From Fragment Screening to Potent Binders: Strategies for Fragment-to-Lead Evolution

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Abstract: To be an effective medicine a drug has to possess many attributes to ensure target potency and specificity, lack of toxicity, bioavailability and duration of action. Discovering a compound with these properties is invariably an evolutionary process. Fragment based drug discovery sets out to identify a starting compound by screening a library of small molecules representing fragments which cover the chemical space of drug like matter. Fragment based screening is increasingly used in the pharmaceutical industry in the early stages of lead identification and optimization. We will provide an introduction into this approach and discuss a number of examples which show how fragment based drug discovery has been used in the discovery of starting points for drug discovery programs and in their optimization.

Key Words: Fragment-based drug discovery, screening, medicinal chemistry.

INTRODUCTION – GENERAL ASPECTS OF FRAGMENT SCREENING

Fragment screening is based on testing compounds with a low molecular weight using detection techniques which are more sensitive than those usually used in a high throughput screening (HTS) bioassay. The molecular weight of these fragments is usually less than 250 Daltons whereas standard compound databases typically contain molecules starting with a molecular weight of 300-400. Since "chemical space" explodes dramatically as molecular weight grows the focus on low weight compounds allows coverage of a larger proportion of the chemical space at this molecular weight compared to the compounds tested in a typical HTS campaign. A major issue is the optimization of the fragments identified to a more conventional range of biological activity. Often structural information is used to guide fragment optimization limiting the target space to those amendable to structure determination. Fragment based approaches using well designed libraries have the potential to complement HTS which according to a recent study by GSK only in 50% of all screens yields leads worth of pursuing [1]. One reason for this observation is probably the lack of suitable compounds in the screening collection. In this case increasing the size of the collection by adding diverse libraries should increase the probability of a successful screening. A second possibility is that the protein is not a target for small organic molecules. It has been shown experimentally that fragment-based screening is a very good predictor for a druggability of a target [2] confirming the assumption that libraries of diverse, small, fragment-like compounds offer a efficient way to cover the chemical space of possible starting compounds for drug discovery.

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DESIGN OF THE FRAGMENT LIBRARY AND THE CONCEPT OF LIGAND EFFICIENCY

Regarding the selection of fragment libraries some general recommendations have emerged. The compounds should be available. It is often helpful if analogs are also available to build up a structure activity relationship (SAR). Since the libraries are small and fragments tend to be diverse it is important that these offer opportunity for further synthetic elaboration in a classical medicinal chemistry fashion. The compounds should be soluble, pure and non-reactive. According to the "rule of three" proposed by researchers at Astex therapeutics the maximum molecular weight should be 300 Da, a complexity filter should be applied and emphasis should be given to compounds with high solubility in water [3]. The molecular weight filter is often replaced by using the number of non-hydrogen atoms (< 22 at GSK) [4] to avoid deselecting compound with synthetically desirable groups such as Br. Using only the molecular weight filter for compound selection would lead to a library skewed towards higher molecular weights. This is due to the increase of chemical space with increasing molecular weight. The effect of complexity on the probability of a compound to score active in a screen, has been demonstrated by Hann *et al*. analyzing data from GSK [5] and by Schuffenhauer *et al*. using data from Novartis [6]. The complexity dependence of finding an active compound appears to be bell shaped. At low complexity the compound often matches but the number of interactions is too low to be measured and at the opposite end of high complexity the compound has the potential to participate in many interactions but the probability that it matches is low. Other considerations include filters for toxic or otherwise undesirable functionality, diversity, druglikeness and the use of privileged scaffolds [7-12]. Library design may also be influenced by the target class as it has been shown that the fragment distribution of actives of different target classes, such as kinases or proteases, varies significantly [13]. Researchers from Vertex showed that two-thirds

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of kinase inhibitors contained at least one aniline fragment and 19% employ two or more of these compared to <20% in compounds published in the Journal of Medicinal Chemistry [14].

Ligand efficiency (LE) is a concept popular in fragment based screening. It refers to the free energy of binding of a compound to a specific protein divided by the number of heavy atoms [15-18]. A molecule that achieves a given potency with fewer heavy atoms is by definition more efficient. It has been realized that larger ligands have an inherent disadvantage in terms of many physicochemical properties using atoms efficiently is important in drug discovery. Non-covalent interactions are often not additive [18] and it has been shown that, on average, the most potent small ligands have higher ligand efficiency than the most potent larger ligands for the same target [17]. There may also be technical reasons for this observation such as the detection limit most assays have in the low nano-molar range.

It is instructive to analyze the results from experiments which take the opposite direction, deconstructing highly optimized inhibitors into their constituent fragments. Hajduk *et al*. have done this for 18 optimized inhibitors showing that the ligand efficiency of the fragments and final compound show similar ligand efficiency [19]. This means that every mass unit added contributes equally and proportionally to the binding affinity suggesting that the constancy of ligand efficiency is a quantitative measure for effective fragment elaboration. Structural analysis of the binding mode of the fragments indicated that it can differ from the same fragment in the reference compound [20]. This may be due to restraints exercised by the whole structure.

The minimum ligand efficiency for a compound obeying Lipinski's rules and with an IC50 < 10 nM (the IC50 corresponds to the quantity of a inhibitor needed to inhibit a biological process by half) can be easily calculated and used to show that a fragment with the same ligand efficiency and a molecular weight around 170 should have an IC50 around 2 mM corresponding to a concentration which can be screened reliably only with biophysical methods. Given that compounds exhibiting a ligand efficiency larger than 0.5 are rare the probability of identifying ligands with a heavy atom count < 12 in an HTS campaign, which typically tests at around 10 μM, is low. Since ligand efficiency not only depends on the fragment itself but also on the binding site, fragment screening has been suggested to be used for testing the druggability of a target [2]. More recently the concept of ligand efficiency has been extended to include group efficiency and ligand lipophilicity efficiency. The latter is related to an observation by Leeson and Springthorpe that more lipophilic compounds tend to have a higher probability of non-specific toxicity [21].

SCREENING TECHNOLOGY

One approach to test fragment libraries for their biological activity is to employ typical biochemical assays which are modified to detect activity at much higher concentration of the ligand, typically 1mM. These assays are fast, only a small quantity of the protein is required and they use widely available technology. The major problem is that ligands often interfere with the assay at high concentration. At high concentration the ligands often aggregate resulting in false positives, they may not be sufficiently soluble or be toxic to cells.

Usually biophysical methods are required to detect the weak interactions between fragments and their target proteins. Nuclear magnetic resonance (NMR) spectroscopy is particularly sensitive to intermolecular interactions and was employed in the first fragment based screening experiment, SAR by NMR [22]. An isotope labeled protein is brought together with mixtures containing up to 30 different compounds in which each compound is present at a concentration of around 400 μM. Disadvantages of this approach are the large quantity of labeled protein which is required and that relatively large libraries need to be tested. More recently a number of NMR methods were developed which do not require labeling of the protein and detect changes in the spectrum of the ligand [23-25]. Other NMR-based methods include target immobilized NMR screening [26] and, using ^{19}F-labelled probes in competition screening [27].

Mass spectrometry-based fragment discovery has been pioneered by researchers at Ibis who used it to identify binders to the ribosome IIA subdomain of hepatitis C (HCV-IRES IIA). This leads were optimized to submicromolar inhibitors [28].

Astex therapeutics and SGX Pharmaceuticals have pioneered the use of X-ray crystallography in fragment screening. The binders can not be ranked with this method but it yields valuable information on the mode of binding which is essential for further optimization of the ligands. Limiting is the need to have a crystal structure of the target protein in which the ligand binding site is accessible. The size of the fragment library is often limited to below 1000 [29-31].

LEAD IDENTIFICATION BY FRAGMENT TETHERING

Fragment tethering is an extraordinarily sensitive technology capable of identifying binders with a Kd greater than 5 mM. The basis of this approach is the formation of a disulfide bridge between a cysteine and a chemically reactive ligand [32-34]. Binding is detected *via* mass spectroscopy. All ligands are required to possess an SH group and a number of cysteine mutations must be introduced into the target protein [35].

The cytokine interleukin-2 (IL-2) is central to activation of T-cells and of considerable medical interest due to its role in the rejection of tissue grafts. Researchers at Sunesis have used tethering to optimize an inhibitor of the interleukin-2n(IL-2)/IL-2 receptor (IL-2Ra) interaction from milimolar affinity to 60 nM. Finding inhibitors of protein-protein interactions is particularly challenging and fragment assembly should be a promising alternative to high throughput screening [36]. The starting point for the optimization was a small molecule compound shown by NMR to be a low micromolar inhibitor [37]. The complex was further characterized by an X-ray structure [38]. This starting compound **1** is composed of two fragments, a biaryl acetylene amino acid and a

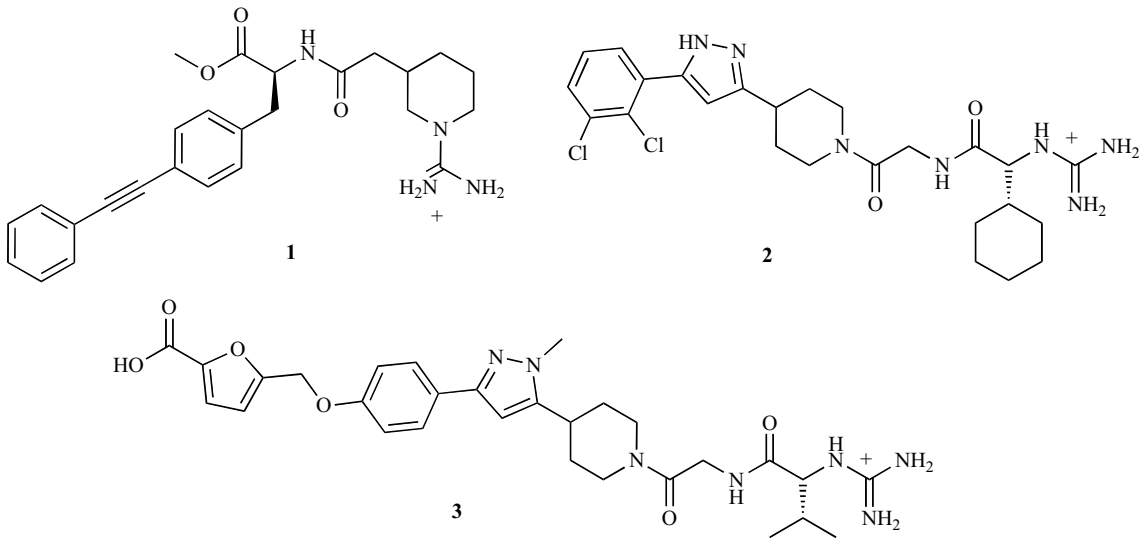

Fig. (1). Evolution of a potent ligand of IL-2 *via* fragment tethering.

piperidinyl guanidine acetic acid Fig. (**1**). Synthesis of several small libraries produced an optimized compound **2** with an IC50 of 10 μM. Throughout chemical optimization compounds were analyzed by surface Plasmon resonance, analytical ultracentrifugation and NMR. Analytical ultracentrifugation was used, not only to determine dissociation constants using the sedimentation curves and the absorbance profile, but also to identify compounds that cause aggregation. NMR spectroscopy was employed to determine the binding site and it was also shown that the degree to which certain resonances shift correlates with affinity. A crystal structure was obtained for this optimized compound.

For further optimization 10 cysteine mutations of IL-2 were constructed around the ligand binding site and screened against a library of 7000 disulfide-containing fragments. Aromatic fragments were preferentially selected and shown to bind at a site close to the 2,3-dichloro substituted phenyl ring. Using modeling it was easy to estimate the distance between the disulfide bound fragment and the phenyl ring. Previous SAR had shown that ether was tolerated in position 4 of the phenyl ring providing a synthetic handle for accessing the region identified by tethering. After synthesis of a small library, compound **3** with a furanoic acid in position 4 of the phenyl ring was identified with an IC50 of 60 nM. Detailed structural studies have shown that the binding surface on IL-2 is adaptive and binds the small molecule using the same residues as the receptor. Electrostatic and surface shape complementarity appear to be central to ligand recognition.

LEAD IDENTIFICATION AND OPTIMIZATION BY FRAGMENT EVOLUTION

This approach is similar to standard lead optimization. Since the starting compound is small there is plenty of scope for elaborating the initial fragment. If structural information is available guidance by structure based design can give rapid direction and progress. In the absence of structural information testing analogs is used to establish a structure activity relationship.

Researchers from Astex have used structure based design approaches to identify a potent CDK2 inhibitor with a favorable pharmacokinetic profile which is now in clinical development. A fragment library of 500 compounds was divided into cocktails of four fragments and soaked into crystals of the apo protein. The library was formed from a kinase set, a drug-like set and a set of compounds from virtual screening against the apoprotein. A number of binders with low potency (40 μM to 1 mM) was identified exhibiting a conserved interaction pattern to key residues at the hinge region of CDK2 (Glu81 and Leu83). Based on ligand efficiency, existence of vectors suitable to access key regions of the binding site, synthetic tractability and novelty three fragments were selected for further optimization.

One of the fragments chosen is the indazole **4** shown in Fig. (**2**). Analysis of the binding pocket of the initial indazole fragment suggested two directions for further substitution indicated by the arrows in Fig. (**2**). Related kinases indicated the possibility to form an additional hydrogen bond to Leu83. Compound **5** exploits two of these opportunities and shows improved potency with the sulfonamide picking up additional interactions. In parallel compound **5** was simplified leading to compound **6** which has a reduced potency but the LE remains the same.

Analysis of the crystal structure of **6** suggested that introduction of a 4-amino group as a synthetic handle would allow accession of an adjacent pocket. These efforts lead ultimately to compound **7** with a significant increase in CDK2 affinity but only moderate antiproliferative cell activity against HCT116 colon cancer cells. The authors suggest as one possible reason for the poor cell activity the high lipophilicity of compound **7**. Introduction of a 3-piperidinyl group in **8** lead to significantly improved cell activity. Further optimization with the aim of improving specificity and pharmacokinetic properties included the replacement of the 2,6-difluorophenyl moiety by a 2,6-diclorophenyl group and the 3-piperidinyl by 4-piperidinyl group leading to a compound which is now in clinical trials.

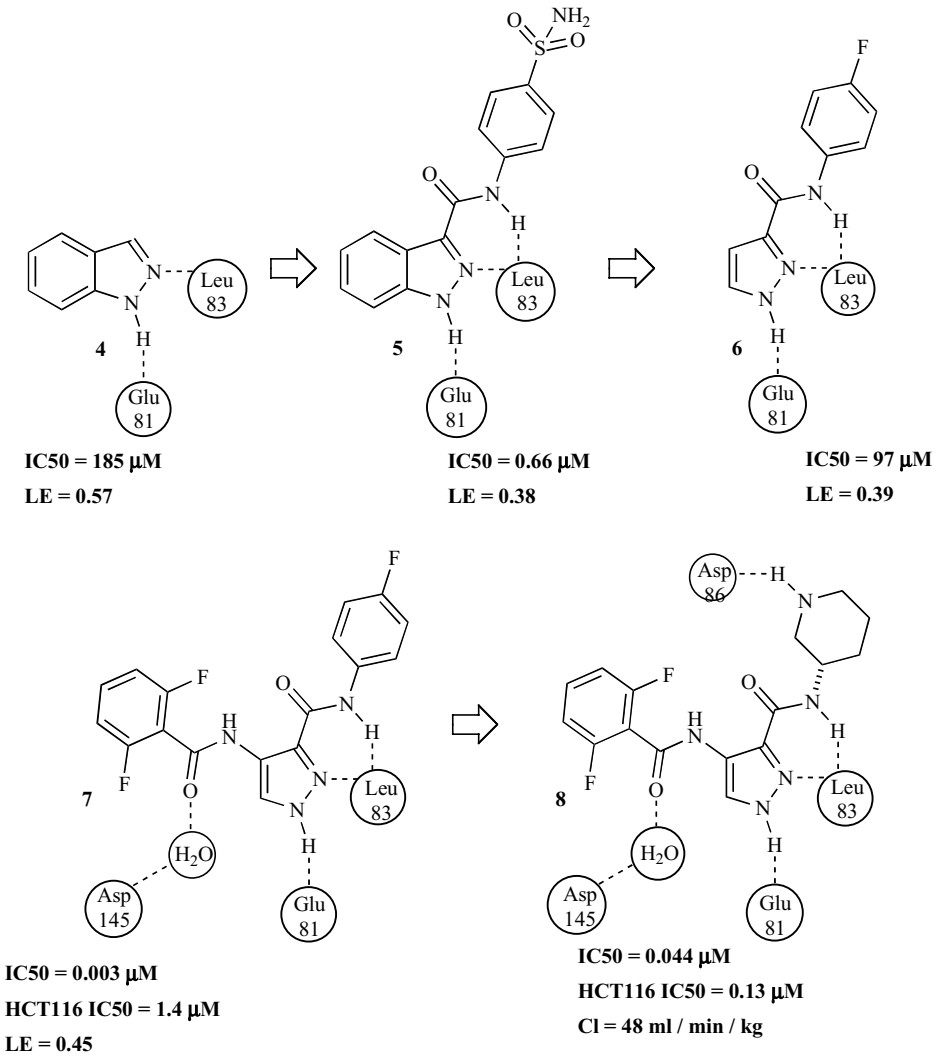

Fig. (2). Fragment evolution leading to a potent CDK2 inhibitor. The arrows indicate directions for further substitution, dotted lines hydrogen bonds.

LEAD IDENTIFICATION AND OPTIMIZATION BY FRAGMENT ASSEMBLY

This approach requires two fragments to bind to adjacent sites. Linking these fragments leads in the best case to a reduction of the loss of translational and rotational entropy upon binding and thus to a large gain in affinity. However, finding the appropriate linker is often very difficult and structural information is almost a prerequisite but no guarantee for success.

Proteases usually have extended binding sites with each aminoacid accessing its own pocket. For this reason proteases are particularly well suited for the fragment linking strategy.

Researchers at Astex have discovered fragment hits for the serine protease thrombin using X-ray crystallographic screening of the protein. The fragment library containing only 80 compounds was biased towards non-basic fragments avoiding well-precedented basic functionality. Hits include a ligand binding to the S2-S4 pockets and another fragment binding to the S1 pocket. The structure of both fragments bound to thrombin was determined and used to design a linker between both. Synthesis of only 40 compounds resulted in a larger, hybrid inhibitor with nanomolar potency [39, 40].

In another example researchers at Abbott identified very high affinity ligands of the antiapoptotic protein Bcl-Xl by identifying and optimizing ligands for multiple hot spots on the protein surface using SAR by NMR [41]. One of these compounds is now in clinical development [42]. The NMR structure of Bcl-Xl with a peptide fragment of the pro-apoptotic proteins, which are the natural interaction partners, has been determined. A library of 10.000 fragments was screened by NMR. A fluoro biaryl acid **9** Fig. (**3**) was identified to bind in the hydrophobic groove with a Kd of ~ 300 μM. Tests of analogs showed that the presence and position of the carboxyacid is essential for activity. Comparison of the structure of the complex with the natural ligand showed the existence of a second proximal site. To identify ligands for this site a second library was screened in the presence of compound **9** in excess. A number of weakly binding naphtol derivatives **10** and a biaryl phenol with activities in the low

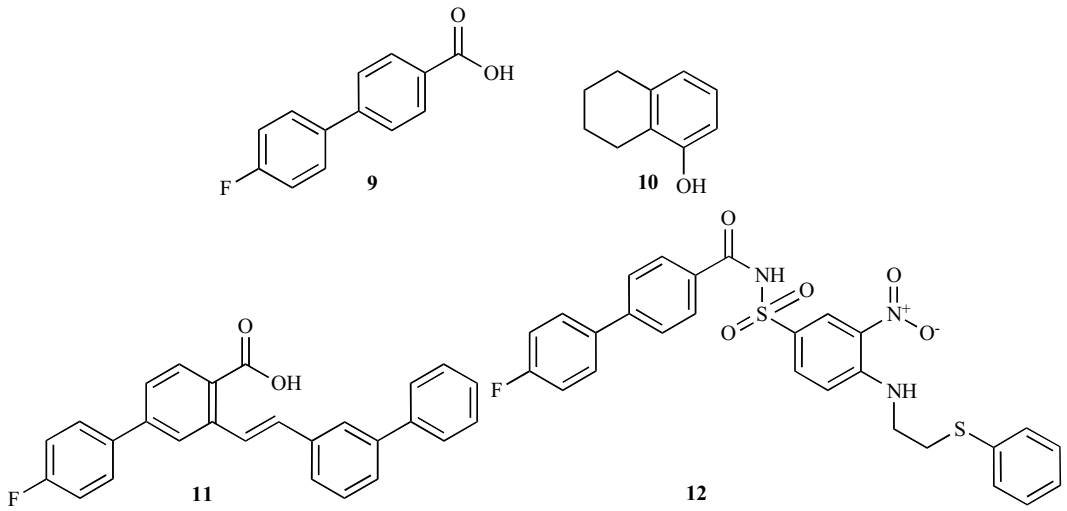

Fig. (3). Evolution of a potent Bcl-Xl ligand *via* fragment assembly using NMR spectroscopy and molecular modeling.

milimolar range were identified and shown to bind in the hydrophobic groove.

To develop a linking strategy the structure of the ternary complex was determined *via* NMR spectroscopy and modeling. The ortho position of the biaryl acid provided the most direct trajectory to the second site. A number of compounds was synthesized. Most inhibited at >10 μM. One compound **11** with a trans olefin linker inhibited with a Ki of ~1.4 μM, a more than 200-fold improvement over the original biaryl acid. A model of compound **11** bound to BCl-Xl was constructed suggesting that the trans-olefin linker was not ideal. Acylsulfonamide linkers were considered as an alternative with the acidic functionality build in. Using commercially available compounds a diverse library was synthesized yielding a potent inhibitor with a Ki of ~0.245 μM. Diversity calculations and molecular modeling was used to construct another library yielding a potent inhibitor **12** with a Ki of ~0.036 μM. The therapeutic use of compound **12** was limited by poor solubility and high protein binding. Several rounds of structure guided design led to a compound without these problems.

CONCLUSION

The number of successful applications of fragment-based drug discovery is increasing rapidly. The majority of these have in common that structural information is available, obtained by NMR spectroscopy or by X-ray crystallography. The considerable synthetic effort necessary to optimize the hits from fragment screening to the level of potency expected from a traditional HTS is a major issue of this approach. Structural information is essential to accelerate the progression from the very weakly binding initial hits to leads with attractive affinities. This progress is simplified by fragment based screening usually providing less complex hits, which are well accessible to synthetic modifications.

Targets for which it is difficult to identify ligands with standard screening methods appear to be particularly attractive for fragment based methods. There are several examples of compounds which disrupt protein-protein interactions having been identified by fragment based methods. Thus fragment based screening will not be universally applicable but has its advantages whenever structural information is available, compound collections are limited and and no potent hits can be identified with more established methods.

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