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Perspective

Fragment-Based Drug Discovery

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1. Introduction

The pharmaceutical industry's ability to produce new medicines is directly tied to its success in identifying druglike molecules that target clinically relevant pathways. As a consequence, the research community is constantly seeking to expand and improve its repertoire of lead identification strategies. Most medicinal chemistry leads (i.e., molecules that demonstrate activity in a relevant in vivo model) are evolved from "hits" obtained by screening collections of compounds against functional assays. Chemical optimization strategies can often improve hits' target affinity by 100- to 1000-fold (corresponding to approximately 2.8−4.2 kcal/mol of binding energy) while maintaining their druglike properties. Validated hits are thus typically required to demonstrate functional activity at low-micromolar to high-nanomolar concentrations (corresponding to 6.8−9.5 kcal/mol of binding energy). Since valid hits must possess nearly two-thirds of the net binding energy of fully optimized leads, the industry has traditionally built large collections of highly functionalized compounds in an attempt to identify sufficient numbers of hits.

The rise of combinatorial chemistry and advances in high-throughput screening (HTS) have allowed pharmaceutical companies to develop increasingly large screening libraries. But even the largest conceivable compound collections fall far short of potential chemical diversity space, estimated to be upward of 10^{60} molecules containing up to 30 non-hydrogen atoms.[1] As molecular size decreases, however, the number of possible molecules decreases exponentially, so in theory it would be more efficient to screen collections of very small molecules (or "fragments") and subsequently expand, merge, or link them. Jencks provided a theoretical framework for this approach more than 2 decades ago,[2] and Nakamura and Abeles provided experimental support in 1985 with their work on the enzyme HMG-CoA reductase.[3] However, the difficulties of identifying weak-binding fragments and elaborating or linking them into high-affinity binders remained formidable challenges.

The 1996 publication by Shuker, Hajduk, Meadows, and Fesik at Abbott Laboratories of the SAR by NMR method (structure−activity relationships by nuclear magnetic resonance)[4] reignited interest in the general idea of discovering drug leads by first identifying discrete components showing molecular recognition at a given target. In these so-called "fragment-based" approaches, low molecular weight chemical fragments are initially selected on the basis of their ability to bind to the target of interest or to inhibit it in a functional assay. These fragments, which can be considered the building blocks of a more complex lead series, are then combined or optimized into compounds that meet or exceed the criteria typically applied to HTS hits. The rationale behind these fragment-based strategies makes intuitive sense; many drug targets contain discrete subsites for binding ligands, substrates, and/or cofactors. Furthermore, many drugs possess modular architectures in which specific components can be replaced by bioisosteres, and optimization strategies often entail the search for these molecular synonyms. When applied to

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well-characterized targets such as enzymes, fragment-based methods offer the possibility of identifying novel molecules with improved affinity, selectivity, and pharmaceutical properties. Moreover, smaller fragments are less likely to contain interfering moieties that block an otherwise attractive ligand−protein interaction, so optimal binding elements are less likely to be hidden by nonbinding elements.[5] In theory, fragment-based strategies also provide a combinatorial advantage relative to preassembling large chemical libraries. To exhaustively explore all two- or three-component combinations of N recognition elements, a total of N^2 or N^3 discrete molecules would have to be constructed and screened. Identifying which of the N recognition elements complement a particular target prior to building a library would reduce this combinatorial complexity. Thus, in principle, fragment-based strategies can sample a larger theoretical "diversity space" than is practical through standard screening methods.

Despite these theoretical advantages, fragment-based drug discovery can be difficult in practice.[6] Both the discovery of fragments and how to advance or link them are areas of intense research. In this Perspective we first review some of the major techniques used to identify and validate weak-binding fragments. We then explore a number of examples that illustrate strategies by which fragments have been developed into chemical lead series.

2. Techniques for Finding Fragments

The first step in fragment-based drug discovery is to develop libraries of fragments. Much has been written about the design of libraries for traditional HTS, and some of these considerations also apply to fragment libraries.[7] However, other considerations are unique to fragment libraries. For example, because fragments will ultimately be elaborated, they should be smaller than typical HTS compounds. Following Lipinski's famous "rule of five",[8] Jhoti and colleagues have proposed a "rule of three".[9] Fragments should have a molecular weight of <300, ClogP ≤ 3, and the number of hydrogen bond donors and acceptors should each be ≤ 3. Scientists at Vertex Pharmaceuticals computationally dissected known drugs into fragments corresponding to molecular frameworks and side chains; these analyses demonstrated that most drugs can be represented by a relatively small set of molecular architectures.[10,11] From this, they constructed a small library of fewer than 200 fragments specifically designed for NMR screening (see below), called a SHAPES library.[12] The compounds were chosen not just to represent fragments found in known drugs but to be soluble at high concentrations, nonreactive, and commercially available. Lewell and colleagues described the use of a "retrosynthetic combinatorial analysis procedure" (RECAP) to identify recurring fragments from known drugs.[13] Fesik and colleagues have proposed enriching fragment libraries with "privileged molecules," such as biphenyls, that have been experimentally shown to bind to proteins frequently.[14] Fragment library design has been reviewed more recently,[15] with the computational deconstruction of drugs into fragments remaining an active research focus.[16]

For targets with fairly rigid binding sites, "virtual screening" methods can be used to augment default libraries with fragments selected on the basis of their structural complementarity to the protein; one of the first methods described was the program DOCK.[17] The predictive utility of docking methods decreases with the conformational mobility of the protein and ligand, so these methods are ideally suitable for analyzing small, inflexible fragments.[18] In fact, one of the pioneering docking programs, LUDI, was designed specifically to identify and subsequently combine fragments that complement a user-specified site on a protein.[19] Other computational approaches include the linked-fragment method proposed by Verlinde,[20] Dean's fragment-based automated site-directed drug design,[21] Karplus's dynamic ligand design,[22] Caflisch's technique for docking both polar and nonpolar fragments,[23] and Leach's "core template" algorithm that connects two or more fragments.[24] Purely computational approaches to drug discovery, including fragment design and discovery, are increasingly important but are beyond the scope of this Perspective. However, many of the applications discussed below rely on computational methods to prescreen fragments or to aid in their optimization and linkage.

With a collection of actual fragments in hand, there are several screening methods available, including functional screening, nuclear magnetic resonance (NMR), mass spectrometry (MS, both noncovalent and covalent), and X-ray crystallography. Each of these methods will be covered separately; their strengths and weaknesses are summarized in Table 1.

2.1. Functional or Direct Binding Assays at High Concentrations. The most conceptually straightforward approach to identifying fragments is through a functional screen. However, since fragments usually have relatively low binding affinities (often in the millimolar range), they are difficult to detect in typical HTS assays where compound libraries are usually screened at low micromolar concentrations. One way to overcome this liability is to screen fragments at high concentrations, as described by the Ellman group.[25] A set of small fragments with a common linkage group was screened in a functional assay at 1 mM to identify inhibitors of the kinase c-Src, an important oncology target. Fragments with activity were then joined via the common linkage group using five different flexible linkers. These were then rescreened to identify the most potent inhibitors. Not only were Ellman and co-workers able to identify inhibitors of c-Src with nanomolar potency, the compounds also displayed greater than 75-fold specificity against the related Fyn, Lyn, and Lck enzymes. Other applications of the technology were demonstrated for the enzymes gelatinase B (MMP-9)[26] and tyrosyl protein sulfotransferase,[27] but in these cases the most potent inhibitors found were only low- to mid-micromolar.

A conceptually related approach has been described by Graffinity Pharmaceuticals. In this technique, each small fragment is immobilized on a microarray, the target protein is added to the chip (usually less than 1 mg of protein), and binding of protein to each fragment is analyzed by surface plasmon resonance (SPR).[28] Selected ligands can be optimized using standard medicinal chemistry approaches. Thus, this approach allows small fragments to be identified in the absence of

Table 1. Strengths and Weaknesses of Fragment Screening Methods Discussed in Text

	functional screening	SAR by NMR	MS methods (Tethering)	MS methods (noncovalent)	crystallography
total protein required	low (<100 μg)	high (>2 mg)	medium-high (0.5−5 mg)	medium-high (0.5−5 mg)	high (typically ≫1 mg)
instrumentation/cost	no special instruments/low	high-field NMR/high	mass spectrometer/high	mass spectrometer/high	X-ray detector/high
throughput	high	medium	medium	medium	low−medium
rate of false positives	high	low	low	low	low
need functional assay to initiate screen	yes	no	no	no	no
requires knowledge of protein structure	no	sometimes (higher resolution methods need NMR protein structure)	partial (requires cysteine residue in or near site of interest; modeling often sufficient)	sometimes (helpful for modeling fragments and predicting binding modes)	yes
requires knowledge of protein function	yes	no	no	no	no
limit to protein MW	no limit	ideally <40 kDa but up to 100 kDa	usually <100 kDa	usually <100 kDa	no limit
amount of information on binding mode obtained	none	medium to high (may not give exact binding mode, depending on preexisting knowledge)	medium (but since fragments covalently linked to target, crystallography facilitated)	low (competition binding experiments and modeling only; newer methods may be amenable to higher resolution)	very high
difficulty of library synthesis	medium (may need to generate library with common linkage group)	low	high (each fragment must contain a disulfide moiety)	low	low

a functional assay, although, like the Ellman method, it does not provide information on the binding site or stoichiometry. No specific examples of the application of this technology have been published.

The advantages of these approaches are threefold. First, in common with most fragment-based approaches, the number of fragments is significantly smaller than typically synthesized for traditional high-throughput screening. Second, the approaches do not rely on knowledge of the structure of the biological target, its mechanism of action, or established leads. Third, functional screening is potentially more likely to provide a functionally relevant inhibitor rather than a molecule that binds to but does not inhibit the target. However, the disadvantages of these empirical approaches are that they require that two fragments bind to adjacent but nonoverlapping sites and that optimizing or linking fragments in the absence of any structural knowledge can be difficult. Moreover, functional screening (especially at high concentrations) is subject to a number of pitfalls, as we will discuss later.

2.2. NMR-Based Screening. The breakthrough discovery approach, SAR by NMR, entails screening by NMR to identify small molecules that bind to a protein target.[4,29,30] First, a library of fragments is screened to identify those that bind to an ^{15}N-labeled biological target. When compounds bind, the resulting changes in amide chemical shifts around the binding site are detected in 2D heteronuclear single quantum correlation (HSQC) spectra. These data, combined with structural information, identify where on the protein a compound binds. Ligands that bind to adjacent sites on the protein are then selected and optimized. The 3D structures of the protein with the resulting ligands are used to either optimize individual fragments or to link fragments that bind in proximal locations. The modified or elaborated fragments are then tested for inhibition in functional assays.

Despite the distinct advantage of pinpointing ligand binding sites, this approach does have drawbacks. The technique requires a high-field NMR spectrometer and significant quantities of pure ^{15}N-labeled protein (the original applications required in excess of 200 mg), the protein targets should be less than about 40 kDa, and the ligands must remain soluble at high concentrations (>0.2 mM). Also, to achieve maximal structural information, the protein backbone residues must be assigned. Despite these limitations, NMR screening has been one of the most productive fragment-based approaches and has identified small-molecule inhibitors of a variety of targets such as FK-506 binding protein (FKBP),[4] stromelysin,[31] the human papillomavirus E2 DNA binding domain,[32] urokinase,[33] and protein tyrosine phosphatase 1B (PTP-1B).[34,35] Moreover, recent adaptations have improved both the efficiency and throughput of the technique. For example, new cryogenic NMR probes increase the signal-to-noise ratio in NMR spectra such that protein concentrations as low as 50 μM can be used to screen a pool of 100 ligands and still detect specific ligand binding with dissociation constants of 0.15 mM.[36] This throughput means that up to 10 000 compounds can be screened in a single day. Sensitivity has also been increased through ^{13}C labeling of the methyl groups of valine, leucine, and isoleucine residues.[37] By use of these improvements, larger proteins may be assayed (for example, dihydroneopterin aldolase, 111 kDa), and protein concentrations as low as 15 μM can be used for screening.

A recent adaptation from Novartis also improves the efficiency of NMR-based approaches by incorporating a "spin label" into one of the fragments whose binding site is known.[38,39] If a second fragment binds simultaneously to an adjacent location, the spin label causes a paramagnetic relaxation enhancement on the second ligand, which can be readily detected by NMR. The authors state that the increased sensitivity of this approach

reduces the amount of protein required by 1−2 orders of magnitude. In addition to Abbott and Novartis, other companies, especially Vertex Pharmaceuticals (see above), Hoffmann-La Roche (see below), and Triad Therapeutics,[40] are also using NMR for fragment-based drug discovery.

2.3. Mass-Spectrometry-Based Methods. Two main approaches have applied mass spectrometry to the discovery of weak binding ligands. Ibis Therapeutics, a division of Isis, has developed a method to detect the binding of noncovalent weak binding fragments to RNA by electrospray ionization mass spectrometry (ESI-MS).[41−43] By optimizing the ionization and desolvation processes, the researchers were able to characterize low-affinity complexes (in the millimolar range) formed between RNA and small molecules. On the basis of the observed mass and abundance of the complexes, the researchers were further able to directly determine both binding affinity and stoichiometry. This fragment discovery process ("SAR by MS") was applied to the discovery of lead compounds that inhibit the function of the bacterial 23S ribosomal RNA (rRNA), the target for the antibiotic thiostrepton. A synthetic 58-mer RNA containing the region the antibiotic interacts with was used for screening. Two series of fragments were identified: a D-amino acid and a quinoxalin-2,3-dione. MS competition experiments showed that the binding sites were nonoverlapping but close to one another. Linking members from both series produced more potent molecules (K_D = 6−50 μM) than any of the individual fragments alone (K_D > 100 μM). This success suggests that the approach may be effective for other targets, and indeed, researchers at Genentech have applied a mass spectrometry screening approach to find fragments that bind to proteins.[44,45] Several labs are exploring mass-spectrometry-based methods for studying protein−ligand interactions,[46] and some of these strategies may be applicable to fragment screening.

Another approach utilizing MS, Tethering,[47] was developed at Sunesis Pharmaceuticals to discover ligands that interact with a protein target at a specific site.[48] The technique relies on the formation of a disulfide bond (e.g., a tether) between the fragment and a cysteine residue in the targeted protein. If a native cysteine does not exist in the region of interest, one can be easily inserted by standard mutagenesis. The target protein is exposed to a library of disulfide-containing fragments (typically <300 Da). Fragments with the greatest affinity for protein sites in the vicinity of the cysteine form the most stable disulfide bonds and are rapidly detected and identified by mass spectrometry. Performing the screening experiments under partially reducing conditions ensures that the intrinsic binding properties of the fragment, rather than thiol reactivity, drive the selection process. Even though the identified fragments have weak binding affinity (often greater than 1 mM), their covalent bonds to the protein facilitate analysis by X-ray crystallography, which in turn facilitates optimization by standard medicinal chemistry and/or linking to fragments that bind to adjacent sites. Tethering has been successfully used to obtain potent small-molecule inhibitors of the enzyme thymidylate synthase[49] and inhibitors of the interaction between the cytokine interleukin-2 and the IL-2α receptor.[50] A related approach using reductive amination of imines and MALDI-TOF mass spectrometry has also been described.[51]

A variation of Tethering uses a known binding element to probe for fragments that bind to an adjacent location. The first binding element, or "extender", contains both a reactive functionality and a possibly masked thiol group. In the presence of the protein target, it covalently modifies a surface residue (such as an active site cysteine) while binding to a specific site on the target. Once the extender is covalently coupled to the protein and the newly introduced thiol group is deprotected, the protein/extender complex is screened against a library of disulfide-containing fragments as in Tethering. Subsequently, hits can be combined with binding elements from the extender to generate reversible inhibitors. This approach has been successfully used to identify potent small-molecule inhibitors of the cysteine proteases caspase-3 and -1.[52,53]

2.4. Crystallography-Based Approaches. X-ray crystallography can yield the most complete picture of fragment binding to a target. Like NMR, crystallography provides not merely a fragment detection method but also a means for optimizing fragments. An early criticism of crystallography as a screening method is that it can be very slow. With advances in robotics, X-ray technology, and computing power, solving crystal structures is becoming increasingly high-throughput, and today many labs are utilizing this technique to aid the discovery of small-molecule inhibitors.[54,55] Ringe and colleagues alleviated an initial concern that small, weakly binding fragments would have insufficient affinity to yield well-resolved electron density in a crystallographic structure by demonstrating that even simple organic solvent molecules could bind to specific sites on protein surfaces.[56,57] Stroud and colleagues then demonstrated that individual fragments of the substrate dUMP bound to the enzyme thymidylate synthase in positions similar to that of the full substrate.[58]

Researchers at Abbott Laboratories have applied crystallography to fragment-based drug discovery.[59] Using the anticancer target urokinase as an example, Nienaber and colleagues soaked a protein crystal with a mixture of diversely shaped compounds and then determined which bound by examining the shape of the electron density map. They successfully identified a hit, 8-hydroxy-2-aminoquinoline and, on the basis of the crystal structure and previous SAR, showed that a single modification would extend the fragment into an adjacent pocket. This modification, generating an 8-aminopyrimidyl-substituted 2-aminoquinoline, increased potency (K_i) from 56 to 0.37 μM.

An additional application of crystal soaking identified phosphotyrosine mimetics by soaking fragments into a crystal of Src kinase.[60] Of 150 small fragments tested, 20 produced crystals of diffraction quality showing the small fragment bound in the phosphotyrosine binding pocket. One of these fragments, a phenyl malonate, was then substituted into a known inhibitor scaffold, replacing its phosphotyrosine, to produce compounds with potency comparable to that of the parent inhibitor. Significantly, the fragment bound in the same manner whether it was free or elaborated into a larger molecule.[61] Plexxikon Inc. and Structural GenomiX are using a similar approach, whereby a target protein is

incubated with small druglike fragments with average molecular weights of 150−300 and crystallization trials conducted. To date, no specific examples have been published.

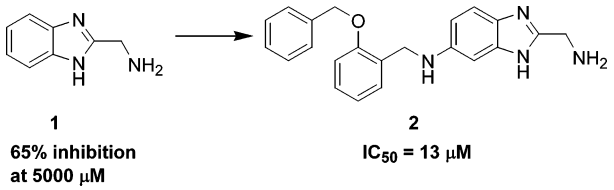
Astex Technology has used crystallography to identify small fragments that bind to cyclin-dependent kinase 2 (CDK2).[62] The researchers realized that two fragments that bind adjacent to each other could potentially self-assemble to generate more potent ligands if they had complementary chemical reactivities. In this approach, called dynamic combinatorial X-ray crystallography (DCX), crystals of a target protein are exposed to a library of small fragments capable of self-assembling, and the fragments that bind are identified by interpreting the electron-density maps. Their initial study was validated using known CDK2 inhibitors of the oxindole series, which could be "broken" into two fragments consisting of a hydrazine and an isatin. When CDK2 crystals were exposed to a dynamic combinatorial library mixture of these fragments, only the expected fully assembled ligands were observed in the electron density maps, and the SAR correlated with the observed in vitro inhibition activity. However, fragments may preassemble in solution before binding to the crystal rather than linking only after binding to the protein (see CDK2 section below). Either way, these results demonstrate the usefulness of X-ray crystallography for the identification of correctly aligned ligands from a dynamic combinatorial ligand (DCL) mixture, and the authors predict the approach will be broadly applicable to drug discovery.

3. Converting Fragments into Hits and Leads

The previous section dealt with identifying fragments. Only in the rarest cases will a fragment be as potent as a hit found through HTS. In this section, we discuss three broad strategies for converting fragments into hits and leads: fragment optimization, fragment merging or linking, and in situ fragment assembly. Fragment optimization closely resembles traditional medicinal chemistry, in which various substitutions or expansions are made to the initial hit (or fragment, in this case) in order to improve affinity and other properties. Fragment merging and linking generally involve combining elements from a fragment with elements from a known substrate, inhibitor, or another fragment to create a hybrid molecule. This approach can improve molecules' potency as well as physicochemical or ADME (absorption, distribution, metabolism, and excretion) properties. Finally, in situ fragment assembly, which encompasses such areas as dynamic combinatorial chemistry,[63,64] uses the target as a template for the synthesis of inhibitors from fragments. These three strategies serve as a useful organizing principle; in practice, they have considerable overlap. For example, fragment linking may involve fragment optimization, and in situ methods may include fragment optimization or fragment linking. In the following sections, we will consider a variety of examples in which fragments are identified and then converted to hits or even leads.

3.1. Fragment Optimization. Chemical optimization strategies typically focus on screening hits with low-micromolar or better affinities, but simpler fragments have also been successfully optimized. While these fragments may have low intrinsic affinities, they typically possess binding specificity sufficient to serve as viable anchors for subsequent derivatization. Once found, a fragment's potency can be directly optimized, blurring the distinction between fragment-based drug discovery and conventional medicinal chemistry. The following examples highlight instances where isolated fragments were successfully optimized to generate compounds that meet or exceed typical HTS criteria.

Scheme 1. Fragment Optimization, Gelatinase B



1 65% inhibition at 5000 μM

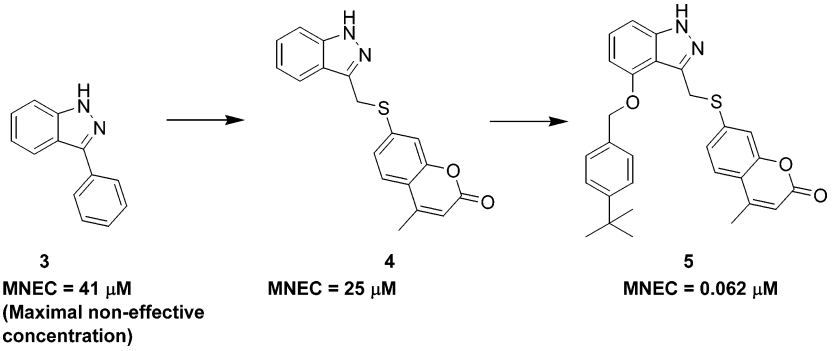
2 IC_{50} = 13 μM

3.1.1. Gelatinase B. The modular active sites of proteases lend themselves to inhibition by compounds that are built from discrete binding elements. Matrix metalloproteases contain a zinc ion that is essential for catalysis, and most inhibitors use metal chelation as a critical component of their binding affinity.[65,66] Ellman and co-workers screened gelatinase B (MMP-9) against 5 mM concentrations of very small (<150 Da) fragments containing a potential zinc-binding functionality and identified the aminomethyl benzimidazole **1** (Scheme 1) as a weak inhibitor.[26] Hypotheses regarding the expected binding mode of this chelator were used to focus the design of a combinatorial library of 176 compounds, of which **2** showed over 100-fold improvement in potency. Importantly, this molecule had attractive solubility and critical micelle concentration properties, suggesting that it inhibited via a specific rather than a pathological mechanism.

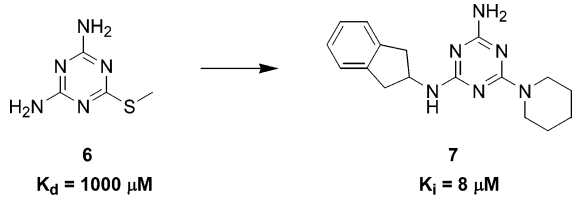
3.1.2. DNA Gyrase. Boehringer and colleagues at Hoffmann-La Roche[67] used computational methods to preselect fragments for functional screening against bacterial DNA gyrase. From an initial pool of 350 000 fragments, a few hundred were selected on the basis of their ability to complement the enzyme structure. Roughly 150 weak hits representing 14 unique structural classes were identified through functional screening. These hits were further characterized using analytical ultracentrifugation, NMR, and X-ray techniques to discard nonspecific inhibitors and to further characterize useful fragments. One of the more potent validated hits (**3**, Scheme 2) was further optimized, again with the aid of crystallography and NMR, ultimately resulting in compounds such as **5** with submicromolar efficacy in an in vitro supercoiling assay.

3.1.3. Erythromycin-Resistance Methylase AM (ErmAM). NMR provides a powerful tool for detecting specific, weak binding fragments and has been used extensively to screen and validate fragments. Fesik and co-workers[29] used NMR to identify fragments that bound to Erm methyltransferase (ErmAM), an important enzyme for antibiotic resistance. They initially discovered a small triazine (**6**, Scheme 3) with a dissociation constant around 1 mM. This scaffold is ideally suited for parallel chemistry, and the team rapidly generated low-micromolar inhibitors such as **7**. NMR structural studies revealed that **7** binds to the same location on the enzyme as the natural inhibitor *S*-

Scheme 2. Fragment Optimization, DNA Gyrase



Scheme 3. Fragment Optimization, ErmAM



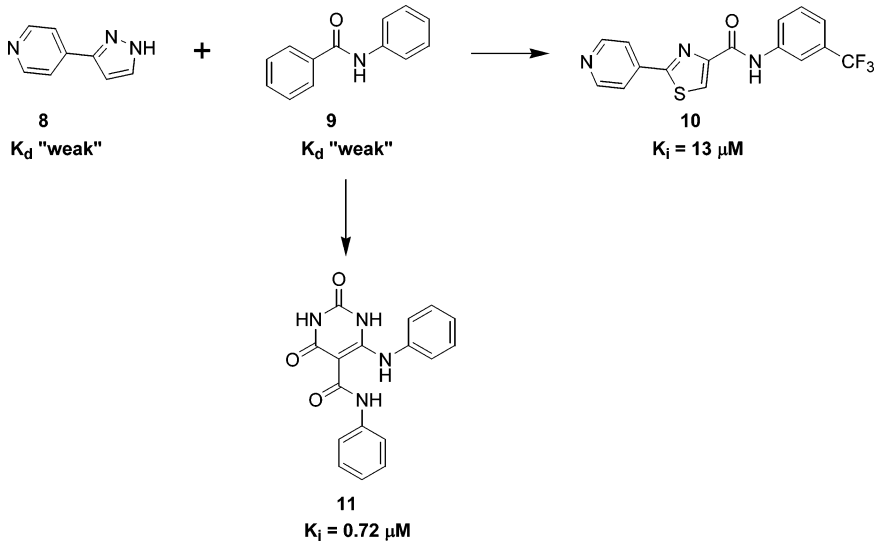
adenosyl-L-homocysteine, which has a K_i of 40 μM. An X-ray structure of a compound related to **7** confirmed this binding mode.

3.1.4. Jun N-Terminal Kinase 3 (JNK3). Vertex used NMR screening to identify inhibitors of the serine/threonine kinase JNK3, a target for neurodegenerative diseases including epilepsy and stroke.[68] An initial HTS screen failed to produce viable hits, so the Vertex SHAPES library[12] (see above) was screened using NMR to identify 17 fragments (such as **8** and **9**) that bound weakly to the enzyme. Competition studies with known inhibitors revealed that 13 of these occupied the ATP-binding site. Docking methods based on the crystal structure of JNK3 were used to propose potential binding models for these fragments. These models were used in turn to select or synthesize a set of several thousand compounds that contained elements from the original fragments. A number of potent hits such as **10** and **11** were obtained, as shown in Scheme 4. The authors note that compounds similar to the final hits were tested but not detected in the initial HTS screen, further illustrating a key advantage of fragment-based approaches: "simple SHAPES scaffolds are more likely to bind (albeit weakly) than the larger, more complex representatives typically found in our HTS library,"[68] in which additional functionality is more likely to disrupt than augment binding.

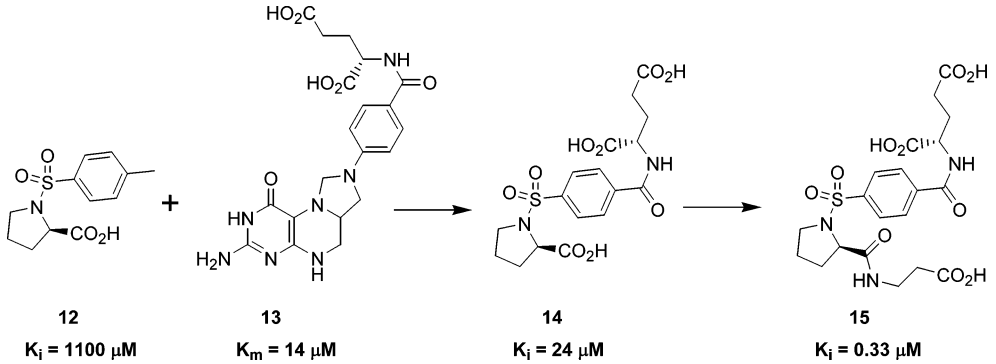
3.1.5. Thymidylate Synthase (TS). Other techniques can identify anchoring fragments suitable for optimization. Researchers at Sunesis used Tethering to identify *N*-tosyl-D-proline (**12**, Scheme 5) as a weak (millimolar) inhibitor of the anticancer and antimicrobial target enzyme thymidylate synthase (TS).[49] X-ray crystallography showed that the tolyl ring binds in a similar fashion as the *p*-aminobenzoic acid moiety of the enzyme's natural cofactor, (6*R*)-5,10-methylenetetrahydrofolic acid (mTHF, **13**). Appending the glutamate group from mTHF onto **12** to produce **14** led to a nearly 50-fold boost in affinity. Further elaboration to yield **15** increased the affinity another 70-fold, to 330 nM. In this example, **12**, **14**, and **15** were shown by crystallography to bind in very similar positions and orientations, illustrating that fragment binding, not disulfide capture, drove the selection and optimization process.

3.1.6. Urokinase. X-ray crystallography has also been used to identify fragments for subsequent optimization. Nienaber and colleagues at Abbott Laboratories screened a small library of 61 compounds against the serine protease urokinase, an oncology target.[59] Pools of six to eight compounds were each screened against nine different crystals. Despite the small library size, a

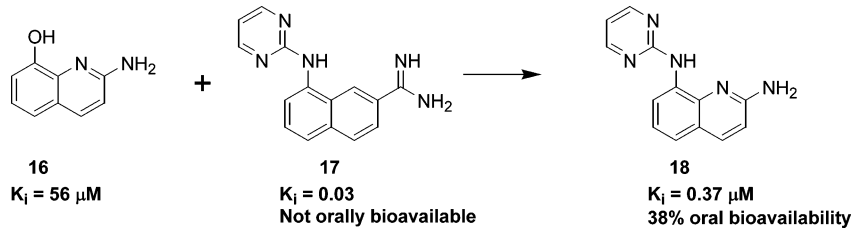
Scheme 4. Fragment Optimization or Merging, JNK3



Scheme 5. Fragment Optimization or Merging, TS



Scheme 6. Fragment Optimization or Merging, Urokinase



number of hits were identified, of which the aminoquinoline **16** (Scheme 6) demonstrated the greatest potency in an in vitro assay. Crystallography revealed that **16** bound in the same location as the naphthyl group of a previously discovered naphthamidine inhibitor **17**. Merging these molecules generated **18**, which shows a dramatic improvement in oral bioavailablility relative to **17**. Although the authors used a fragment-based approach, traditional medicinal chemistry could have arrived at a similar end point, illustrating that differentiating between fragment optimization and medicinal chemistry can sometimes be semantic.

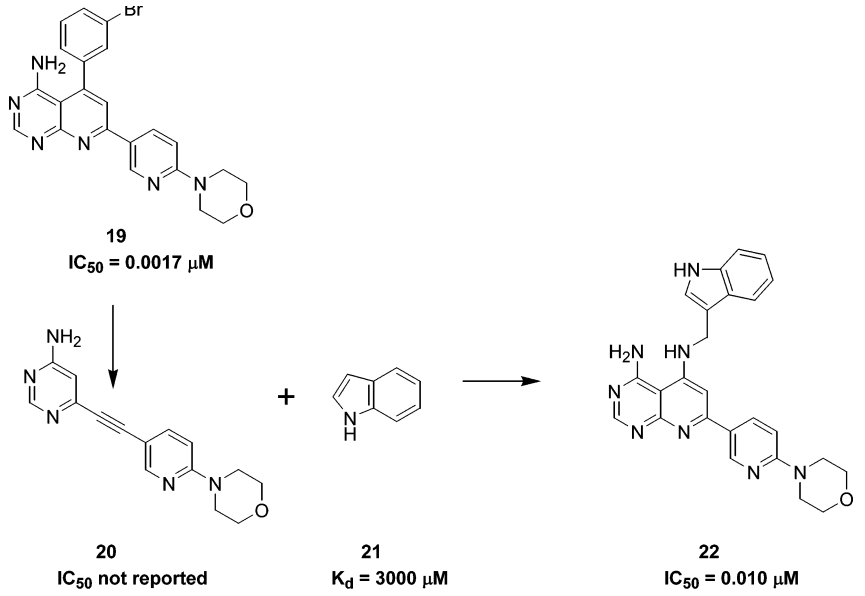
3.2. Fragment Merging and Linking. In most successful examples of fragment optimization, the anchoring fragments bound using discrete, specific contacts, and their binding modes were preserved throughout the optimization process. Known inhibitors or substrates also aided in the selection of initial fragments for screening and guided optimization, often by helping to circumvent undesired qualities. In the case of JNK3, the use of simpler fragments bypassed the steric interference that prevented more complex relatives from binding in an HTS screen. In the case of urokinase, fragment screening provided a strategy for improving the pharmaceutical properties of an existing lead series. In fact, some of the most successful applications of fragment-based methods have involved deconstructing known leads and reassembling them to generate a new chemical series with improved properties. The next several examples of fragment merging and linking illustrate such reconstruction processes.

3.2.1. Adenosine Kinase (AK). In general, the adenosine kinase (AK) inhibitors exemplified by **19** (Scheme 7) have poor solubility.[69] On the basis of known SAR, **20** was chosen as an anchoring pharmacophore and NMR was used to screen a library of 2000 small fragments (each at 5 mM) to identify alternative companion fragments that bound to AK in the presence of 1 mM **20**. The simple indole **21** bound with a low millimolar dissociation constant in both the presence and absence of **20** but was in competition with **19**. Chemical shift data further suggested that the soluble fragment **21** binds in the bromobenzene binding pocket of AK, and merging it onto the initial anchor produced the low nanomolar compound **22**. This hybrid molecule had lower potency than the starting molecule **19** but better pharmacokinetic properties.

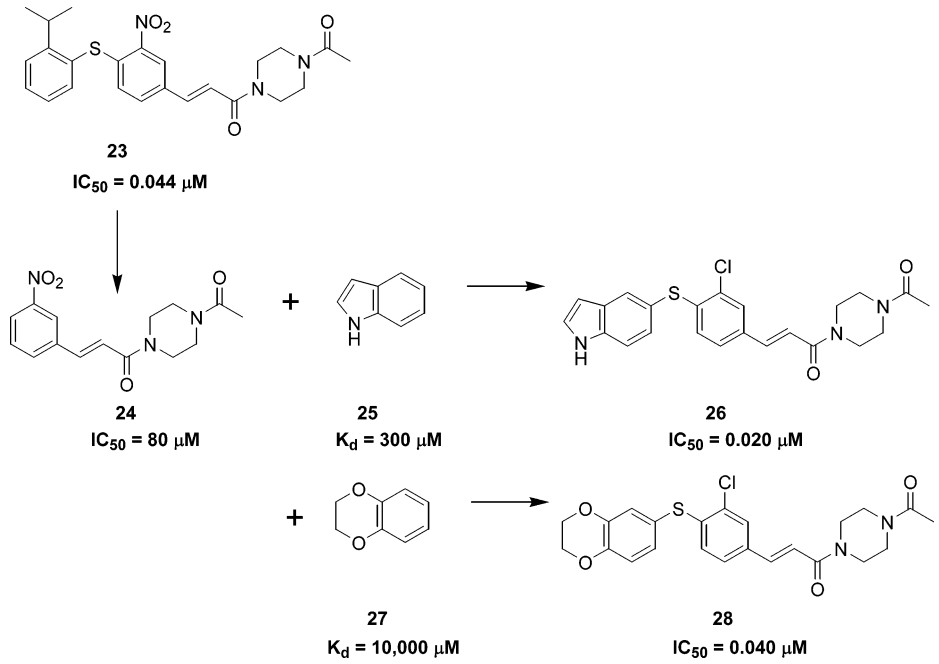
3.2.2. Leukocyte Function-Associated Antigen-1 (LFA-1). Liu, Huth, and colleagues at Abbott Laboratories used fragment merging to improve the properties of a series of LFA-1/ICAM-1 inhibitors.[70] A known diaryl sulfide lead **23** (Scheme 8) was found by NMR to bind to an allosteric site on the I domain of LFA-1. A model constructed from NMR-derived distance constraints pointed the isopropyl group of **23** toward two lysine residues, suggesting that the isopropylphenyl moiety could be replaced with a more hydrophilic alternative. In an NMR screen, 2500 fragments (each less than 150 Da) were tested in pools of 5 at 5 mM each against the I domain in the presence of 0.3 mM **24**. A number of fragments were identified, two of which (**25** and **27**) are shown in Scheme 8. When these were appended onto **24** to produce **26** and **28**, the full potency of **23** was recovered. The similar inhibition constants of **26** and **28** are interesting given the 30-fold difference in binding affinities of their constituent fragments. The solubility of **26** was similar to **23**, but that of **28** was 4-fold better. Moreover, while **23** showed no oral bioavailability, **26** had an oral half-life of 4.7 h, and **28** could be delivered both orally and intravenously because of its increased solubility.

3.2.3. Protein Tyrosine Phosphatase 1B (PTP-1B). The protein tyrosine phosphatase PTP-1B regulates phosphorylation of the insulin receptor and is a promising target for diabetes and obesity therapy. However, finding advanceable small-molecule hits for this target has been challenging. PTP-1B is readily expressed in large amounts and has been extensively characterized both enzymatically and structurally. In addition to the catalytic pocket, the substrate recogni-

Scheme 7. Fragment Merging, AK



Scheme 8. Fragment Merging, LFA-1

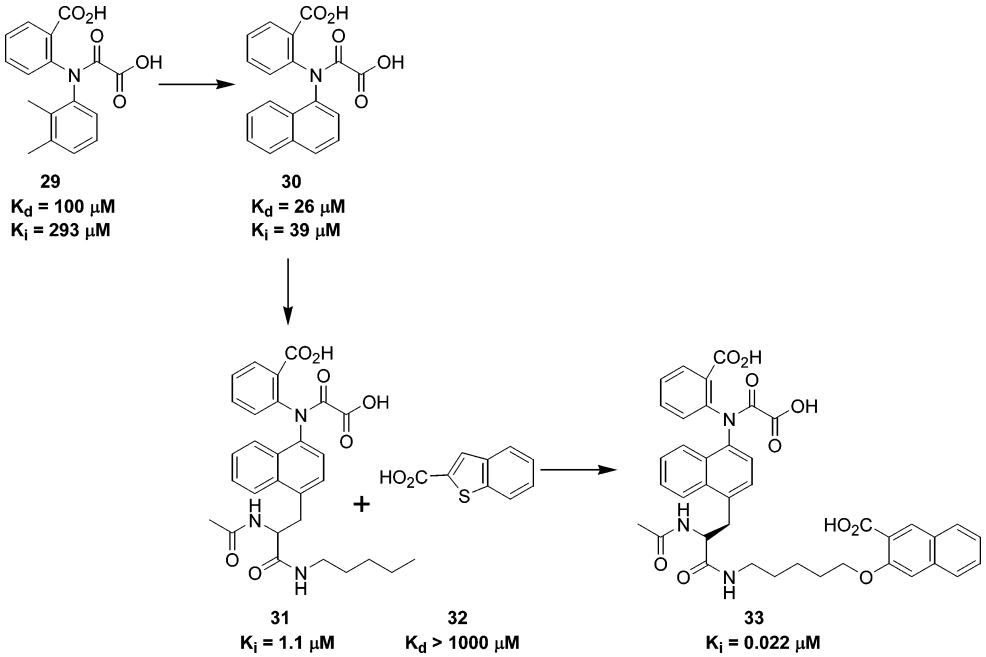


tion surface of PTP-1B contains an additional phosphotyrosine binding site,[71] and insulin receptor-derived peptides bind in a manner where adjacent phosphotyrosine side chains occupy these two sites.[72] Simple flexible compounds that link two phosphotyrosine groups (or close surrogates), while potent, have not proven to be robust starting points for drug discovery because of their excessive ionic character and sometimes unpredictable binding modes.[73−77]

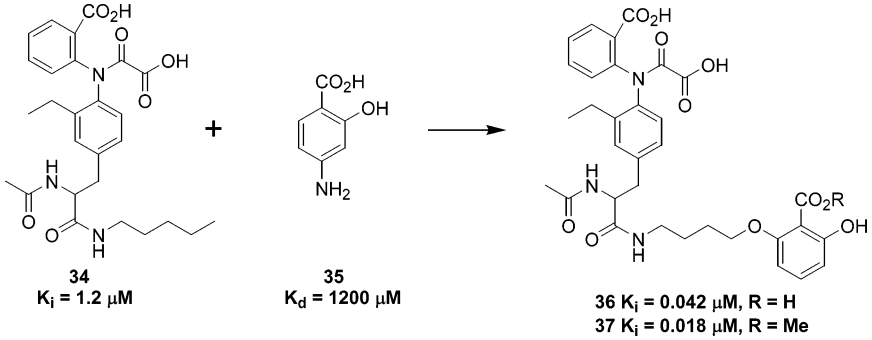
NMR-based methods have been used to identify potential lead series that occupy both sites and overcome the pharmaceutical liabilities of phosphotyrosine-like inhibitors. Szczepankiewicz and colleagues at Abbott Laboratories used NMR to screen a library of 10 000 compounds and identified **29** (Scheme 9) as a weak binder that showed inhibition in an enzymatic assay.[34] A limited optimization effort led to **30**, which was shown to be competitive and reversible. Structure-based optimization led to **31**, which extends a pentyl chain toward the second phosphotyrosine binding site. Again, by use of NMR, PTP-1B was rescreened against a library of 10 000 fragments to identify compounds that bound to this second site. A number of small acidic molecules were identified, including **32**. A closely related analogue of **32** was appended to **31** to generate **33**, which has an inhibition constant of 22 nM. Moreover, **33** demonstrated very high selectivity for PTP-1B over the phosphatases LAR, SHP-2, CD45, and calcineurin. In fact, a small (2-fold) selectivity was even observed over the very closely related TC-PTP.

In a related study,[78] Liu and colleagues used NMR to screen a 10 000 member library of <200 Da fragments to identify the salicylic acid **35** (Scheme 10) as a second site binder. When **35** was coupled to the active site binding fragment **34**, the resulting molecule **36** showed a nearly 30-fold boost in affinity. The methyl ester **37**

Scheme 9. Fragment Linking, PTP-1B



Scheme 10. Fragment Linking, PTP-1B

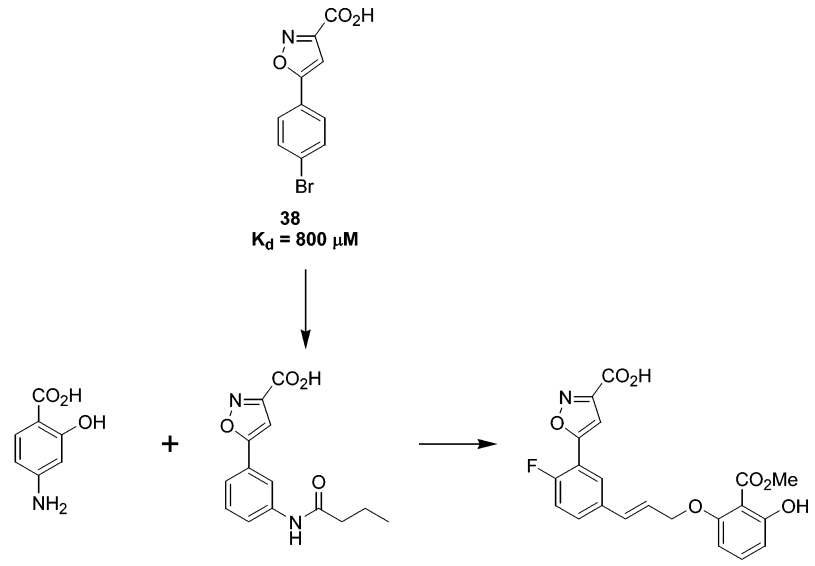


showed even greater potency. This is particularly important from an ADME perspective because any reduction in the highly anionic nature of most PTP-1B inhibitors is likely to improve their bioavailability. Unfortunately, **37** still showed very low Caco-2 cell permeability. To increase cell permeability, heterocyclic carboxylic acids were screened by NMR and the singly charged fragment **38** (Scheme 11) was found to bind to the active site.[35] Limited optimization led to **39**, and a crystal structure of **39** bound to PTP-1B suggested a linking strategy to the methyl ester of **35**. The resulting compound **40** displayed low micromolar potency. Moreover, this molecule showed more than 30-fold selectivity over the closely related target TCPTP and no inhibition of other phosphatases such as LAR, CD45, cdc25, and SHP-2 at 300 μM. The structure of **40** bound to PTP-1B validated the design: the heterocyclic acid binds in the active site, while the methyl salicylate binds in the second pTyr binding site. Significantly, **40** displayed cell permeability in a Caco-2 permeability assay and cell activity in a phosphorylation assay because of its improved physicochemical properties. Together, these papers describe a powerful iterative approach in which NMR screening identified an initial fragment, SAR by NMR was used to identify a second fragment to link to the first, and NMR screening was then used again to replace the initial binding element. A similar approach is described below with the target MMP-3.

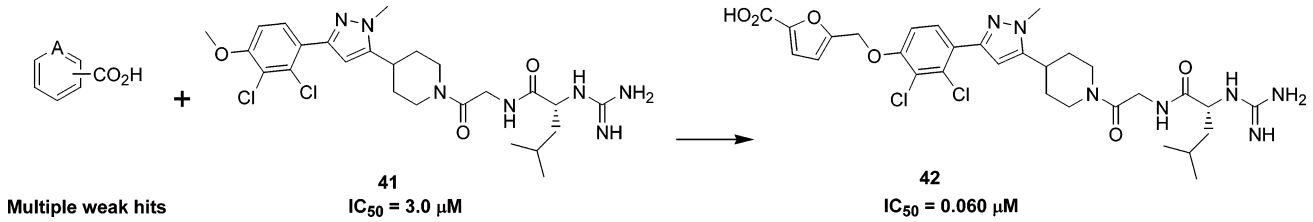
3.2.4. Interleukin-2 (IL-2). The extended and flexible binding interfaces of extracellular protein−protein interactions present thermodynamic challenges for high-affinity small-molecule binding. Inspired by the discovery by Tilley and colleagues of small-molecule inhibitors of IL-2/IL-2Rα,[79] a modular medicinal chemistry strategy was initiated that produced a low-micromolar inhibitor **41** (Scheme 12).[50] Considerable optimization attempts failed to generate analogues with improved affinity for IL-2, so Tethering was used to interrogate the surface of IL-2 surrounding the binding site for **41** in an attempt to identify additional contact points. The screening revealed a preference for small aromatic acids near the hydrophobic terminus of **41**. A simple library was constructed in which aromatic acids were appended to **41**, yielding analogues with 50-fold improvements in binding (e.g., **42**). These compounds also demonstrated activity in cell-based assays, illustrating the potential use of fragment-based approaches to direct chemical optimization strategies of primitive lead series.

The above examples illustrate various ways in which fragments can be merged with portions of known inhibitors to yield molecules with improved overall properties. Perhaps the most ambitious application of

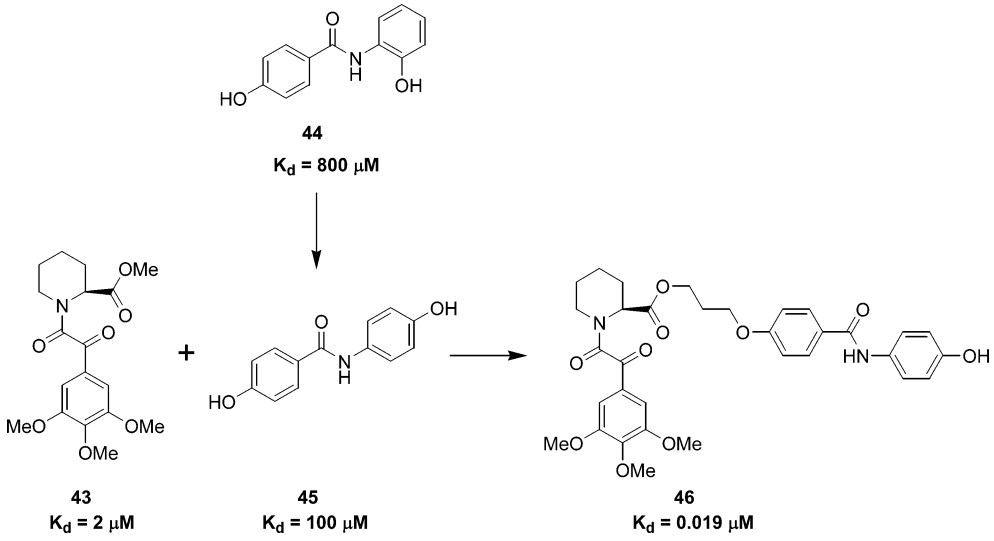
Scheme 11. Fragment Linking, PTP-1B



Scheme 12. Fragment Merging, IL-2



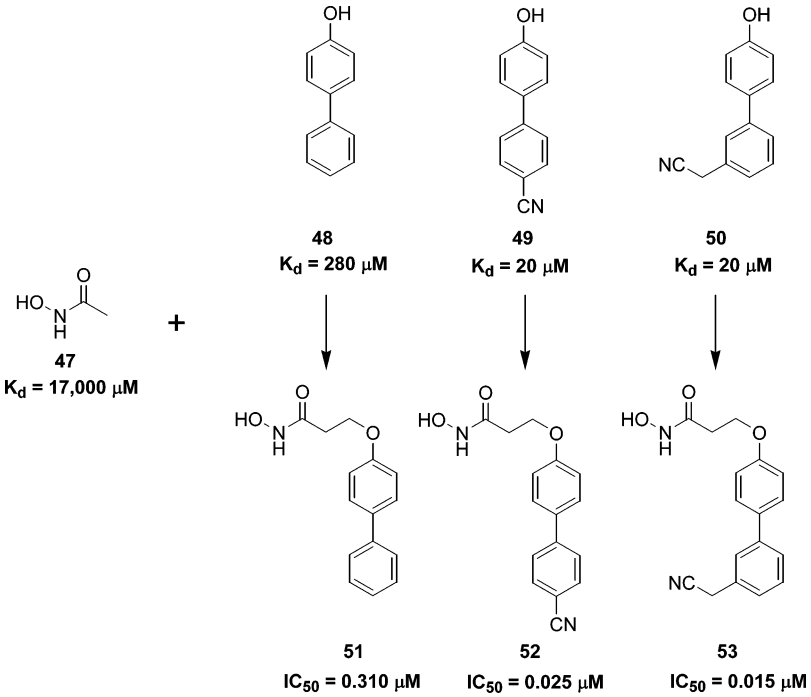
Scheme 13. Fragment Linking, FKBP



fragment-based methods has been in the de novo discovery of novel lead series by linking two fragments. This application best fulfills the theoretical promise of using fragment assembly as a vehicle for sampling enhanced chemical diversity space. The following several examples approach, and in some cases achieve, this ideal, using NMR, crystallography, functional screening, and MS screening.

3.2.5. FK506 Binding Protein (FKBP). The SAR by NMR technique was first applied to a study of inhibitors of FK506 binding protein (FKBP).[4] After using NMR to screen a library of about 1000 compounds, Fesik and co-workers identified the pipecolinic acid derivative **43** (Scheme 13), which binds with low micromolar affinity. Similar molecules had previously been characterized as FKBP inhibitors, and it was straightforward to determine the binding site using NMR chemical shift changes in the protein. The authors next screened the library again in the presence of saturating levels of **43** and identified **44** as a weak binder at a nearby site. A brief SAR study improved the affinity of **44** to generate **45**, and an NMR-based model of the ternary complex suggested a linking strategy. The resulting molecule **46** was found to have nanomolar

Scheme 14. Fragment Linking, MMP-3



affinity for FKBP, representing a binding enhancement of more than 100-fold over **43**. In addition to **46**, four other molecules were synthesized with varying linker lengths or positions, all of which showed high-affinity binding to FKBP and some linker-length SAR.

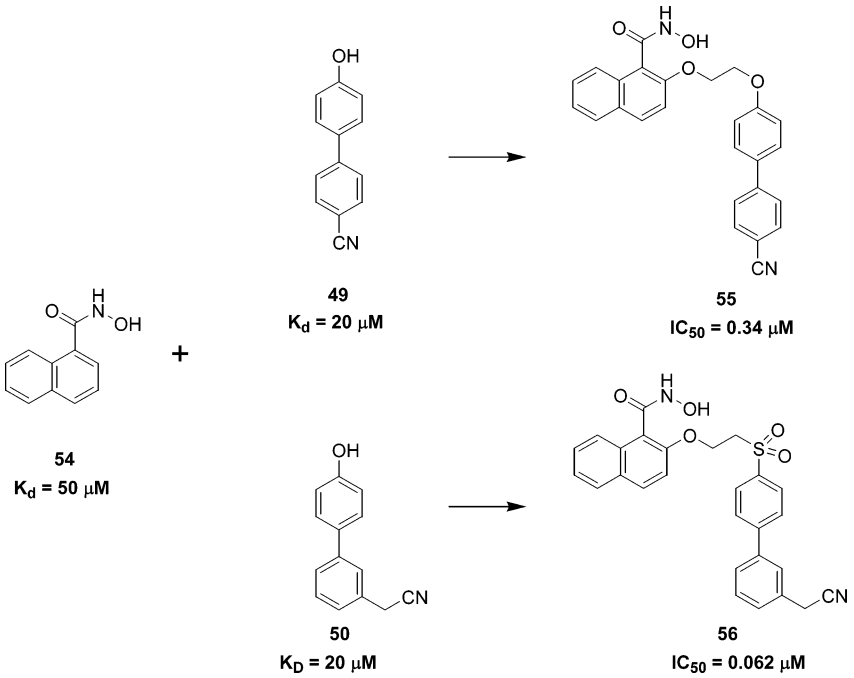
3.2.6. Matrix Metalloproteinase-3 (MMP-3). SAR by NMR was also used to screen the metalloproteinase stromelysin (MMP-3), an oncology target.[31] A previous HTS screen of 115 000 compounds had failed to identify any non-peptide inhibitors with a potency better than 10 μM. Acetohydroxamic acid (**47**, Scheme 14) was selected as an initial fragment based on its known ability to serve as a zinc chelator. Despite its low affinity (17 mM), **47** completely inhibited enzyme activity at concentrations of 500 mM and could therefore prevent autoproteolysis during the subsequent screening process. When an NMR screen was conducted in the presence of **47**, a number of companion biphenyl fragments were selected, including **48** and **49**. Thirty-three additional biphenyls were prepared to refine the SAR, resulting in the identification of **50** as another moderately active fragment. NMR structural determination helped guide the design of a series of linked compounds, resulting in the potent molecules **51**−**53**. In each case, the linked compounds bound roughly 1000-fold more tightly than the biphenyls alone, while compounds with shorter or longer linkers showed a much lower increase in potency. The NMR structure of **52** bound to MMP-3 revealed that the linked molecule binds in a fashion similar to that of the discrete fragments and that the linker itself makes hydrophobic contacts with a valine residue of MMP-3. Unfortunately, the nanomolar inhibitors **51**−**53** lacked oral bioavailability because of rapid hydrolysis of the hydroxamate moiety.

In a follow-on study,[80] a small collection of alternative zinc chelating fragments was screened by NMR and **54** (Scheme 15) was identified as a reasonably potent surrogate that bound to stromelysin in the presence of the biphenyls previously identified. Linked compounds were synthesized and validated structurally by NMR, ultimately resulting in compounds such as **55**, which exhibits an oral half-life in rats of nearly 2 h. Further modification of the linker recovered the potency of the earlier compounds (e.g., **56**). Similar to the case of PTP-1B (above), these two papers together describe a powerful sequential approach in which a first fragment was chosen using pre-existing knowledge, NMR was used to identify a second fragment, and with the selection of a second fragment, NMR was used again to replace the initial binding element.

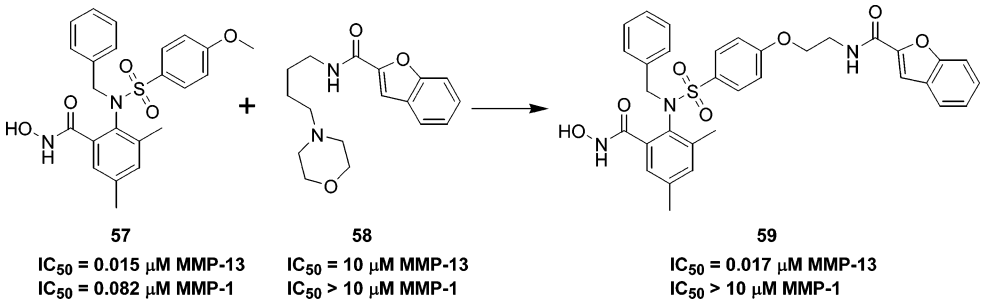
3.2.7. Matrix Metalloproteinase 13 (MMP-13). A conceptually similar process on another MMP was employed by researchers at Wyeth, who first identified a nonmechanism-based inhibitor of MMP-13 (also known as collagenase-3) (**58**, Scheme 16) in a high-throughput screen of 58 079 compounds.[81] This compound possessed an attractive structure and did not inhibit the related metalloproteases MMP-1, MMP-9, or TACE. Structural determination by NMR revealed that the compound binds largely in the S1′ pocket of MMP-13 and does not interact with the catalytic zinc. The S1′ pocket of MMP-13 is deeper than that of other MMPs, providing an explanation for the observed specificity. This suggested a design strategy in which a relatively nonspecific mechanism-based inhibitor WAY-152177 (**57**) could be linked to the S1′-pocket binding element of **58**. The resulting compound, WAY-170523 (**59**), has high affinity for MMP-13 while maintaining selectivity against MMP-1, MMP-9, and TACE.

3.2.8. Human Papillomavirus E2 Protein. In another early use of NMR, Fesik and co-workers successfully merged discrete fragments to generate molecules that disrupted the DNA-binding domain of the human papillomavirus protein E2.[32] Armed with both crystallographic and NMR-derived structures of the protein, the team screened a library of fragments at 1 mM concentration and identified biphenylcarboxylic acids (e.g., **60**, Scheme 17) and biphenyl ether carboxylic

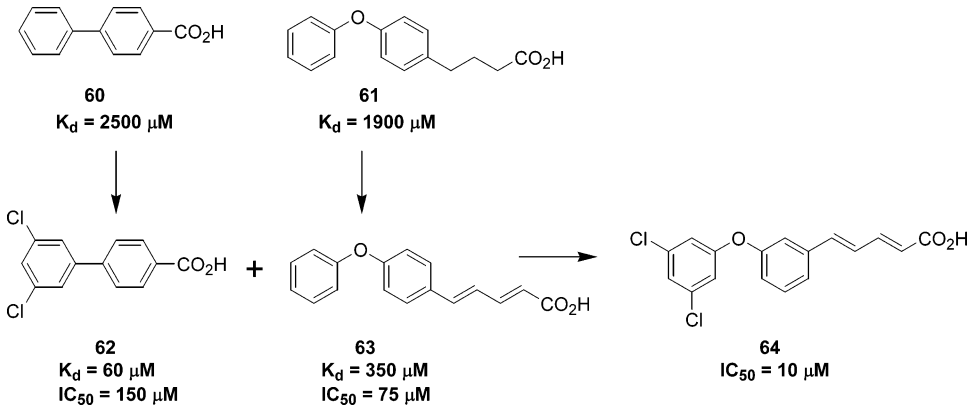
Scheme 15. Fragment Linking, MMP-3



Scheme 16. Fragment Linking, MMP-13



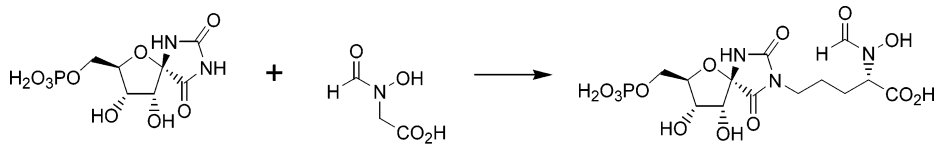
Scheme 17. Fragment Merging, E2



acids (**61**) as weak binders that inhibited DNA binding to E2. Small sets of analogues were made around these molecules, resulting in **62** and **63**, which show greater binding affinity and improved inhibition of E2−DNA binding. NMR studies suggested that these classes of fragments bind to a common location on the protein. Merging the fragments into a single compound produced **64**, which demonstrated potency greater than either of its two components. By contrast, a previous screen of 100 000 compounds had failed to produce any hits more potent than 10 μM.

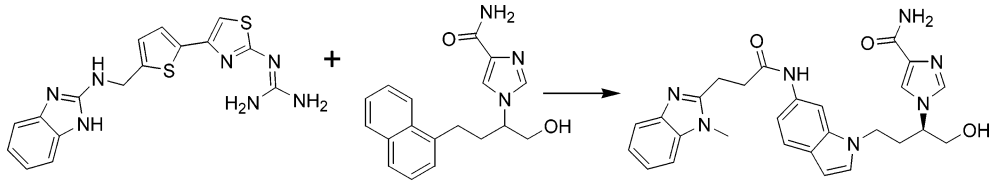
3.2.9. Adenylosuccinate Synthetase (AdSS). Known inhibitors that can be cocrystallized present clear opportunities to improve affinity using linking strategies. Hanessian and co-workers[82] utilized a previous structure of the herbicidal target adenylosuccinate synthetase (AdSS) complexed with the natural products hydantocidin 5′-phosphate (**65**, Scheme 18) and hadacidin (**66**) (Scheme 18).[83] When a hybrid molecule combining both of these fragments was synthesized (**67**), a greater than 10-fold boost in affinity was observed. Moreover, the researchers were able to verify the

Scheme 18. Fragment Linking, AdSS



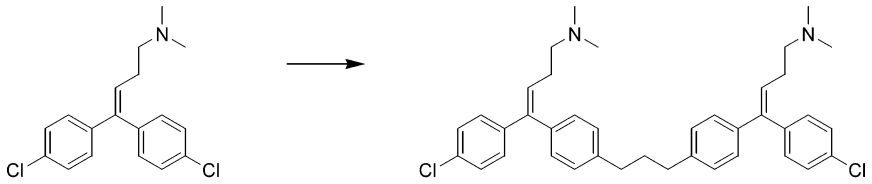
65 IC_{50} = 0.675 μM **66** IC_{50} = 3.5 μM **67** IC_{50} = 0.043 μM

Scheme 19. Fragment Merging, Adenosine Deaminase



68 K_i = 1.2 μM **69** K_i = 0.68 μM **70** K_i = 0.0077 μM

Scheme 20. Fragment Linking, Bcl-xL



71 IC_{50} = 180 μM **72** IC_{50} = 10 μM

binding mode of **67** using X-ray crystallography, demonstrating that the hybrid molecule binds as the sum of its parts.

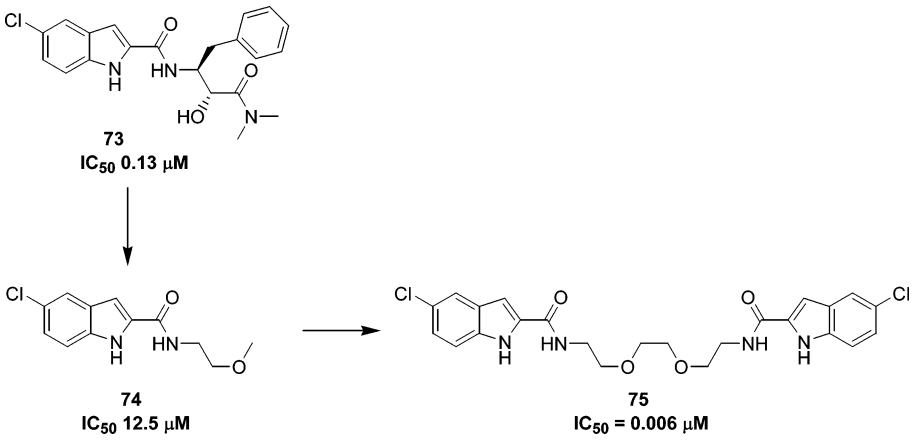
3.2.10. Adenosine Deaminase. In a recent publication, Nakanishi and colleagues describe the design and synthesis of potent non-nucleoside inhibitors of the anti-inflammatory target adenosine deaminase[84] that were derived by merging fragments **68** and **69** (Scheme 19). Independently derived crystal structures of these fragments bound to adenosine deaminase revealed conformational changes within the active site that would have hindered attempts to generate potent inhibitors using a simple linking strategy. Instead, a more complex design process was undertaken in which the original fragments were re-engineered to preserve their essential binding contacts while introducing productive vectors for linking. The resulting merged molecule, **70**, shows dramatically improved potency relative to its constituent fragments.

3.2.11. B-Cell Lymphoma xL (Bcl-xL). When multiple copies of the same fragment can bind simultaneously to adjacent sites, linking can be used to generate bivalent molecules with enhanced affinity. Jahnke and colleagues at Novartis used NMR to show that the weak inhibitor **71** (Scheme 20) binds to the oncology target Bcl-xL in the Bak-peptide binding region.[38] The molecule was then spin-labeled to facilitate identification of a second ligand that binds in the presence of the first. Surprisingly, the authors found that a second molecule of **71** binds nearby. Several dimeric molecules were constructed, resulting in the synthesis of **72**, the most potent in the series.[39]

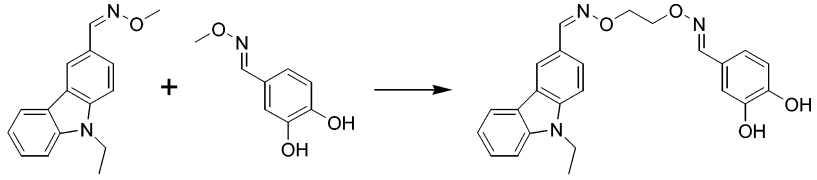
3.2.12. Glycogen Phosphorylase. As with Bcl-xL, two individual copies of the potent glycogen phosphorylase inhibitor **73** (CP-91149, Scheme 21)[85] were found to bind simultaneously at the dimer interface between the two enzyme monomers.[86,87] Rath and colleagues proposed that a dimeric molecule could be made that would target both sites, and they generated **75**, which showed a dramatic increase in potency. A crystal structure of **75** bound to glycogen phosphorylase, an important diabetes target, confirmed the expected binding mode.

3.2.13. c-Src. While the several preceding examples made extensive use of high-resolution structural information, empirical fragment assembly methods have also been successful. In an approach termed "combinatorial target-guided ligand assembly", Ellman and co-workers used a functional assay to identify fragments that weakly inhibited the protein kinase c-Src, an oncology target.[25] Each fragment was equipped with a common chemical linkage group (in this case, an oxime) to facilitate rapid library construction. A set of 305 oximes was screened at a concentration of 1 mM each to identify 37 weakly inhibiting fragments. A library was then constructed in which all pairwise combinations of these fragments were linked using five different linker lengths. From this library, compound **78** (Scheme 22) was identified as a mid-nanomolar inhibitor, representing a significant increase in potency over either of the precursors **76** and **77**. The catechol moiety was found to be essential for activity in subsequent SAR studies. Moreover, the linker length was found to be very important: an increase of one methylene unit decreased the potency by more than an order of magnitude.

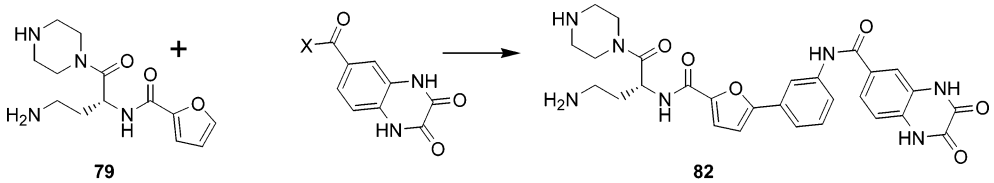
Scheme 21. Fragment Linking, Glycogen Phosphorylase



Scheme 22. Fragment Linking, c-Src



Scheme 23. Fragment Linking, U0161A RNA

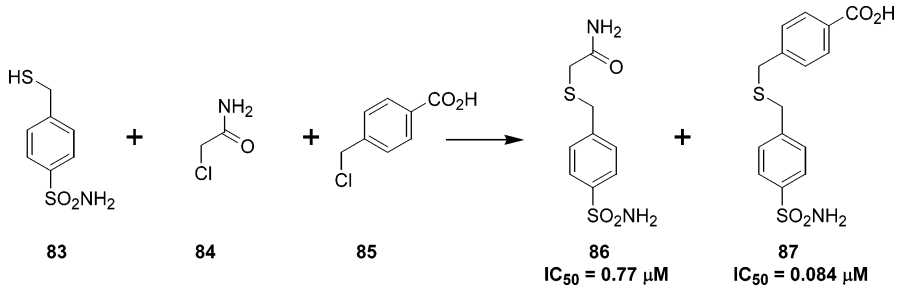


3.2.14. U1061A RNA. Another interesting case of a largely empirically driven fragment assembly approach was described by Swayze and colleagues at Ibis Therapeutics.[42] The method utilizes a mass spectrometry screen to identify compounds that bind to U1061A RNA, an antibiotic target. Mass spectrometry provides both binding information as well as stoichiometry, therefore obviating potential artifacts that can plague screens performed at high compound concentrations. Furthermore, mass spectrometry can be used to conduct competition experiments. Here, the authors demonstrated that the weak binders **79** and **80** (Scheme 23) could bind to the target RNA concurrently, while **79** and **81** were competitive. These data suggested that the binding sites for **79** and **80** are distinct but adjacent, leading to the synthesis of **82**, which shows a much greater affinity for the target than either of the component fragments.

3.3. Emerging Strategies: In Situ Fragment Assembly. The process of using fragment assembly to generate de novo leads is greatly facilitated when techniques such as NMR or crystallography can be used to identify fragments that can bind to nearby sites in a mutually compatible manner. Competition studies can also provide useful indirect evidence to select combinations of fragments that can bind concurrently as potential candidates for linking. But even with this type of information, productively linking or merging fragments remains a significant technical challenge. This challenge is further magnified when the target protein has a flexible binding surface. Several labs are now exploring ways of using the target protein both to *select* and to *combine* pairs of fragments in situ. In effect, the protein assembles its own inhibitor by selecting fragments that can cross-link to each other when brought into mutual proximity. The final set of examples illustrates this emerging area of investigation.

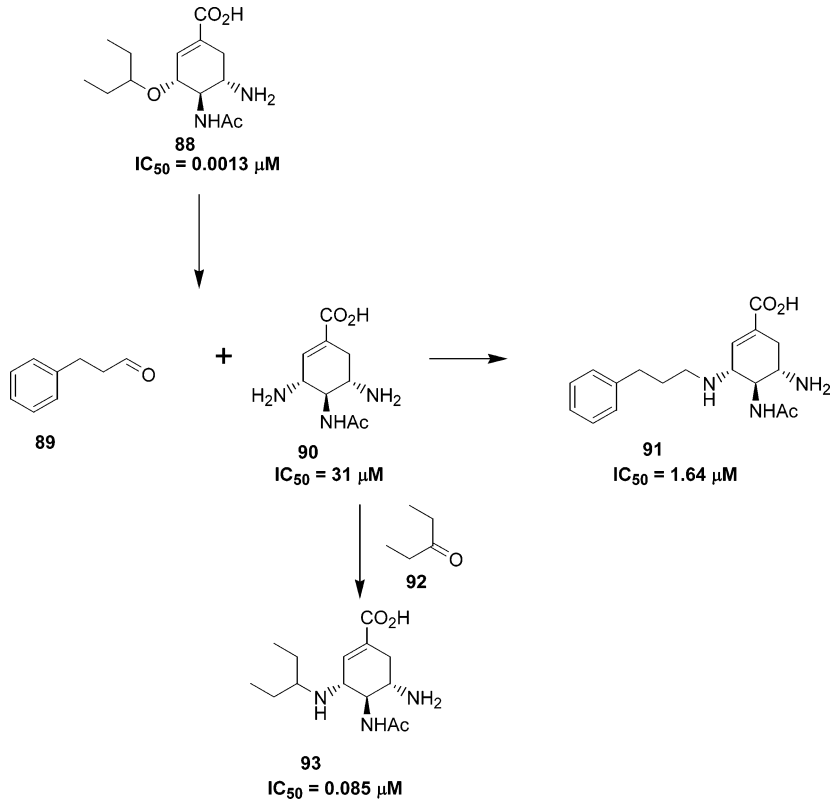
3.3.1. Carbonic Anhydrase. Techniques that use proteins to select their own inhibitors stem from observations that certain products are enriched when chemical reactions occur in the presence of a protein. Huc and Lehn conducted reductive aminations in the presence or absence of carbonic anhydrase and noticed enhanced formation of products containing the known para-substituted arylsulfonamide recognition element.[88] In a related study,[89] five carbonic anhydrase inhibitors with inhibition constants ranging over 1 order of magnitude were deconstructed into their thiol and alkyl chloride precursors. When the thiol **83** (Scheme 24) was reacted with a 1:4 mixture of alkyl chlorides **84** and **85** in the absence of enzyme, equimolar yields of **86** and **87** were observed. In the presence of 1 equiv of carbonic anhydrase, the product ratio shifted to 8:92, indicating that the enzyme preferentially templates the formation of **87** in accordance with its higher affinity for the

Scheme 24. In Situ Fragment Linking, Carbonic Anhydrase



83 **84** **85** **86** IC_{50} = 0.77 μM **87** IC_{50} = 0.084 μM

Scheme 25. In Situ Fragment Linking, Neuraminidase



88 IC_{50} = 0.0013 μM

89 **90** IC_{50} = 31 μM **91** IC_{50} = 1.64 μM

92

93 IC_{50} = 0.085 μM

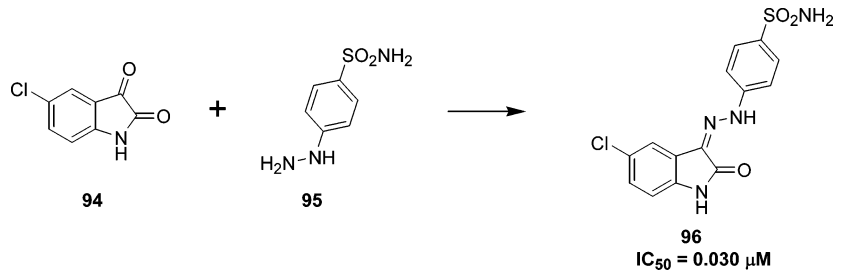
enzyme. Significantly, the preferential formation of **87** was strongly suppressed when the carbonic anhydrase inhibitor methazolamide was added to the reaction, suggesting that the templating occurs within the active site of the enzyme.

3.3.2. Neuraminidase. In similar studies at Alantos Pharmaceuticals AG, reductive amination was conducted in the presence of the influenza A viral enzyme neuraminidase.[90,91] Here, a known inhibitor (Tamiflu, **88**, Scheme 25) was deconstructed into an anchor fragment (**90**) that was reacted with a series of aldehydes (such as **89**) and ketones (such as **92**). Although inhibitors with enhanced potency were generated (**91** and **93**), the correlation between inhibitor potency and product distribution was not always straightforward.

3.3.3. Cyclin-Dependent Kinase 2 (CDK2). Congreve and co-workers at Astex Technology have used crystals of target proteins to select and/or promote inhibitor formation.[62] To validate the method, the researchers deconstructed a previously reported oxindole inhibitor of the oncology target kinase CDK2 (**96**, Scheme 26)[92] into precursors **94** and **95**. A cocktail of six different hydrazines (including **95**) was incubated with five different isatins (including **94**) in the presence of CDK2 crystals, and electron density corresponding to **96** was observed. In a control experiment in which **95** was mixed with an isatin that was sterically incompatible with CDK2, no inhibitor density was observed. Because the condensation between hydrazines and isatins occurs readily in aqueous buffer at room temperature,[93] it is unknown whether the presence of the crystal actually changed the product distribution by bringing selected reactive groups together or if the crystal extracted the high-affinity product **96** once it had been formed in solution (see also above).

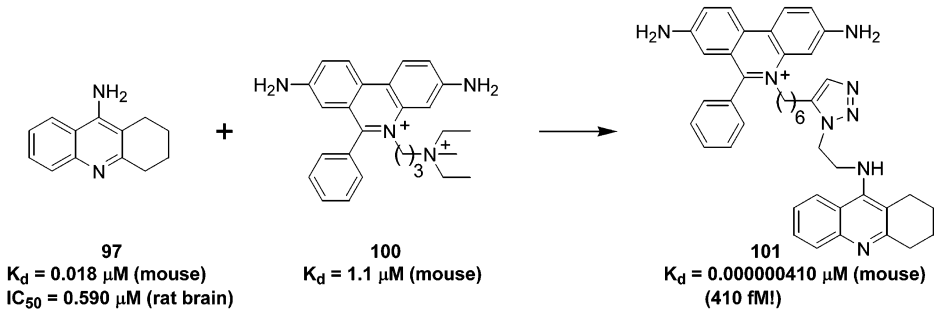
3.3.4. Acetylcholinesterase (AChE). Studies of the Alzheimer's disease target aceytlcholinesterase (AChE) provide one of the most successful examples of using a protein to assemble its own inhibitor. This enzyme contains a deep and narrow catalytic "gorge" as well as a more solvent-exposed "peripheral site." Dimeric molecules that span both sites have dramatically enhanced affinities compared to their monomeric components, as illustrated with **97**−**99** (Scheme 27).[94,95] Sharpless, Finn, and colleagues sought to generate bivalent inhibitors of AChE in situ using 1,3-dipolar cycloaddition of

Scheme 26. In Situ Fragment Linking, CDK-2



94 **95** **96**
IC_{50} = 0.030 μM

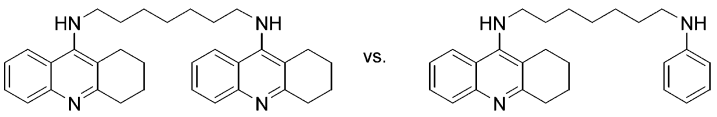
Scheme 27. In Situ Fragment Linking, AChE



97
K_d = 0.018 μM (mouse)
IC_{50} = 0.590 μM (rat brain)

100
K_d = 1.1 μM (mouse)

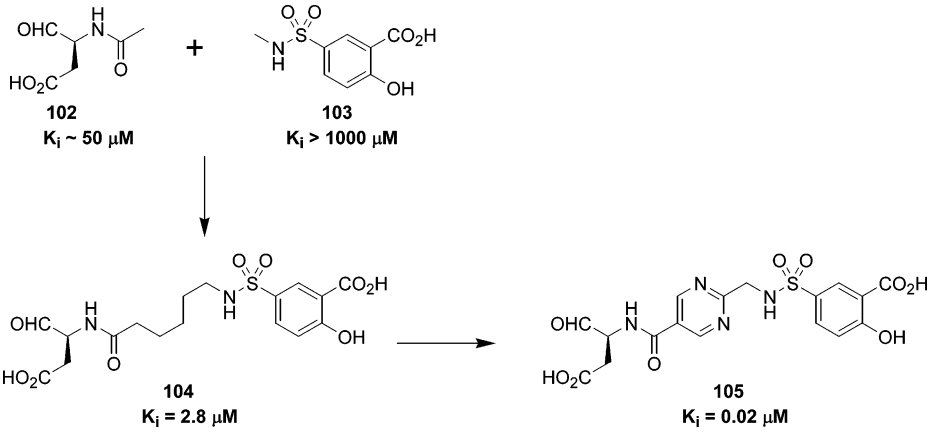
101
K_d = 0.000000410 μM (mouse)
(410 fM!)



98
IC_{50} = 0.0004 μM (rat brain)

99
IC_{50} = 0.390 μM (rat brain)

Scheme 28. In Situ Fragment Linking, Caspase-3



102
K_i ~ 50 μM

103
K_i > 1000 μM

104
K_i = 2.8 μM

105
K_i = 0.02 μM

azides and acetylenes to generate 1,2,3-triazoles.[96,97] A small set of compounds derived from known monomeric inhibitors **97** and **100** (Scheme 27) was prepared with reactive azide and alkyne side chains. These were then incubated with one another in the presence of AChE at room temperature for 1 week, and the reaction mixtures were examined by mass spectrometry. In one case, the cycloaddition produced the femtomolar (!) inhibitor **101**, by far the most potent noncovalent inhibitor of AChE described in the literature. The production of **101** was found to be completely dependent on the presence of functional AChE. Interestingly, only the syn isomer (shown) of **101** was formed in the presence of AChE, while the anti isomer was formed in equal amounts in a thermal reaction; the anti isomer was also found to be at least 10-fold less potent in an enzymatic assay. Thus, although the anti isomer is still a very potent inhibitor, assembly within the enzyme favored the highest affinity isomer, suggesting an exquisite level of product discrimination.

3.3.5. Caspase-3. Another example of in situ fragment assembly uses disulfide exchange to assemble inhibitors in the active site of caspase-3, an anti-inflammatory target.[52] An initial extender was derived from the obligate aspartyl group (**102**, Scheme 28) recognized by the S1 subsite of the enzyme. Following screening, the salicylic acid fragment **103** was selected and shown by crystallography to bind in the S4 subsite. The extender was then replaced by a simple aspartyl aldehyde and linked to the S4-binding fragment by a

linear chain. The resulting bivalent molecule (**104**) inhibited at low-micromolar concentrations, while the salicylic acid fragment showed no detectable inhibition by itself. Subsequent structure-assisted optimization improved the potency more than 100-fold to yield **105**.[98] A similar process was applied to caspase-1, yielding compounds that inhibited the enzyme at low-nanomolar concentrations and also showed reduced IL-1β secretion in cell-based assays.[53]

These emerging in situ fragment assembly methods must meet certain practical criteria before they can be widely applied toward new lead discovery. Obviously, the chemistries employed must be compatible with the chemical functionality present in protein amino acid side chains. The reactions should be fast enough to enable a reasonable number (>1000) of potential products to form and be identified within a day or so. The coupling reaction would ideally be under thermodynamic control to prevent highly reactive intermediates from dominating the product distribution. Most importantly, the products themselves need to be attractive starting points for further optimization and thus should be reasonably compact, soluble, and stable.

4. Discussion and Conclusions

The preceding examples demonstrate that fragment-based methods of drug discovery are widespread in industry and academia and that these methods have been successful in generating new drug leads with high potency and improved pharmacokinetic properties. Given the diversity of approaches, it is easy to get lost in the details. Here, we consider some of the broad lessons and current limitations of fragment-based drug discovery.

Once fragments are identified, the most conceptually straightforward approach of advancing fragments, optimization through chemical elaboration, generally requires highly specific or energetically favorable neighboring contacts to succeed. This is most likely either when the nucleating fragment itself supplies much of the total necessary binding energy or when adjacent potential contacts on the protein can greatly supplement the binding energy of the initial fragment. These contacts can involve metal coordination (the metalloproteases), mechanism-based transition-state analogues, or very deep, well-defined pockets, as were observed in thymidylate synthase and urokinase. Certain classes of protein targets possess "anchoring sites" that provide the requisite binding energy to enable high-affinity association with a small molecule. Clearly this is most likely to be the case for enzymes, especially those that have evolved to recognize small-molecule substrates. However, even protein−protein interactions show potential. For example, although the potent IL-2 inhibitors discussed above are fairly extended molecules, a recent report from Hoffmann-La Roche describes a class of small molecules, "nutlins," that potently inhibit the interaction between the tumor suppressor protein p53 and the tumor promoter MDM2.[99] These molecules were discovered in a high-throughput screen, but their compact nature suggests that they, or similar molecules, could be discovered through a fragment optimization strategy.

Fragment merging strategies, and especially fragment linking strategies, are generally more challenging than fragment optimization. They may also be less widely applicable, since they require two fragment-binding sites in proximity. However, these strategies can be greatly facilitated by methods that ensure that fragments bind noncompetitively with one another. In general, these strategies are driven by structural methods. For example, both SAR by NMR and SAR by X-ray can reveal if two fragments can bind simultaneously and in some cases even facilitate linking by providing the orientation of the fragments. However, structural information is not always essential, as seen in the case of SAR by MS on RNA, where the technology was used to identify two molecules that could bind simultaneously. Moreover, emerging dynamic methods such as target-guided reductive amination and Tethering with extenders can also succeed in the absence of direct structural information.

To date, most successful applications of fragment-based methods, particularly those involving fragment linking, have taken advantage of existing knowledge of the system, such as known cofactors, ligands, and mechanistic considerations. In most cases, structural information has been used to guide the process. Thus, even in the dynamic methods, structural information was used to design the fragments and, in the case of Tethering with extenders, to optimize the potency of the leads. So far, there have been no published examples using fragment-based approaches to discover de novo leads against targets with no known leads, although we have presented many cases where the leads from fragment assembly differ considerably from known leads. This is likely because fragment-based drug discovery methods are still new, and their development and validation have generally relied on using targets that often have known structures and that have already been subjected to other methods of lead discovery. Moreover, much of this research is occurring in industry, and so many of the most exciting results are likely being withheld until suitable patent protection is established.

Another hurdle for all lead discovery, including fragment-based methods, is identifying which hits are worth pursuing; not all inhibitors can become leads. Researchers have long realized that certain chemotypes are prone to yield false positives either because they react irreversibly and nonspecifically with target proteins or because highly colored or fluorescent compounds can interfere with colorimetric or fluorescent assays.[100] More recently, Shoichet and colleagues have demonstrated that many small molecules, even those lacking obvious pathological moieties, can form aggregates that nonspecifically inhibit proteins.[101−103] Although identification of such artifacts is usually possible,[104] it is still unclear exactly what molecular properties lead to this behavior, and therefore, researchers need to be vigilant during both fragment discovery and optimization that the evolving leads preserve the integrity of the fragments. Indeed, even when molecules are designed using the best available data and are demonstrated to bind legitimately at low-nanomolar potency, there is room for surprise: crystallography of bis-difluorophosphonate molecules bound to PTP-1B demonstrated that the molecules bound to a site not predicted by modeling or design.[77]

In conclusion, we believe that fragment-based methods will continue to evolve to complement other discovery approaches. Indeed, since this Perspective was submitted, at least two other reviews have appeared describing fragment-based drug discovery.[105,106] Moreover, many of the tools developed for fragment assembly, such as NMR-based screening methods, have found their way into the general repertoire and are now used to characterize and validate hits from traditional screening. Ultimately, fragment-based methods will be absorbed into a holistic approach to drug discovery, where fragments will be expanded and combined into libraries for functional screening, HTS hits will be dissected into component fragments for individual optimization, and technologies such as crystallography, NMR, and the modeling techniques underpinning structure-based drug design will be called upon routinely.

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Biographies

Daniel A. Erlanson is a Senior Scientist at Sunesis Pharmaceuticals, Inc. Dr. Erlanson has been with Sunesis from its inception in 1998 and has been instrumental in inventing and developing the core technologies as well as in discovering and progressing leads against a variety of targets. Prior to joining Sunesis, Dr. Erlanson did postdoctoral work with James A. Wells at Genentech, Inc. where he developed methods to conformationally constrain peptides. Dr. Erlanson received his Ph.D. in Chemistry from Harvard University in the laboratory of Dr. Gregory L. Verdine, where he studied protein−DNA interactions using both chemical and biological techniques and developed technologies for site-specifically introducing modifications into nucleic acids and proteins. Dr. Erlanson received his BA in chemistry from Carleton College in Northfield, MN.

Robert S. McDowell received his Ph.D. in Organic Chemistry from the University of California at Berkeley in 1984. He started his medicinal chemistry career at Syntex Corporation and moved to Genentech, Inc. in 1988 as one of the founding members of the Bioorganic Chemistry Department. During his career at Genentech, he helped to develop novel approaches for discovering non-peptidyl drug leads starting from three-dimensional structures of bioactive peptides. In 1998, Dr. McDowell joined Axys Pharmaceuticals (now Celera) as Director of Structural Chemistry. Dr. McDowell has been at Sunesis Pharmaceuticals, Inc. since February 2000 and currently is the Vice President of Discovery Chemistry.

Tom O'Brien was educated at Trinity College Dublin, Ireland, and continued his studies at Cornell University in the laboratory of Dr. J. Lis where he received his Ph.D in Genetics and Development. He then conducted postdoctoral studies at the University of California at Berkeley with Dr. Tjian where his project focused on dissecting the molecular events underlying gene expression. While at University of California at Berkeley, Dr. O'Brien was also a Special Fellow of the Leukemia Society of America. In 1999 he joined the Biology Department at Sunesis Pharmaceuticals, Inc., where he is currently a Staff Scientist. At Sunesis he has played a pivotal role in developing novel technologies to aid the identification and advancement of small-molecule leads against a number of therapeutic targets.

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