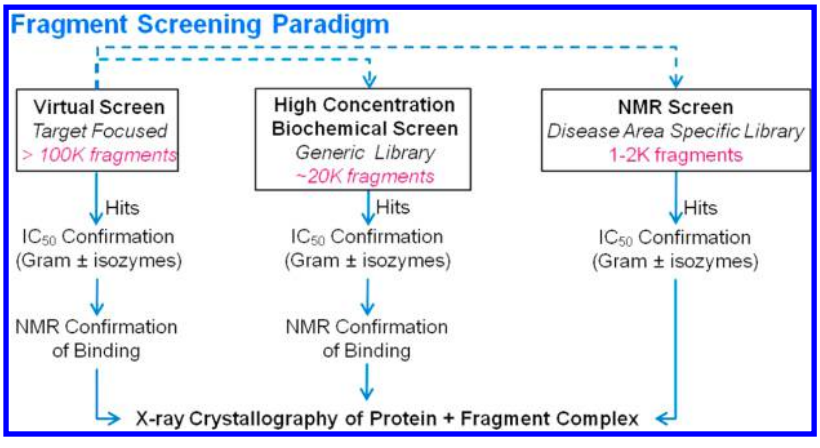

Figure 4. Potential combinations of fragment screening approaches. If a broad spectrum antibacterial agent is ultimately desired, gram-positive and gram-negative isozymes of the bacterial target are screened.

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compound concentration (typically 1 mM) poses technical problems that can often lead to an increased false positive hit rate due to interference with the assay detection system (absorption, fluorescence of compounds). Interference can be reduced by a correction method at the single-point screening stage.[93] In addition, at high concentration compounds may lack solubility and form aggregates that can nonspecifically inhibit enzyme function and thus produce false positive hits.[94] It is therefore desirable to filter hits with a secondary assay, ideally by one-dimensional (1D) NMR binding confirmation. This very high throughput combination approach of using HCS followed by 1D NMR has been applied successfully to several targets.

(III) FBLG for Drug Discovery. *Fragment Growing, Linking, Merging.* Once fragment binders have been identified in the binding site, in drug discovery the objective is then to design potent inhibitors by either growing the fragment, linking fragments, or merging fragments onto existing scaffolds for the target (Figure 5). A theoretical basis for the benefits of linking fragments was described by Jencks as early as 1981.[95] On the experimental front, this was confirmed and exploited by scientists at Abbott, using "SAR by NMR" to detect and link fragment positions.[96] While fragment growing is by far the more common option pursued with fragment hits, a number of groups have been developing automated computational approaches for linking fragments. Computational fragment connecting methods for inhibitor design first emerged in the mid-1990s, shortly after the first fragment positioning methods; examples include Caveat,[97] HOOK,[98] and Concerts.[99]

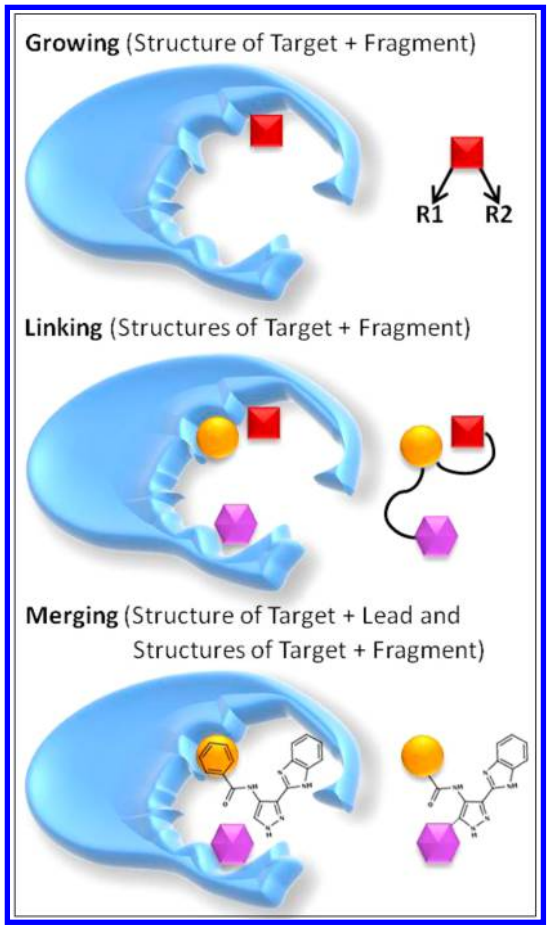

Figure 5. Strategies for using fragment positions in inhibitor design.

While the concept of fragment-based design is simple, in practice it is quite a difficult problem.[100,101] Almost all de novo design programs suffer from a lack of full synthetic accessibility of the output. Newer methods try to address this issue by restricting the combinatorial problem in some way when connecting the fragments. BREED,[102] for example, tries to generate novel inhibitors by fragmenting a set of known ligands and recombining the fragments to generate hybrid molecules. ReCore[103] can be used to link or grow fragments using the recap synthesis rules for connection. As an initial test of ReCore, at AstraZeneca we took an early lead for a given program and showed that the approach was able to generate later stage, more potent compounds. Alternatively, the CONFIRM[104] approach uses a database of bridge molecules derived from existing compounds to increase the likelihood of synthetic accessibility of the output molecules, by retaining information about atom types to which the bridges can connect. Using the CONFIRM method for linking fragments, a search query is derived from two bound fragments by identifying points of connection on the bound fragments. The library of bridge molecules is then searched to identify those molecular bridges that can potentially be used to link the fragments within the binding site. The set of unique molecules are then enumerated and docked into the target binding site. The entire process was automated within Pipeline Pilot.[119] Other de novo design approaches can be used for fragment-based inhibitor design including GANDI,[105] Med-SuMo/Med-Portions,[106] AlleGrow,[122] MOE 2010.10 modules[123] and BROOD.[124]

Case Studies. A number of successful examples of using fragment-based approaches to develop a clinical candidate have been reported.[100] One well-known early fragment linking example was carried out at Abbott using SAR by NMR to design inhibitors for the FK506 binding protein.[96] Howard et al. used a novel fragment-linking approach to develop a thrombin inhibitor.[107] The approach involved virtual screening to select a small, target-focused subset of fragments for screening by X-ray crystallography. Protein-fragment structures revealed a clear opportunity for fragment linking, resulting in compounds with dramatically increased potency and selectivity versus trypsin. There are also a number of recent examples of successful fragment growing involving either structure-based design alone[108,109] or in combination with virtual screening[110].

Fragment-based lead generation has been successfully applied across a number of therapeutic areas at AstraZeneca.[57] As an example, a novel series of cyclic amidine-based inhibitors of β-secretase were discovered via a fragment based approach.[111,112] NMR screening was used to identify the initial hits that were subsequently evolved into submicromolar inhibitors using a combination of X-ray crystallography, molecular modeling, surface plasmon resonance, and functional enzyme assays. In another example, inhibitors for bacterial type II topoisomerase were generated from initial hits from an NMR fragment screen through the structure-based design and synthesis of a library of compounds linking the fragments and related ones.[113]

The examples described above all involve the use of 3D structures of protein−fragment complexes to aid the subsequent design of inhibitors. In certain instances, FBLG can also readily progress in the absence of a 3D structure of the target, especially if additional structural information from NMR

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experiments is available. As described above, ILOE-NMR can, for example, be used to identify pairs of ligands that can bind simultaneously and potentially be linked. The approach probes the size and topology of the binding pocket and provides directional information to guide subsequent synthesis to link or optimize fragment binders. Abell and co-workers refined the approach, and used it to design a potent inhibitor for PtS from *Mycobacterium tuberculosis*.[42] The binding of the pantoate substrate which had been well-characterized was used as a starting point, and the relative binding modes of fragments were derived from ILOE observations. Guided by ILOE-NMR results, two weak fragments (with K_ds of 1.0 and 1.1 mM, respectively) were modified and linked, ultimately to generate an inhibitor with a K_d of 860 nM. These NMR results were subsequently confirmed by crystal structures, as a further proof of concept for the approach.

Challenges and Future Directions. Fragment screening is a powerful approach for lead generation that has yielded clinical candidates and has great potential to generate future drugs. Virtual fragment screening also has proven useful and even for difficult test cases it can yield results that are significantly better than random screening. A number of challenges still remain, though.[114−116] Weak fragment hits are still often overlooked in favor of more potent HTS hits with poorer physical properties and ligand efficiencies. This bias is largely due to the fact that there is increasing pressure to shorten drug discovery timelines. The timelines for success to the next milestone with fragment-based screens can be relatively long, since fragment hits may require more optimization cycles. Furthermore, a robust system for crystallography to aid in the optimization of hits can be difficult to obtain. Fragment docking, especially if enhanced with directional information about the binders from NMR, can aid in the optimization process.

The novel aspects of fragment-based approaches compared to lead-like methods are in terms of the relatively rapid determination of the energetic hot-spots within a binding site, greater coverage of chemical space in screening, and ultimate identification of high quality lead matter. The greatest limitations still lie in accurately predicting fragment positions and in optimizing fragment connections, in particular in the absence of iterative structural input. Furthermore, the larger and more complex the fragments are, the more dependent the results will be on the choice of fragments within the library; this issue is generally dealt with by the requirements set for inclusion in the fragment screening library in terms of physical properties, number of functional groups, etc. The thermodynamic binding parameters of small fragments are likely more sensitive to protein flexibility and solvent effects than those for larger, more drug-like molecules. With respect to predicting computationally the binding of fragments, protein flexibility and solvent effects are often handled inadequately, though the tendency of FBLG approaches to exploit information-rich experimental approaches such as X-ray crystallography and NMR may overcome some of these limitations.

Enhancements to the technology aimed at increasing the accuracy of fragment docking, possibly through more accurate inclusion of solvation, entropy effects, and protein flexibility, are desired. The power of fragment-based approaches going forward, however, will be realized through the use of computational approaches to integrate and take full advantage of the increasing wealth of experimental information. The use of computational approaches to aid in experimental design, include the strategic development of fragment libraries and the biased selection of chemotype from HTS libraries for efficient screening as two recent examples. Increasingly, computational tools are providing models that enable data from one regime to inform the design of experiments in another, ultimately resulting in more efficient drug discovery.

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Notes

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