

Fragment-Based Drug Discovery

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Learn from yesterday; live for today; hope for tomorrow. The important thing is not to stop questioning. **Albert Einstein**

Fragment based drug discovery (FBDD) has played an important role in the past decade, helping in many cases to streamline the complex process of drug discovery from target validation to new drug application. A total of eighteen drug candidates discovered by FBDD have been advanced to clinical trials so far, and Zelboraf[®] (vemurafenib, PLX4032) is the first FDA-approved drug discovered using fragment-based approaches. X-ray crystallography or NMR spectroscopy was used to identify thirteen of the eighteen FBDD drug candidates.

I. LIGAND–PROTEIN INTERACTIONS: FIRST PRINCIPLES

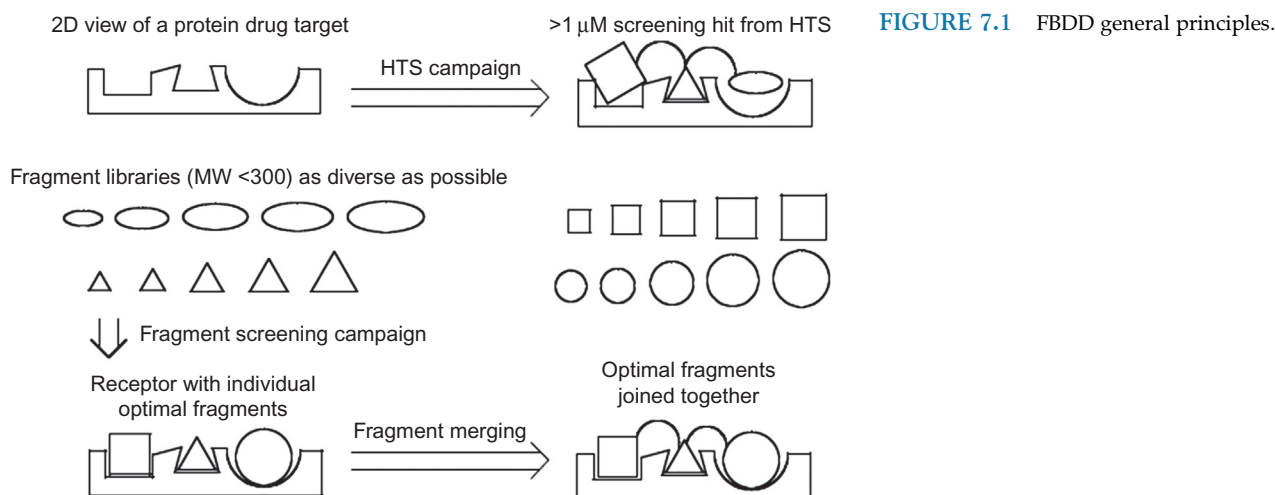
When Paul Ehrlich conceived of a drug as a magic bullet interacting as a key in a lock, he was not far from understanding the reality of ligand–protein interactions. Although target proteins are flexible and can adopt one or more of a manifold of induced conformations, binding sites on proteins have evolved to recognize a limited number of endogenous modulators and substrates and to exclude others.

A. Binding Energy as the Sum of the Parts

The Gibbs free energy (ΔG) of multivalent ligand–protein binding is the sum of the energies involving each of the substructures or fragments that comprise the ligand [1,2]. To the extent that we understand ligand–protein interactions on as small a scale as possible, we can design inhibitors and modulators from first principles more easily than the hit-or-miss approach implicit in high-throughput screening (HTS, see Figure 7.1). Fragments are typically clustered in diverse, low molecular weight libraries. Non-FBDD HTS screening libraries can be considered as combinations of fragments in various ways, but only sampling a small fraction of the total possible diversity space because of their larger molecular weight range. As ligands become larger and more complex, the probability of finding useful information for any randomly selected compound is exponentially smaller [3]. As hits are identified from HTS, typically with 1–10 μM potency, the individual fragments encompassed within those hits are typically not optimized for optimal interaction with the biological target. In cases where it is possible to screen the fragments separately in advance, one can design more efficient ligands *a priori* by further synthetic manipulation such as by linking different fragments together. Therefore, less complex and smaller molecules are better starting points early on in the drug discovery process. This is the essence of FBDD – determining which smaller molecular substructures or fragments interact optimally with targets of interest and how they bind, and then using that information to obtain better hits and leads for further consideration [4–17]. FBDD represented a paradigm shift in understanding the lead generation process in drug discovery, and is an attempt to get more information rapidly while doing the same amount of work overall.

B. Historical Development

Automation and miniaturization of biological assays led to the implementation of HTS in drug discovery in the early 1990s at most of the major pharmaceutical companies using their large internal compound libraries. With the advent of combinatorial chemistry, even larger numbers of compounds were synthesized and screened to determine activity against a variety of biological targets. Diversity in corporate screening libraries varies from



small molecule fragments to the combination of many such small fragments linked together to form large and complex structures, prepared in the context of different programs. Libraries for HTS vary in the range of 10^4 – 10^6 compounds, and hit activity is often in the potency range of 1–10 μM , which is then followed by hit to lead (HTL) medicinal chemistry for further optimization [18,19].

However, many compound libraries prepared using combinatorial chemistry incorporate common chemical features suitable for automated synthesis, which limited overall diversity. Hits generated through HTS are often overly lipophilic with poor thermodynamic solubility. Many HTS libraries represent a relatively small fraction of possible chemical space, thus limiting the chance to identify reasonable starting points for lead optimization [20]. In addition, significant cost and time has to be dedicated to creating and enriching the diversity and quality of these libraries. Also, HTS demands a validated assay system, operational as an industrial process with a reasonable throughput and sufficient miniaturization to lower the cost per assay is often challenging and require huge amounts of resources that are usually not available for smaller companies.

During the 1990s, researchers at Abbott demonstrated structure–activity relationship development by nuclear magnetic resonance (SAR by NMR), which enabled the discovery of active fragments to complement those obtained by HTS [21]. High- or medium-throughput X-ray crystallography of compound libraries against protein targets were also initiated in 2000 in seminal work at Abbott [22]. Both of these techniques have provided valuable insight in early drug discovery research, with FBDD evolving to complement HTS in an attempt to discover more chemically-tractable smaller hits prior to starting chemical synthesis of analog libraries [6–20].

C. First Principles: Ligand Efficiency

Ligand efficiency or LE is a powerful tool to rank order hits from any screening campaign and is defined as:

$$\text{LE} = (\text{pKi or pIC}_{50}) / (\text{N}_{\text{hev}} \text{ or number of heavy atoms}) \quad [23]$$

$$\text{BEI} = (\text{pKi or pKd}) / \text{MW}$$

$$\text{SILE} = \text{pIC}_{50} / (\text{N}_{\text{hev}})^{0.3}$$

$$\text{LLE} = \text{pKi} - \text{Log } P \text{ (or Log } D)$$

$$\text{LELP} = \log P / \text{LE}$$

A retrospect analysis performed over a wide range of protein targets and ligands by Reynolds et al has shown that protein–ligand binding affinities are strongly influenced and vary by molecular size [23]. Binding Efficiency (BE), Fit Quality (FQ), and Size Independent Ligand Efficiency (SILE) can be used as alternate efficiency indices, while Ligand Lipophilic Efficiency (LLE) and Ligand-Efficiency-Dependent Lipophilicity (LELP) can be used as lipophilicity guiding indices. These metrics can be used to assess the relative merits of hits after screening and guide further optimization [24].

Changes in LE for analogs in lead optimization as measured by effects on MW as potency is increased for eighteen FBDD programs was investigated by Ferenczy and co-workers [24]. Biological data from the optimized compound and for one or several analogs in the optimization process were considered (Figure 7.2). The effectiveness of optimization was gauged by the slope of the line, as a smaller slope represented a more efficient process of optimization where there is greater improvement of affinity by a smaller increase in the molecular weight. The slope varied from values of 10.3 to 138.5, with 61.4 being the average with a standard deviation of 34.3. The broad extent of the variation of the slope suggests that optimization will in some cases be more dramatic than for others when using FBDD approaches.

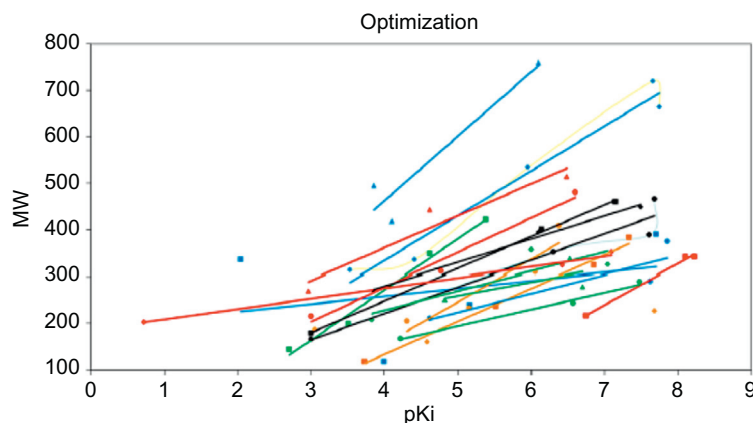


FIGURE 7.2 Ligand affinity (pKi) vs molecular weight for 18 FBDD programs. Reprinted with permission from Ferenczy GG, Keseru GM. How are fragments optimized? A retrospective analysis of 145 fragments optimizations. *J Med Chem* 2013;56:2478–86. Copyright (2013) American Chemical Society.

II. WHAT IS FRAGMENT-BASED DRUG DISCOVERY?

FBDD evolved as a different way to start the lead generation process in drug discovery, with the idea to collect more information while doing the same amount of work overall. FBDD provides the opportunity to design more efficient ligands *a priori*, as smaller molecules with less complex structures are chosen as starting points. These smaller fragments are often chosen in a way so that they provide synthetic handles to support further chemical manipulation and to grow the molecules either by linking, merging, or by substitution, with the aim of increasing binding potency and selectivity and optimizing ADME properties. Smaller molecules have the potential to access those binding sites that are inaccessible to the larger ones. Also, smaller compounds could provide more operational freedom for structural manipulations during multiple optimizations.

A. Overview of FBDD

Drug discovery research since 1990 has been heavily influenced by HTS technology and the combinatorial chemistry preparation of large compound libraries [19]. Large screening libraries and advances in screening automation have made HTS easier to perform. Projects that are initiated via HTS are of necessity focused on limited functional group manipulations based on the nature of the core scaffold and the substitution available or allowed [20]. Though many large, hydrophobic compounds—whose overall characteristics were not drug-like—emerged as hits, they did not serve as good lead generation starting points for lead optimization, and HTS in general has not yielded a high rate of return in terms of NMEs approved for therapy [25,26]. Because of this, consideration of drug-likeness of compounds in libraries was stressed to a much greater extent, and it was recognized that many inappropriate compounds are found in current screening collections that often result in false positives that waste time and effort during follow up. Hann et al related the complexity of a compound to the probability that it will experience a binding event [3]. In short, because the compounds in the current screening collections were both highly complex and overly hydrophobic, being derived mainly from previous LO campaigns, HTS provided only limited success in identifying novel hits that could be progressed into lead candidates, and then into marketed drugs.

One of the keys in FBDD is to use alternate biophysical read-outs of the binding of ligands with target proteins suitable for detecting low-affinity interactions to complement biochemical and functional assays. A seminal article by Fesik et al in 1996 coined the phrase “SAR by NMR,” using NMR spectroscopy, as such a method is not dependent upon the detection of biological activity [21]. The SAR by NMR approach is to screen small, highly polar fragments, as opposed to larger members of screening libraries, and then to create lead candidates through the linking of multiple independently optimized fragments. An early example of the FBDD approach was the development of potent inhibitor **1** against stromelysin, where NMR screening had identified that **2** and **3** bound to stromelysin in two distinct, neighboring locations, namely the catalytic site and the S1' pocket, respectively (Figure 7.3) [27].

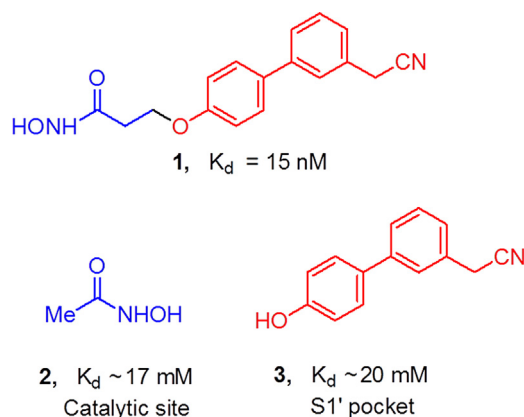


FIGURE 7.3 Fragment merging with dramatic improvement in potency.

B. The Concept of FBDD

Many HTS hits that are sufficiently potent to be considered further and the majority of lead candidates during lead optimization represent complex molecular structures comprised of multiple, interconnected ring systems, onto which any number of substituents are grafted. These structures are complex because of their size, extent of conformational freedom, and array of diverse chemical substitution. A careful analysis of such structures reveals that they can often be deconstructed into a set of chemical building blocks, such as heterocyclic and phenyl rings, and linkers comprised of amide, urea, ketone, and methylene functionalities (Figure 7.4). From these basic building blocks and linkers, one can envision constructing molecular structures that present only a limited number of optimized pharmacophores and degrees of conformational freedom.

These basic starting structures are called fragments, and Figure 7.4 shows hits generated from HTS compared to those from FBDD [5]. Smaller fragments with high ligand efficiency are more relatively useful as starting points in SAR development, although with fewer points of interaction than HTS hits with lower ligand efficiency and more points of interaction.

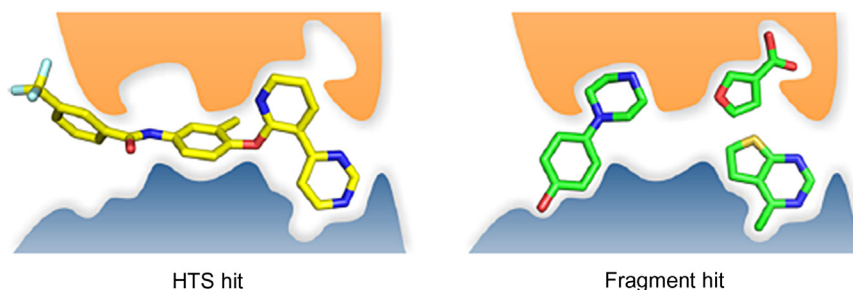


FIGURE 7.4 Interactions made by HTS hits vs. fragment hits where smaller fragments have better overall LE. Reprinted with permission from Scott DE, Coyne AG, Hydson SA, Abell C. Fragment-based approaches in drug discovery and chemical biology. *Biochemistry* 2012;51:4990–5003. Copyright (2013) American Chemical Society.

C. Fragments in FBDD

A fragment is a small molecule characterized by researchers at Astex as falling within the Rule of 3™ [28,29].

- Molecular weight < 300 Da
- Number of hydrogen bond donors ≤ 3
- Number of hydrogen bond acceptors ≤ 3
- cLogP (predicted) ≤ 3
- Number of rotatable bonds ≤ 3
- Polar surface area (PSA) $\leq 60 \text{ \AA}^2$

Although FBDD is simple in principle, many factors play a significant role in converting fragments to leads, such as synthetic tractability, location of the binding site relative to the active site or the potential for allosteric modulation, and whether the particular binding site is unique to the original target protein or present in many different proteins [30].

The process of FBDD starts with the selection of a library of fragments. Common techniques used to detect a fragment binding event are NMR [31,32], surface plasmon resonance (SPR) [33,34], mass spectrometry (MS) [35,36], and X-ray crystallography [22,37]. X-ray crystallography is the only general technique that can reliably demonstrate the binding location and orientation of the fragment hit. While NMR can achieve these two outcomes in a step-wise fashion, X-ray crystallography provides both at once. Further stages in the FBDD process are shown in Figure 7.5, which shows it may take two to three years for any particular FBDD project to achieve validated or advanced lead status or entry into preclinical development.

D. Fragments Hits vs. HTS Hits

FBDD and HTS aim to discover lead candidates that have the desired potency with favorable selectivity and ADME properties. HTS seeks to identify one or more mature chemical starting points for hit-to-lead medicinal chemistry, whereas FBDD seeks to incrementally construct leads, often starting with less initial potency but equivalent or better LE. ADME (Adsorption, Distribution, Metabolism, and Excretion) properties play a very important role in determining the fate of the compounds *in vivo*. Metabolically liable and chemically reactive

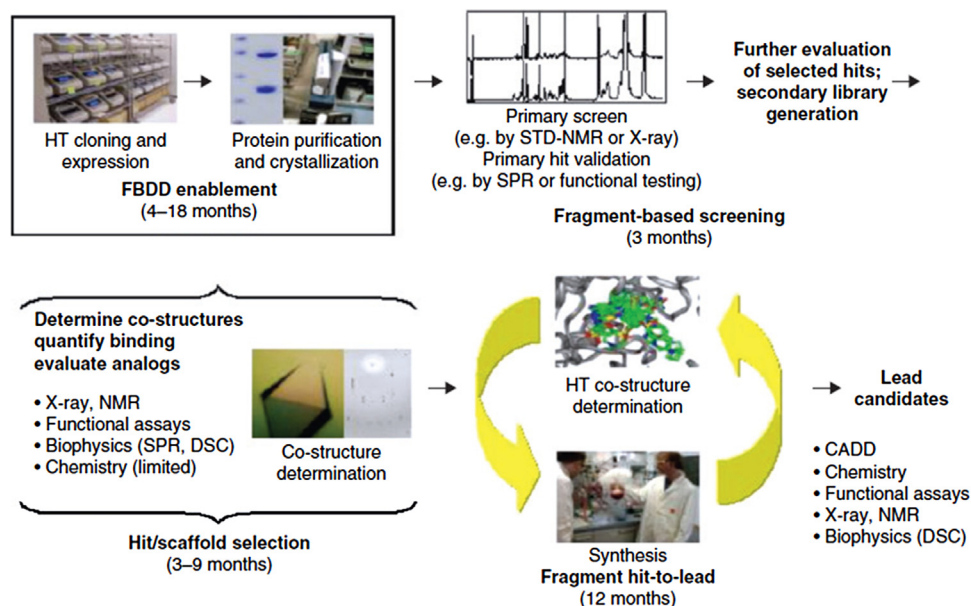


FIGURE 7.5 FBDD workflow with predicted timelines.

groups can be avoided during fragment elaboration in the hit-to-lead stage, unlike in complex HTS hits where they are already incorporated in structures from screening libraries.

III. CREATION AND ANALYSIS OF FBDD LIBRARIES

A. Fragment Library Design

Typical fragment libraries consist of 100–1,000 members and are generally much smaller than libraries used in HTS campaigns. Even with this lower number, greater diversity of structure and topology for the fragment MW range can often be achieved relative to the larger libraries. The potency of fragments upon screening is often weak (in the mM range), which can be dramatically improved during SAR development since a linear relationship frequently exists between MW and binding affinity [38].

Fragment libraries are typically selected with specific attributes in mind. For example, certain techniques require covalent attachment to either protein targets or solid support, which necessitates the incorporation of suitable functionality for that purpose, such as the sulfhydryl group. Some libraries for X-ray crystallography are designed to incorporate heavy atom substitution, such as with bromine, in order to solve the X-ray structures more rapidly [39]. Computational methods have been developed by both pharmaceutical companies and academic groups to prescreen fragment libraries as a way to select members for further study and consideration [40]. Hubbard et al utilized an *in silico* screening method to analyze fragment screens against twelve diverse targets to direct fragment library design [41]. A significant number of fragments overlapped as active compounds irrespective of target. For example, hits for protein–protein interactions tended to be more hydrophobic and heavier than fragment hits from other programs. Of the fragments hits against three kinases, 11 percent were common and at least 52 percent were unique to each kinase screened. An *in silico* screen using molecular graph theory to design fragment libraries was used by Nunez et al at Abbott to identify fragments against human trypsin [42]. Similarly, Hung et al have included non-aromatic sp^3 -rich fragments in their fragment library, to generate fragment hits with high topological diversity [43]. It is a common strategy to create fragment libraries that bear representative substructures known to have favorable drug-like properties or are found to a high degree in currently marketed drugs [39]. Chemical novelty is not as important a criterion for inclusion in a primary fragment library, because unique composition of matter suitable for patent protection would be added later during elaboration and merging hit to lead medicinal chemistry.

B. Analysis of Fragments

Once a fragment has been determined to bind to a particular target protein, the magnitude of its binding must be established. Functional inhibition is routinely determined on initial fragment hits, although it is generally understood that interpretation of weak inhibition ($> 10 \mu\text{M}$) can often be confounded due to artifacts such as aggregation. The quality of binding is assessed based on stoichiometry, dose dependence, and location and orientation of the fragment hit. However, a fragment hit may exhibit a component of binding that involves one or more desired pockets or regions on a target protein. Competitive displacement experiments, using a potent inhibitor known to occlude the desired pockets or regions, can be used to determine sites of interaction [44].

X-ray crystallography and—to a lesser extent—NMR spectroscopy are the only general techniques that can reliably determine the binding location and orientation of fragment. Insight into the orientation of binding of a fragment hit helps to identify synthetic opportunities for accessing a desired neighboring pocket or region, or for linking with a different fragment hit that already occupies that neighboring pocket or region.

The crystals to be used for X-ray crystallography in an FBDD campaign must present a higher diffraction quality than is typically required by conventional structure-based drug design. A minimum resolution of $< 2.5 \text{ \AA}$, typically on the order of $2 \text{ \AA}$, is required to reliably orient a fragment that is bound to the target protein, because fragments are deliberately chosen to be small in size and therefore—in contrast to most HTS hits and LO compounds—typically present a rather compact and minimally asymmetric 3D structure. In contrast, reliable orientation of an HTS hit can often be achieved at a resolution of $3.2 \text{ \AA}$.

Previously, prosecuting a greater number of less validated targets has been promulgated as a way to minimize the downside risk to the HTS/HTL approach. In contrast, the downside risk to the FBDD approach can be minimized by prosecuting a smaller number of targets that have achieved at least strong preclinical therapeutic validation. Such targets virtually guarantee that any quality LO compound will proceed into development, justifying the significant up-front resources required by the FBDD approach. Such targets are also typically pursued at many pharmaceutical companies and have many associated patents describing a sundry of inhibitor chemotypes and scaffolds, typically derived either directly from HTS/HTL campaigns or from patent-busting operations on previous HTS/HTL-derived compounds. Under these circumstances, the downside risk inherent to the FBDD approach is further reduced because the very strength of FBDD lies in its proven ability to span effectively a wider chemical space, which plays to chemical novelty. Also more polar chemical starting points can be selected, thereby avoiding at least the initial lipophilicity of many HTS hits that may later on become associated with specific ADME liabilities.

C. The Role of the Medicinal Chemist in FBDD

The fundamental differences between FBDD and HTS also mean that medicinal chemists play a different role in each approach. In the HTS follow up, the medicinal chemist is tasked to determine—often with limited chemistry resources—whether any HTS chemotypes represents chemical matter that can be progressed in a timely fashion into LO. Much data must be analyzed and synthetically challenging analogs are generally avoided. The HTS chemist is part informatician, part medicinal and synthetic chemist. In FBDD, the medicinal chemist plays the role of both a synthetic and structural chemist. The predictive power of the medicinal chemist for rational design is much enhanced by the use of NMR or X-ray structure studies, complemented by any functional assay data that is available on the target of interest. Since many starting fragments are commercially available and possess similar physicochemical properties, it is important to understand the nature of target. Knowledge of toxicity, stability, and bioisosterism can be used in the selection of appropriate fragment libraries [45,46].

IV. FRAGMENT SCREENING METHODS

After the original discovery of weakly biologically active or inactive hits from FBDD, the challenge for the medicinal chemist is to take this information and generate novel leads suitable for development into clinical candidates. Detecting a fragment inhibitory event is accomplished by increasing the concentration of the fragment in the functional assay, typically to $0.2\text{--}1.0 \text{ mM}$ depending on the maximal solubility allowed. Once a fragment has been detected to bind to the target protein, the magnitude of its binding should be established, if possible.

A. Nuclear Magnetic Resonance

Ligand-based NMR techniques are based on detecting the ligand in a way that can be changed by the binding of that ligand to the protein target. One-dimensional ^1H NMR experiments such as CPMG (Carr-Purcell-Meiboom-Gill), water-ligand observed via gradient spectroscopy (WaterLOGSY), and saturation transfer difference (STD) are commonly used to detect ligand binding [5]. In addition to Abbott Laboratories, these techniques have been used in many other pharmaceutical companies such as at Astex Therapeutics, Evotec, Schering-Plough, and Vernalis [47–55].

Saturation transfer difference (STD) NMR is probably the most common NMR technique used to screen fragments for binding to protein targets. STD-NMR takes advantage of the enhanced cross-relaxation rate in a large protein system, and the fact that protons on the bound-state ligand become part of a larger proton–proton relay network [56,57]. Saturation of the protein methyl resonances is therefore quickly propagated through this relay network to the bound-state ligand, leading to a saturation of the proton resonances on the ligand. To this end, protein methyl resonances ($\sim 1\text{--}0.5$ ppm) are chosen because many ligands of pharmaceutical interest have few protons that resonate in that region. In addition, methyl groups serve as relaxation sinks in proteins and are efficient propagators of proton saturation throughout the protein and hence the bound-state ligand. Protein methyl groups are at the terminus of amino acid side-chains protruding into the interior of most binding pockets, and are more likely to be proximal to the bound ligand [58]. STD-NMR studies are carried out such that ligand saturation, effected when the ligand is bound to the protein, is measured on the free ligand, not the bound-state ligand. Many factors such as ligand-to-protein ratio, absolute protein concentration, amino acid composition of the target binding site, and flexibility of the bound-state ligand contribute to the sensitivity of the STD-NMR experiment [58]. Often, competitive binding of the protein to ligand is required to eliminate false positives that can arise from precipitation or denaturation of the protein due to high ligand concentration. Information regarding fragment binding sites, occupancies, and the affinity of binding can be identified by using competitive displacement of a ligand with the known binding mode. STD-NMR methods were used in identifying fragment hits from a small library of $\sim 1,400$ fragments, where ten to twelve fragments were employed in the presence and absence of the previously known ATP-competitive ligand PU3 [59]. A total of eighty-two displaceable hits were identified as fragments binding to the ATP pocket of Hsp90 by Brough et al [60].

Researchers at Abbott also utilized NMR methods to identify fragment hits on the protein stromelysin, which is implicated in arthritis and tumor metastasis, whereas HTS screening of 115,000 compounds failed to generate lead compounds with potencies better than $10\text{ }\mu\text{M}$ [27]. Acetylhydroxamic acid (**2**) binds the catalytic zinc site, preventing metalloproteinase-3 (MMP-3) from autolytic degradation, with a dissociation constant of 17 mM (Figure 7.6). Upon selective screening of fragment libraries, a series of biphenyl compounds such as **4** were identified, which was further confirmed as the best compound after the synthesis of biaryl fragments that bound stromelysin. NMR was utilized to solve the structure of these fragments bound to stromelysin, which not only showed the chelation of **2** to the active site zinc but also confirmed a binding pocket that accommodated the biaryl functionality. Thus, by linking the two fragments, **5** was designed and prepared, which showed a good increase in potency and ligand efficiency. NMR-based structure determination provided evidence to support a particular binding orientation for compound **5**. These studies coupled with further SAR development led to the discovery of **6** (ABT-518) as a lead candidate, which was tested in Phase I trials for the treatment of cancer [61,62].

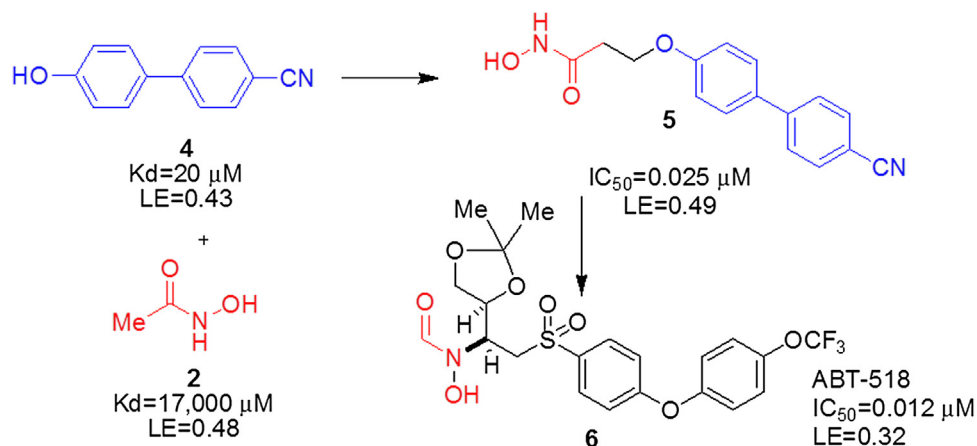


FIGURE 7.6 SAR by NMR in the discovery of ABT-518(**6**).

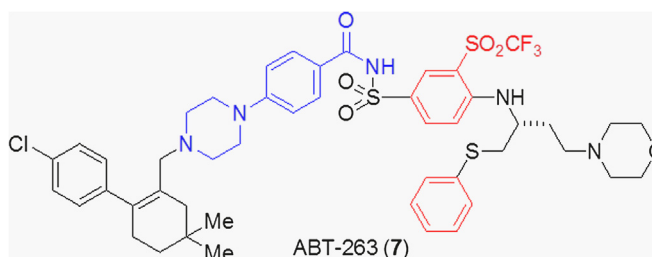


FIGURE 7.7 Fragment linkers were identified using NMR in the discovery of ABT-263(7).

NMR methods have also been used in identifying fragments that bind at the protein–protein interface of Bcl-xL. Fragment hits of potency 2 mM were identified from a library of 10,000 fragments. Subsequent synthetic elaboration led to the discovery of a compound whose NMR based structure bound to Bcl-xL helped in identifying an acyl sulfonamide linker. Further LO methods using this linker led to the discovery of ABT-263(7). (Figure 7.7) [63].

B. 2D (protein-based) Screening

Protein-based NMR techniques are more powerful than ligand-based techniques in characterizing ligand binding, but are much less widely applicable because the protein must be either ^{15}N or ^{13}C isotopically labeled. In cases where both these requirements are met, the binding of a ligand can be monitored based on the bound-state perturbation of the chemical shifts of specific proton and heteronuclear protein resonances. The specific protein resonances whose chemical shifts are thus perturbed serve to localize the ligand binding site, thereby eliminating the need to use competitive displacement experiments for this purpose, which helps to minimize false positives generated due to nonstoichiometric, nonspecific binding caused in ligand-based NMR methods [64,65]. ^1H – ^{15}N HSQC was used by Fresh et al to identify fragment hits that bind to a surface pocket of the Y220C p53 mutant [66].

C. X-ray Crystallography

The use of X-ray crystallography in FBDD has become more prevalent over the past fifteen years. Having a ligand–protein crystal structure provides both validated fragment hit and structural binding information in a single step [67]. X-ray crystallography is performed in FBDD by soaking and co-crystallization of the ligands with proteins. The hit rate found in typical examples when using X-ray crystallography in FBDD is c. 0.5–10 percent, and the most valuable information is obtained when multiple and unanticipated binding sites are identified. When proteins do not form robust crystals with added substrate or inhibitor, then fragments can be evaluated for their ability to displace a known ligand that co-crystallizes with the protein of interest upon soaking [68].

Though a powerful technique used to generate lead candidates, X-ray crystallography has several practical drawbacks. Only 20–30 percent of soluble proteins are amenable to crystallization, and membrane-bound targets such as G-protein-coupled receptors (GPCRs) and ion channels are generally excluded from screening in this way. It is important to have high resolution crystal structures ($<2.5 \text{ \AA}^2$) to properly interpret the position or confirmation of ligand binding. In certain cases, ligands can cause conformational changes that can take place during the interaction of the crystalline state of the protein [69,70]. Astex Pharmaceuticals has generated fragment hits using X-ray crystallography and has identified multiple efficient, synthetically tractable small molecules for lead optimization (Figure 7.8) [71]. For example, a screen of 500 fragments was conducted against cyclin-dependant kinase 2 (CDK2) by soaking the apo CDK2 crystals against combinations of four fragments. The CDKs are a family of serine-threonine protein kinases that regulate elements in cell cycle progression. A common ATP kinase binding site was observed in ~ 30 co-crystal structures generated against the screening hits. Four low molecular weight fragments were identified as weak potency hits with good ligand efficiencies and a suitable synthetic handle for elaboration. Key hydrogen bond interactions to the backbone residues at the hinge region of CDK2 (Glu₈₁ and Leu₈₃) were conserved in all the bound fragments.

Having co-structures of fragment hits provides a greater opportunity to select the best such hits to be considered for lead optimization. Suitable vectors to access key regions, synthetic flexibility, and novelty are a few considerations in selecting fragments for further consideration. Compound **8** had an amine group that was suitably functionalized for picking up interactions with other residues on the protein. Hydrophobic substitution via an aryl group resulted in **9** with a 150-fold increase in activity. Further introduction of a sulfonamide at the 4-position yielded **10**, which showed a hydrogen bond interaction with Asp₈₆ and a further increase of activity ($1.9 \mu\text{M IC}_{50}$, $\text{LE} = 0.43$) [71].

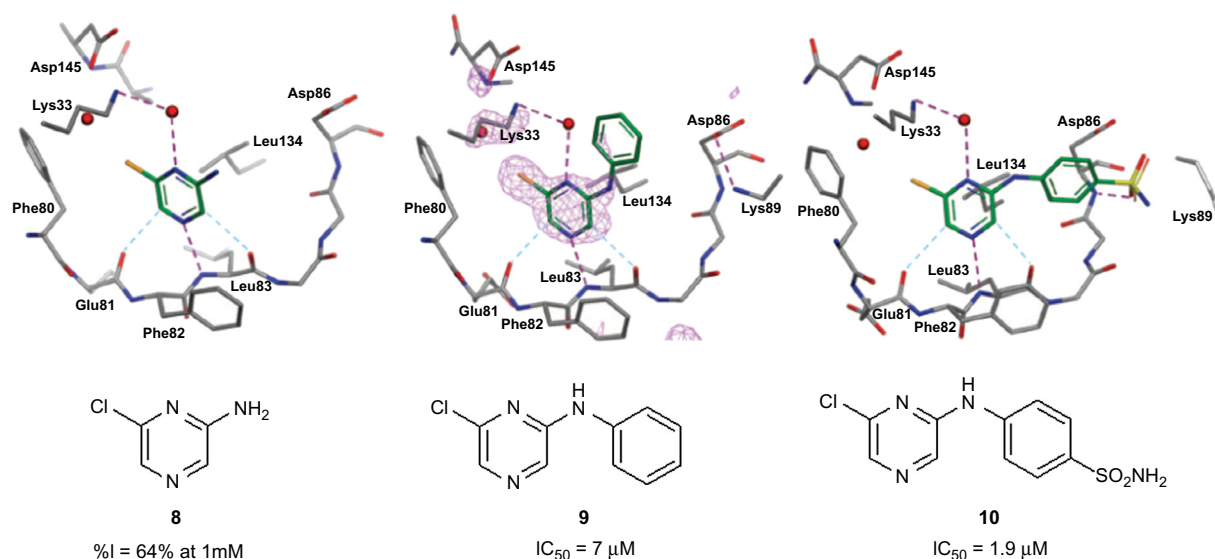


FIGURE 7.8 Lead optimization using fragment 8 guided by X-ray crystallography. Reprinted with permission from Wyatt PG, et al. Identification of *N*-(4-piperidinyl)-4-(2,6-dichlorobenzoylamino)-1*H*-pyrazole-3-carboxamide (ABT7519), a novel cyclin dependent kinase inhibitor using fragment-based X-ray crystallography and structure based drug design. *J Med Chem* 2008;51:4986. Copyright (2013) American Chemical Society.

Similarly, substitution of **11** at the 7-position with a hydrogen bond donor picked up an interaction with the protein back bone at the hinge region through Leu83 (Figure 7.9), increasing the potency 700-fold as in **12**. Further introduction of basic functionality off the 5 position led to **13** with a further increase in potency (0.03 μM IC₅₀, LE = 0.45). An X-ray crystal structure revealed that the 4-amino group formed hydrogen bonds with the carboxylates of Asp₁₄₅ and Asn₁₃₂.

Structure-based lead optimization improving biological activity and drug-suitability ADME properties led to the discovery of ABT-7519 (**14**) (Figure 7.10), which is currently in Phase I/II clinical trials [4]. X-ray crystallography in FBDD has been extensively employed in additional drug discovery programs [72–75].

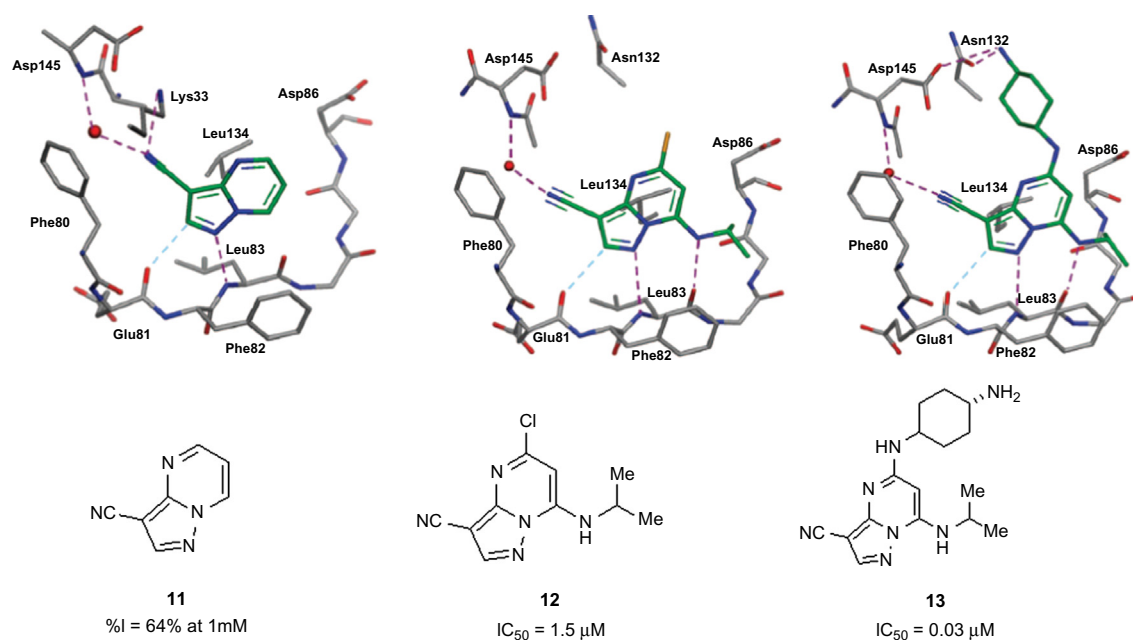


FIGURE 7.9 Lead optimization using fragment 11 guided by X-ray crystallography. Reprinted with permission from Wyatt PG, et al. Identification of *N*-(4-piperidinyl)-4-(2,6-dichlorobenzoylamino)-1*H*-pyrazole-3-carboxamide (ABT7519), a novel cyclin dependent kinase inhibitor using fragment-based X-ray crystallography and structure based drug design. *J Med Chem* 2008;51:4986. Copyright (2013) American Chemical Society.

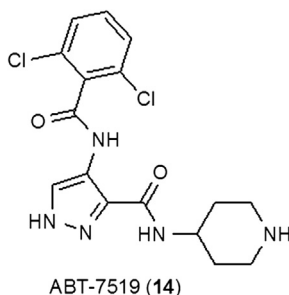


FIGURE 7.10 Structure of ABT-7519 (14).

V. OTHER BIOCHEMICAL AND BIOPHYSICAL METHODS

The value of screening fragments to obtain hits of low molecular weight and high LE is somewhat intuitive. However, the key has been to identify and exploit screening technologies that can detect the weak activity or binding that is inherent in the fragment-based approach. In addition to NMR and X-ray crystallography, several other approaches are now adapted for FBDD.

A. Substrate Activity Screening

Substrate activity screening is a fragment screening strategy in which substrates for a particular target protein are identified and then optimized rapidly [76,77]. For example, substrates for the cysteinyl protease cathepsin S bearing a fluorogenic group were optimized for cleavage [78]. Enhancement of substrate activity determines what structural features improve the binding interaction on the C-terminal side. After substrate activity is optimized, the scissile bond is modified to obtain an inhibitor, such as by the use of bioisosteric replacement of the scissile bond. For cathepsin S, a 15 nM K_i inhibitor was identified that was selective ($>1,000$ -fold) relative to other cysteinyl proteases such as cathepsins B, L, and K.

B. SPR Spectroscopy

In most SPR spectroscopy cases, a protein is immobilized onto a metal-coated chip and ligands are allowed to flow past on it. Once the binding occurs, the change in the ligand-protein mass causes a change in the reflective property of the metal. An SPR biosensor chip is usually made of gold and has a target biomolecule covalently bound. The solutions of single fragments are then passed over it sequentially and as they bind to the biomolecule an increase in the surface mass is detected in real time [79–83]. Binding affinity and kinetics of fragments with molecular weight <100 Da can be calculated from the time-dependent fragment association-dissociation. A known ligand is tethered on the surface and the protein is allowed to complex, then a competing fragment is identified when decomplexation of the protein from the surface is detected. This technique can screen fragment libraries with as little as 25–50 μ g of protein [79]. An alternate technology has been reported where the ligands are immobilized and assessed for the binding of the protein [84,85].

C. SAR by Mass Spectroscopy

Mass spectrometry techniques have been used as a fragment screening method where covalent or relatively-strong noncovalent ligand–protein complexation is detected using soft ionization electrospray mass spectrometry. In one variant, libraries of sulfhydryl-group-containing (–SH) fragments are incubated with protein targets mutated to incorporate Cys residues near the active site [86,87]. Those that demonstrate affinity for the protein may orient in such a way as to form a covalent disulfide bond with neighboring cysteine residues, which are then detected by MS analysis. This approach has a limitation where a sulfhydryl group may itself perturb the binding.

D. Interferometry and Isothermal Titration Calorimetry

Isothermal titration calorimetry is similar to SPR, involving changes in refractive index after binding to fragment leads [88,89]. ITC allows determination of the enthalpic and entropic contributions of a ligand when it

binds to the protein and significant amounts of energy is released. Greater ligand efficiency is observed for fragments that bind largely via enthalpic interactions. This technique requires larger quantities of protein compared to other techniques, and has a low throughput [90].

E. Virtual Screening

Computational methods can be used to predict structural information and understand the molecular interactions of ligands and proteins. Several companies (BioLeap, BiosolveIT, and MEDIT) extensively use computational methods for FBDD [91]. A range of scoring functions and docking methods examine the binding of fragments and drug-like compounds with multiple targets. This docking technology was used to predict a pool of compounds that consisted of fragments whose binding with X-ray crystal structures were already known, and ~50 percent of fragments and drug-like compounds were correctly docked compared with their X-ray crystal structures. Factors such as the free energy change of the ligand, conformational entropy, water interactions, desolvation, and protein conformation changes are to be considered for producing the correct binding mode of the ligand that enables this technology to be used for screening [92].

VI. FRAGMENT MERGING/LINKING/GROWING

Once fragment hits are validated, they need to be elaborated to improve potency, selectivity, LE, and novelty. Often X-ray crystal structure data provide the ideal place to start the elaboration campaign. When no crystal structure data are available, a balanced approach of *in silico* docking and validation using biochemical experiments has to take place. Three main approaches that are followed in the elaboration cycle are:

- fragment merging;
- fragment linking; and
- fragment growing.

A. Fragment Merging

Fragment merging involves combining the common structural features of fragments, substructures, or known ligands that complex with the protein [16,93]. A hybrid series can be generated based on different compounds in a series that help to identify important binding patterns and interactions. One such approach is seen in the discovery of fragments targeting acetylcholine binding protein (Figure 7.11).

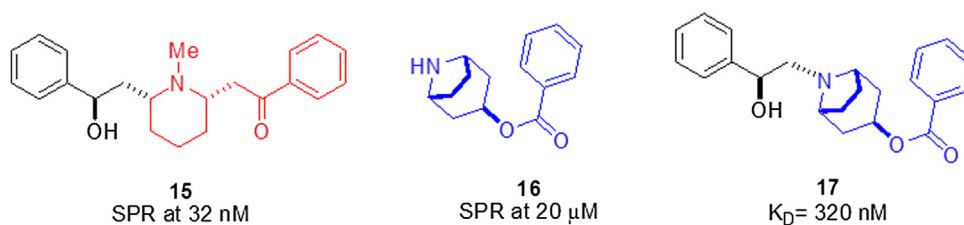


FIGURE 7.11 Fragment elaboration by fragment merging of 15 and 16 to give 17.

From the crystal structure, merged fragment 17 was identified with a K_D of 320 nM, identified by overlaying the crystal structure of the natural alkaloid lobeline (15, 32 nM) and a benzoate substituted nortropine fragment (16, 20 μ M) [93]. Other FBDD programs have utilized this approach to identify fragments against Hsp90, Jun N-terminal kinase JNK3, and P13 γ kinases [60,32,94].

B. Fragment Linking

Nonoverlapping fragments that bind at different sites (preferentially adjacent sites) can often be linked together to generate a new chemical series. This strategy, known as fragment linking, is the most common

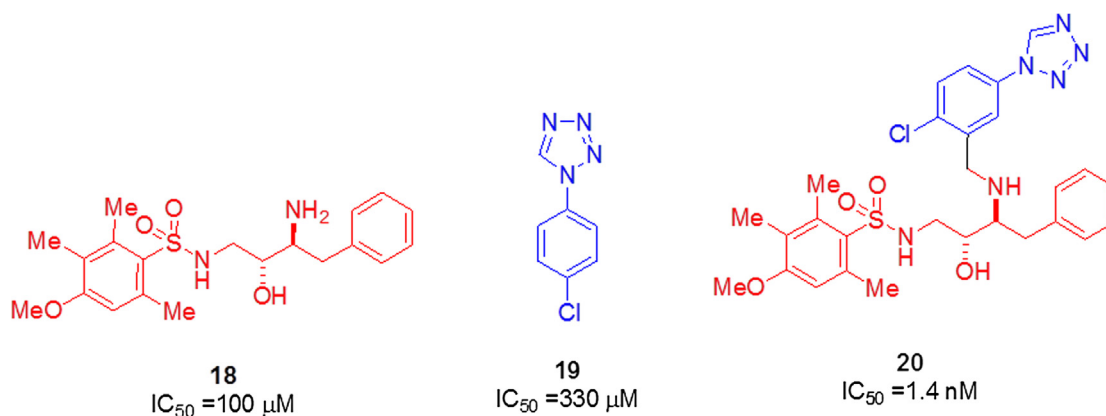


FIGURE 7.12 Fragment elaboration by fragment linking of 18 and 19 to give 20.

method of fragment elaboration. A compound derived by connecting the fragments should have a Gibbs free energy of binding better than the sum of the individual fragment binding energies. Fragments bind to the target with a particular conformation, and an ideal linker will allow the fragment in the combined derivative to still adopt the same conformation. Rigid linkers can reduce degrees of freedom and the entropic penalty upon binding event. On the other hand, flexible linkers can impart a greater number of rotatable bonds, causing an increase in the overall conformational space accessible both in the bound and solution phase, which can lower the selectivity for the particular target [95]. Often this strategy can help to identify hybrid series that are more potent than the fragments from which they were created. Howard et al identified 20, having a 1.4 nM IC_{50} against thrombin (Figure 7.12), designed by combining *p*-chlorophenyltetrazole 19 ($IC_{50} = 330 \mu M$) bound at the S1 site of thrombin to that of amino alcohol 18 ($IC_{50} = 100 \mu M$) bound at an adjacent pocket [96].

C. Fragment Growing

It is a common practice to use traditional medicinal chemistry methods of elaboration to build on the insight provided by the crystal structure coordinates of particular fragments bound into protein targets. Hydrogen bond donors or acceptors are used for growing at a particular site on the fragment to exploit possible electrostatic interactions with the protein, and the value of substitution is corroborated by the potency of this elaborated fragment. The choice of fragments and substitution is influenced and guided by the local environment around the binding pocket. Factors such as potency, LE, synthetic tractability, and other physicochemical properties are generally taken into consideration [97]. Finding an optimal balance of substitution during the elaboration step is always a challenge. Though addition of hydrophobic functionality is often preferred for increasing potency, factors such as solubility, lack of aggregation, nonspecific binding, and poor bioavailability are to be considered. Nevertheless, any change in a particular series of compounds should be validated with biological experiments and preliminary ADME studies.

For example, Potter et al disclosed that improved potency ($2 \mu M$ IC_{50}) was achieved for 22 via fragment growing starting from 21 (Figure 7.13). The initial phenyl imidazole fragment (180 μM IC_{50}) was elaborated by adding chlorine and a phenyl ethylamine group to give 22 with an IC_{50} of $2 \mu M$ [98].

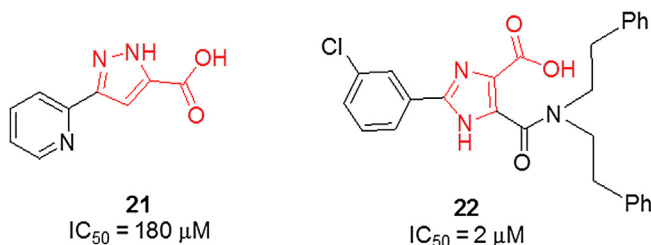


FIGURE 7.13 Fragment elaboration by fragment growing of 21 to give 22.

VII. FRAGMENT HIT FOLLOW-UP, AND PITFALLS TO AVOID

A. How to Best Reduce False Positives (NMR, MS) and False Negatives (X-ray)

Ligand-based NMR suffers from nonspecific (i.e., nonstoichiometric) fragment binding. Although protein-based NMR is much less sensitive to such false positives in fragment-based screening, this technique is limited to the particular proteins to which it can be practically applied. Functional assays suffer from the effects of both high fragment concentration (0.2–1 mM) in the presence of nM protein concentrations, and from an inability to determine protein integrity after the assay is complete.

Past experience would suggest that the intersection of fragment hits from ligand-based NMR and those from a functional assay screen can produce a set of fragments with a rate of false positives suitable for immediate characterization by X-ray crystallography. However, fragment hits identified by only one screening method have been observed to present a significantly higher rate of false positives, and may therefore benefit from further biophysical characterization prior to crystallography, which may take the form of assessing dose-dependency, estimating the stoichiometry of binding, and estimating the potency of binding. Protein-based NMR, SPR, and differential scanning calorimetry (DSC) are potentially capable of providing information on one or more aspects of the further characterization.

B. Isothermal and Isothermal Titration Calorimetry and Further Secondary Analysis

SPR uses only unlabeled protein but requires that the protein retains binding upon being tethered to the SPR surface. ITC uses only unlabeled protein but may suffer in reliably quantifying weakly binding interactions. Protein-based NMR requires labeled protein and a high-resolution 2D ^1H – ^{15}N or ^1H – ^{13}C correlation spectrum. Both ITC and protein-based NMR methods require substantially more protein than SPR. All three methods can measure the dose-dependency of fragment binding and could therefore in principle yield an estimation of binding potency. For protein-based NMR, such measurements are extremely time consuming and have an implicit sensitivity that is maximally constrained by the extent of a net protein chemical-shift perturbation induced by 100 percent fragment occupancy. For ITC, the overall weak sensitivity of the measurement is often such that even establishing binding at a single fragment concentration is difficult. In general, SPR appears to be the most universally applicable technique for establishing dose-dependent binding or a dose–response binding curve. SPR sensitivity is governed by changes in molecular weight upon fragment binding, and is therefore expected to be more predictable from fragment to fragment for a given protein. Only small amounts of unlabeled protein are required, typically <1 mg for 100 fragments.

In terms of the stoichiometry of fragment binding, the most important requirement is that a sufficient level of fragment binding occurs at a single protein site; how many additional protein sites experience some level of occupancy is largely irrelevant. Therefore, only approaches that can discriminate between different populations of either bound fragments or fragment binding sites can provide such information. Protein-based NMR is one such approach, and competitive displacement ligand-based NMR is another. However, neither SPR nor ITC can provide such discrimination. While it is true that the total signal intensity measured in an SPR experiment can provide some insight as to whether fragment binding is a single or multiple event, this information provides no insight into either the total number of binding events or the relative proportion of each such event.

While the above discussion is focused mainly on approaches to triage a set of primary fragment hits down to select fragments most likely to yield an X-ray co-crystal structure, there is the added complication that the further optimization of any such fragment, whether it yields an X-ray co-structure or not, is dependent on readily being able to measure some parameter that is highly correlated with the binding potency of that fragment. An interesting point to realize is that a comparison of two different fragment co-structures with a given protein does not allow one to infer the relative binding potency of these two fragments. Therefore, developing fragment SAR based solely on co-crystal structures is not possible.

Once binding potencies of <10 μM have been achieved, the ability to develop fragment SAR is more likely, as this is the potency range in which most HTS hits fall already. The challenge lies in developing SAR for those select fragments whose binding potency remains >10 μM . As fragments bind to proteins with a dissociation constant of 1 mM, many of them are not soluble at these concentrations. It is important to confirm that the fragments are soluble at the concentrations in the buffer solutions used for screening.

C. Pitfalls to Avoid

Lack of structural integrity of the fragment library can generate a false positive signal, and even 1 percent of a reactive impurity can contribute up to 10 μ M in a fragment screen performed at 1 mM. False positives can be obtained with certain reactive functionalities and metal impurities that are known to interfere with the biochemical and biophysical methods used in the screening process. Certain compounds in screening libraries act as oxidizers generating hydrogen peroxide under the assay conditions, inactivating the protein. For example, **23**, **24**, and **25** are known to inhibit PTP1B by generating hydrogen peroxide under certain buffer conditions, which keeps the protein in a reduced state (Figure 7.14) [99,100].

These compounds can be reoxidized by ambient oxygen to generate hydrogen peroxide. Structurally similar compound **26** was not checked for hydrogen peroxide generation and was claimed to be a novel protein–protein inhibitor [101]. Later it was discovered that the activity was due to the generation of hydrogen peroxide [102–104].

Many small molecules tend to aggregate at higher concentrations and can affect the inhibition of proteins. Often, screens are conducted at higher concentrations to identify low affinity binders, and aggregation can lead to a false positive signal. It can be difficult to predict aggregation *a priori* based on the structure, and in fact approved drugs benzyl benzoate (**27**) and mefenamic acid (**28**) form aggregates at higher concentrations (Figure 7.15) [105].

It may be possible to prevent aggregate formation by adding small amounts of non-ionic detergent to the buffers used in compound-library screens [106].

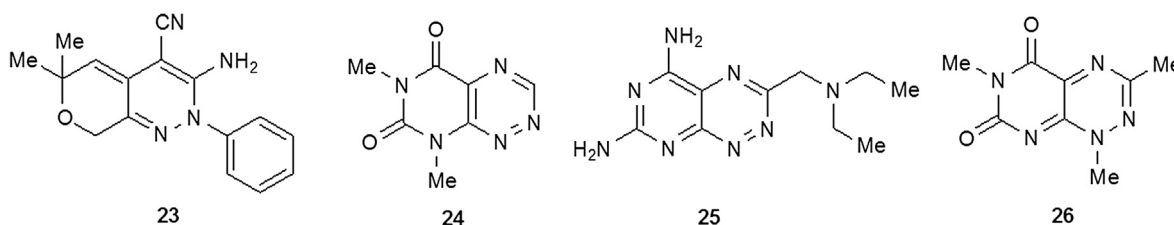


FIGURE 7.14 Agents known to generate hydrogen peroxide under biochemical assay conditions.

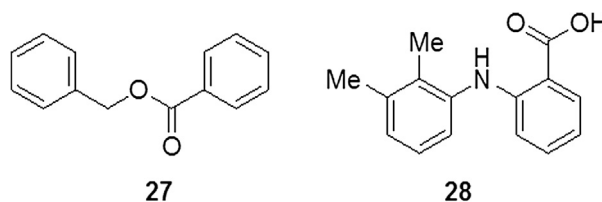


FIGURE 7.15 Drugs known to form aggregates at high concentrations.

VIII. ZELBORAF[®], FIRST APPROVED DRUG FROM FBDD

FBDD has achieved a major milestone with the approval of the first drug approved using this method, Zelboraf (PLX4032, **32**, Figure 7.16) [107]. It is a selective kinase B-Raf enzyme inhibitor that is used for the treatment of metastatic melanoma. The goal of the PLX4032 project was to develop inhibitors that were selective for Raf kinases, particularly the oncogenic V600E mutation. Fragment screening initially showed activity against other kinases like Pim-1, but hit to lead medicinal chemistry led to the desired selectivity for the Raf kinases. A selected library of 20,000 scaffolds with MW 150–350 Da were screened at a concentration of 200 μ M in functional assays against well-characterized kinases, and the initial hits were used for co-crystallography. 7-Azaindole **29** was elaborated using the bound co-crystal structure that appeared to have multiple binding orientations. Compound **30** was identified as the next fragment with increased potency and single binding mode. Subsequent medicinal chemistry led to the discovery of PLX 4720 and PLX 4032 with good selectivity against B-Raf and the V600E mutant, and were selective compared to other kinases [108]. Zelboraf (PLX4032) is a good example for fragment elaboration where selectivity was achieved during the lead optimization process, which may be the result of the appropriate selection of fragments during FBDD.

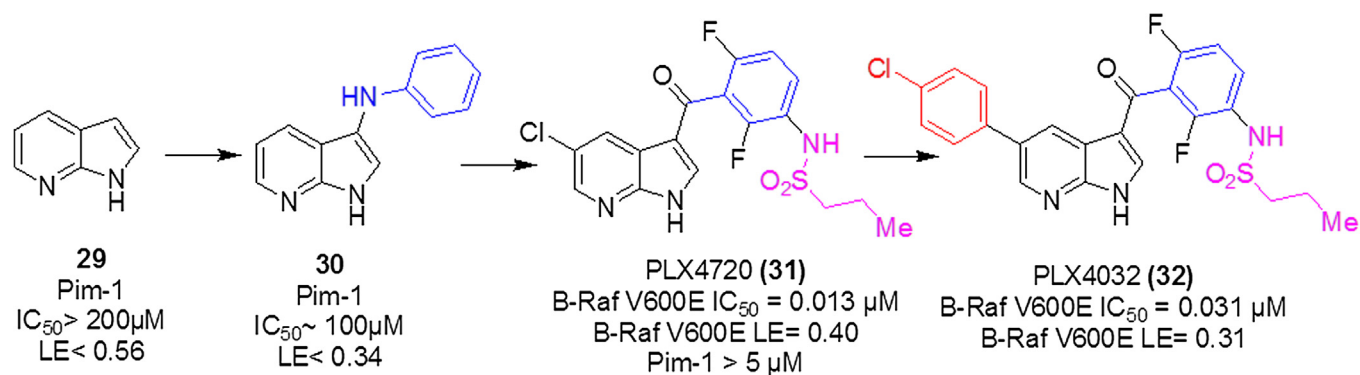


FIGURE 7.16 Zelboraf (PLX4032) as the first FDA approved drug discovered by FBDD.

IX. LIMITATIONS OF FBDD

An important caveat to FBDD is that fragments will not always orient in the same way individually as when combined together in an optimized structure. For example, β -lactamase inhibitor **33** was conceptually deconstructed into fragments **34**–**37**, which were prepared and individually evaluated for functional activity and binding mode by ligand–protein X-ray crystallography (Figure 7.17). Fragments **34**–**37** were found to bind to the protein in very different orientations when compared to the binding mode of **33**. In fact, **34** and **35** induced a conformational change in the protein itself and mapped into a previously unidentified tunnel carboxylate site. Fragment **34** was also seen to adopt a second binding mode that, along with **36**, bound into a different new distal carboxylate recognition site [109].

Low potency fragments are unsuitable for both whole cell screening and kinetic assays as an mM potency would require high fragment concentrations, which can result in false positives. Though widely applicable, FBDD failed to generate validated hits against an antibacterial target shikimate kinase obtained from *M. tuberculosis* [5].

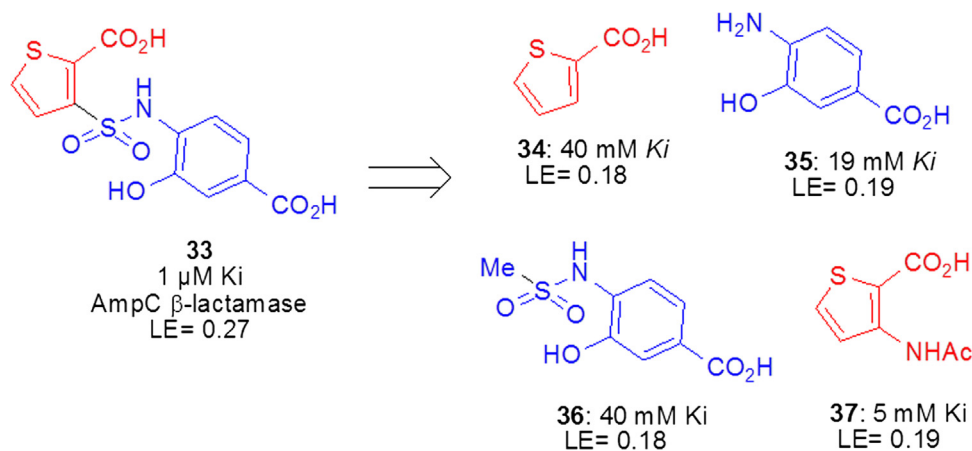


FIGURE 7.17 Elaborated fragment **33** lacked expected orientation as seen by individual fragment **34**–**37**.

X. TRENDS FOR THE FUTURE

FBDD has become an accepted approach at the early stage of many drug discovery programs. The past decade has seen major advances in fragment screening technologies, such as X-ray crystallography, NMR spectroscopy, and SPR. All of these seek to achieve a sustainable delivery of high-quality lead candidates that can be reliably progressed into the lead development and clinical pipeline. While new technologies facilitated fragment screening, the concepts of FBDD are still being applied on complex biological targets. In the future, FBDD will be adapted to more challenging targets, such as oligonucleotides, allosteric inhibitors, and protein–protein interactions.

Fragment libraries from both in-house and commercial sources have to be evaluated carefully. Most of these libraries lack extensive structural diversity and can contain flat heterocyclic molecules with modest overall complexity.

Limitations of NMR and X-ray screening methods are to be kept in mind when evaluating shallow surface exposed and highly hydrophobic pockets. A single-pocket binding site with one large, diffuse pocket is less preferred than a multi-pocket binding site with several smaller, more constrained subpockets. Better understanding of the target binding sites either through NMR or X-ray is needed to evaluate the quality of hits in guiding the lead optimization process. Extensive use of certain fragments, substructures, and substitution patterns has assisted in identifying novel fragment core structures for intellectual property protection.

Though structural data plays a crucial role, the techniques involved have some limitations. Characterization of targets like membrane proteins is difficult, as they are tough to crystallize and subject to functionally-relevant conformational changes not likely to be picked up by FBDD [110]. While identification of novel drugable targets and the application of FBDD to challenging targets is highly innovative, it is often challenged by an unpredictable and low success rate, limited resources, funding, and luck. X-ray crystallography remains the best tool for obtaining structural information of protein–ligand interactions and is an essential tool for FBDD.

While 2012 saw thirty-five new drugs entering the market, these would have entered clinical development three to seven years ago. Many pharmaceutical companies have downsized research operations and have outsourced jobs to low-cost providers as the result of many discovery programs not yielding the expected FDA approvals. This has resulted in greater opportunity for smaller biotech companies, academic labs, and research facilities to contribute and perform with limited resources. The concept of FBDD to start smaller and simpler has been validated, allowing smaller companies to address more versatile and challenging targets in the field of drug discovery.

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