Structure-based screening and design in drug discovery

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Structure-based screening represents an integrated approach for the identification and optimization of hits by the combined use of nuclear magnetic resonance (NMR) spectroscopy, homology modeling and X-ray crystallography. A general feature of the methodology is the introduction of structure-based methods (NMR, modeling and X-ray) early in the drug discovery process to optimize hits in terms of their affinities and specificities. This approach promises to deliver leads with improved physicochemical properties as compared with leads generated from a traditional HTS program. This review presents examples of structure-based screening from published and in-house drug discovery projects.

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▼ In a traditional drug discovery effort the basis for the identification of modulators of the function of a target is HTS. With this approach, a specific assay is developed for the target protein, which is then transferred to a format that enables screening of a large set (10^5–10^6) of compounds. Despite the fact that this approach has, in many cases, proven successful, it has some inherent challenges and limitations:

- The development of an assay could be tedious and time-consuming;
- The transfer of the assay to an HTS format could prove difficult;
- For some target classes the HTS hit-rate is low, and results in few good chemical starting points [1]; and
- The hits identified from a historical compound collection usually have moderate affinity (1–10 μM), with relatively high molecular weight and lipophilicity, complicating the lead optimization process considerably [2].

In this review we will discuss an alternative to the traditional HTS approach for hit generation and drug discovery – structure-based screening (Fig. 1).

Structure-based screening integrates several biophysical techniques for the identification and optimization of small-molecule modulators. The goal is to identify lead compounds with improved profiles compared with typical compounds identified from screening against a historical compound collection (Fig. 1). The lead compounds should be relatively small (M_w = <400 Da) and should match the polar interactions in the active site of the target. Such lead compounds enable the introduction of lipophilic groups (to further improve affinity and the physicochemical properties) without increasing molecular weight and lipophilicity to an extent that will cause the resulting compounds to lose their drug-like properties [2].

The structure-based screening approach rests on a nuclear magnetic resonance (NMR)-binding assay as the primary tool for the detection of hits. Binding is detected by NMR either by observing the signals from the ligand (ligand-based screening) [3,4] or signals from the target (target-based screening) [5]. Following the selection of promising scaffolds, analogs are identified from commercially available sources and/or in-house compounds. To guide this process, structural information is crucial. A crude structure–activity relationship (SAR) for the identified hits can be based on a simple ranking of binding affinities for different ligands. In addition to the SAR, structural information describing the interaction with the target protein is important. The structural data could be low-resolution information, such as epitope mapping by NMR of the binding surface [6,7], or automated docking of ligands [8–10]. Most desirably, high-resolution NMR or X-ray structures of the complexes between hits and the target should be determined.

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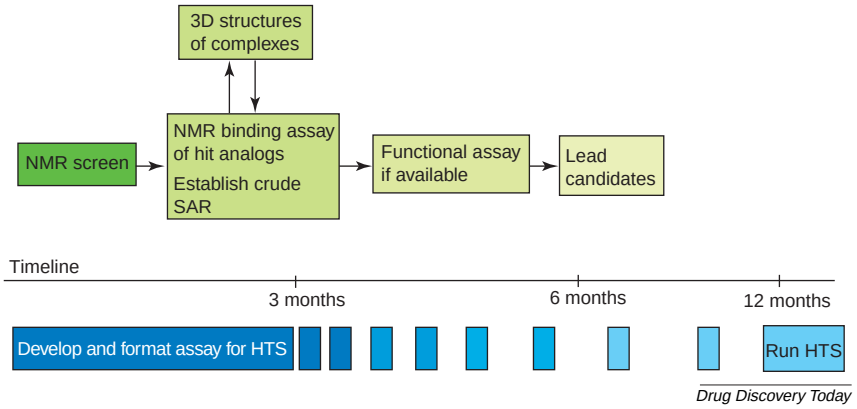

Figure 1. Strategy and timeline for a structure-based screening program compared with a high-throughput screening (HTS) program. The development and formatting of an assay suitable for HTS usually takes 3–12 months. In our laboratory the throughput in nuclear magnetic resonance (NMR)-based screening is currently 100–200 compounds per day, but the recent introduction of cryo-probe technology allows significantly higher throughput [53].

Because most compounds used in NMR screening are highly soluble, the probability of obtaining crystal structures is reasonable, despite their rather low affinity. Based on the available structural information, a set of promising analogs are selected and tested for binding to the receptor to confirm or reject the direction of the design. The process is then repeated in an iterative manner. In a successful iterative design cycle, the probability of obtaining high-resolution structural data increases in each step, along with a better understanding of the SAR and improved binding strength(s) of the compound(s).

NMR-based screening

NMR has a long tradition in the study of molecular interactions [11] and is an excellent method to identify weak, but specific, binding between small molecules and drug target molecules (i.e. proteins) [12]. Binding studies by NMR represent a generic binding assay that can be applied as soon as a target protein is available, without the need for extensive assay development. In addition, it has been shown in many cases that modification of hits from HTS screens produces analogs with significantly higher lipophilicity and molecular weight compared with the profile of orally available drugs [2]. Small polar scaffolds (which are typically used in NMR-based screening) could in this context provide attractive starting points for further chemical modification. The design of NMR-based screening libraries has recently been reviewed by Lepre [13], and will not be discussed here. However, it should be noted that the focus of compound library design has recently shifted from maximizing library size and chemical diversity towards incorporation of more lead- and drug-like physicochemical properties in smaller libraries [2,14].

Ligand-based screening

There are several ligand-based NMR methods that can be used to monitor the interaction of a small compound with a target protein. These have been reviewed extensively by others [12,15], and will only be mentioned briefly in this review. Two main classes of ligand-detected NMR-binding assays can be distinguished:

- Methods that rely on the detection of an altered hydrodynamic property (i.e. molecular tumbling rate or diffusion rate) upon target binding; and
- Methods that rely on transfer of an NMR signal (magnetization) between target and ligand.

Experiments that do not fall into either category are the use of paramagnetic spin labels to increase the relaxation of nearby spins [16,17], and fluorine (^{19}F) NMR to detect binding of fluorinated compounds to a target [18].

Diffusion and relaxation filter-type experiments belong to the first class of methods [4,19–25]. When a small ligand binds to a macromolecule its apparent rates of diffusion and reorientation are decreased. Such decreased diffusion rates are easily measured by gradient-enhanced NMR spectroscopy [25]. The decreased rate of molecular tumbling (increased rotational correlation time) is directly manifested in an increased transverse relaxation rate of the NMR signals, which is easily measured. The transferred nuclear overhauser enhancement (NOE) technique also falls into this class [26,27]. It relies on detecting intra-ligand NOEs that develop in the bound state – where the dipole–dipole interaction caused by the decreased molecular tumbling rate is much more efficient than in the free state – by observing the free ligand.

Methods that represent the second class of experiments are NOE pumping [28,29], saturation transfer difference (STD) [30] and water–ligand observed via gradient spectroscopy (WATERLOGSY) [31,32]. These techniques are closely related in that they rely on dipole–dipole interaction between ligand and target spins. NOE pumping and WATERLOGSY represent coherent methods, because net magnetization is transferred. In this context, STD should be considered an incoherent method because it relies on transfer of saturation, for example, absence of coherence. This set of experiments represents the most attractive

NMR-based techniques for binding detection currently available because it enables a lower protein concentration than other techniques. The STD technique has been demonstrated to also be applicable to membrane protein targets [33].

The use of ligand-based screening is exemplified by our internal efforts on protein tyrosine phosphatase 1B (PTP1B). This protein dephosphorylates the insulin receptor, thereby attenuating the insulin signal. Inhibition of PTP1B is, therefore, predicted to increase glucose uptake. Figure 2 shows the results from a one-dimensional ligand-based screening of PTP1B (the reference spectrum of compound cocktail in (**a**) and with protein added in (**b**) identifying one of the compounds as a hit (unpublished).

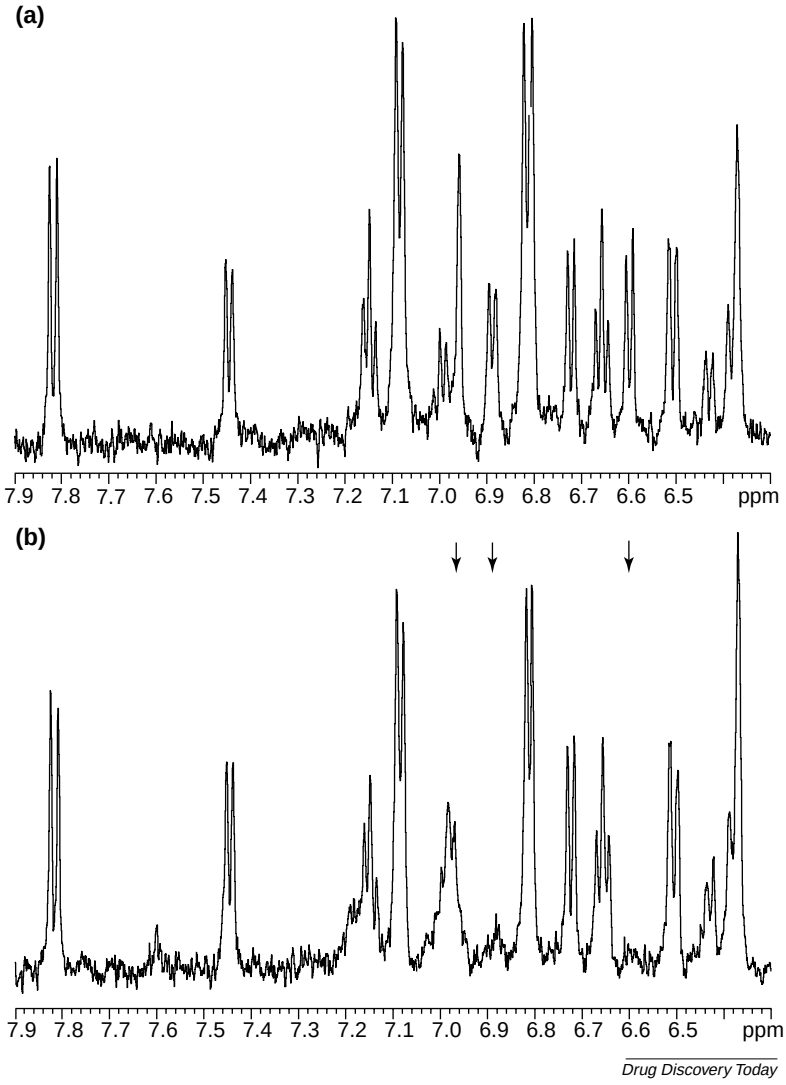

Figure 2. The aromatic region of $T_{1\rho}$-filtered ^{1}H one-dimensional spectra of cocktail O in the absence **(a)** and presence **(b)** of PTP1B. The concentrations for each compound in the cocktail were 100 μM and 50 μM for PTP1B. The three signals (at 6.59, 6.88 and 6.95 ppm) (which originate from the one compound in the cocktail that binds) disappear when PTP1B is present in the sample, whereas the signals from the other nine non-binding compounds in the cocktail remain unaffected.

Target-based screening

Target-based NMR screening relies on the detection of perturbations of target resonances upon the binding of ligands. The most well-known technique is the SAR-by-NMR scheme introduced by Fesik and co-workers in 1996 [5]. Ligands are identified by monitoring alterations of target signals in a two-dimensional (2D) ^{1}H-^{15}N-correlation spectrum. The method requires isotopically enriched target protein, but has the benefit (provided sequence-specific resonance assignments have been obtained) of being able to identify the binding epitope on the target, and to assess whether any significant conformational changes occur upon binding. This approach is limited to smaller proteins (<35 kDa) but the introduction of transverse relaxation optimized spectroscopy (TROSY)-type experiments potentially enables the study of larger protein targets [34].

The incorporation of ^{13}C in the target protein can also be used for the detection of binding of small molecules to targets. For instance, selective ^{13}C labeling of the methyl groups of Val, Leu and Ile has been demonstrated as an attractive approach [35].

Another method for screening larger protein targets has recently been developed in our laboratory. This approach, termed site-selective screening [36], relies on a sequence-specific labeling technique previously applied in the study of protein–protein interactions [37,38]. Amino acid X_n is labeled with ^{13}C and amino acid Y_{n+1} with ^{15}N. Provided that only one XY pair occurs in the amino acid sequence, only one signal will be detected in an HNCO-type [heteronuclear (^{1}H,^{15}N)-amide to ^{13}C-carbonyl correlation] correlation spectrum [39]. The choice of the XY combination is directed by the need to identify compounds interacting with a specific binding site. The labeling strategy is, of course, only sequence-specific in an indirect sense; that is, the occurrence of a unique pair of labeled amino acid residues confers the sequence specificity. Using this labeling strategy, it is possible to screen selectively for binding to a selected epitope without the need for sequence-specific assignments. Chemical shift perturbations upon addition

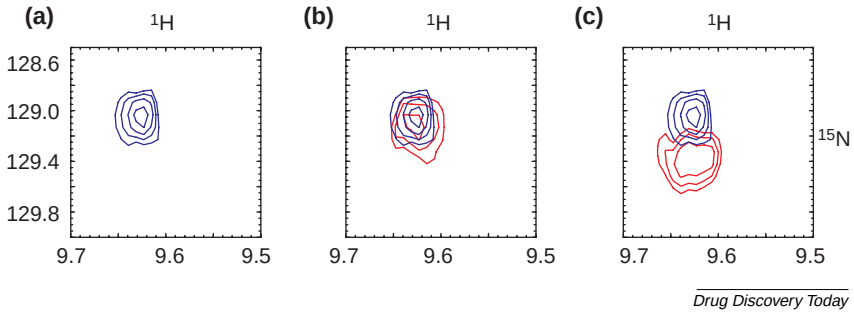

Figure 3. Plots of ^{1}H-^{15}N fast-heteronuclear single quantum coherence (HSQC) spectra recorded at 600 MHz showing the region corresponding to the Val115 cross-peak [54]. The ^{13}C-^{15}N Val FABP-4 concentration was 0.1 mM and the samples contained 1% deuterated-DMSO. **(a)** Reference spectrum of free FABP-4 (blue). **(b)** 1:9 mixture of FABP-4 and compound cocktail omitting BVT.1960 (red). **(c)** 1:9 mixture FABP-4 and compound cocktail including BVT.1960 (red).

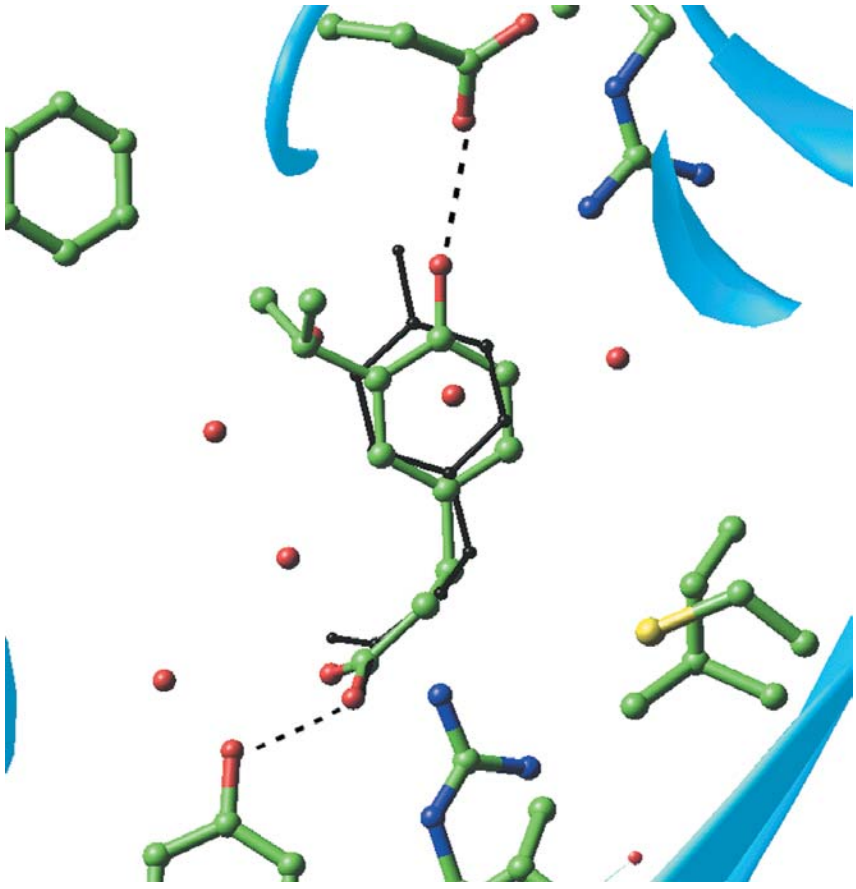

Figure 4. X-ray crystal structure of the active site of FABP-4 showing the initial nuclear magnetic resonance (NMR) hit BVT.1960 (black) and the optimized analog BVT.1961 with the isopropyl substituent (green).

of a potential ligand are easily detected, even for large proteins, because of the reduced spectral complexity resulting from the use of a selectively labeled sample.

The principle of the site-selective screening method was demonstrated for the human adipocyte fatty acid-binding protein, FABP-4. FABP-4 has been implicated in insulin resistance [40] and is thus a potential drug target. Potential inhibitors need to be specific for FABP-4. In particular, binding to FABP-3, which is expressed in heart and muscle, should be avoided. Val114 and Val115 line the fatty acid-binding pocket in FABP-4. In FABP-3, the corresponding amino acids are Ile and Leu [41]. A ligand that binds to this epitope could, therefore, potentially be selective for FABP-4. Further analysis shows that Val114 and Val115 comprise a unique amino acid residue-pair in the amino acid sequence of FABP-4, and are thus suitable for selective labeling.

A sample of FABP-4 with selectively ^{13}C- or ^{15}N-labeled valine was produced and, as expected, only one peak was visible in a 2D HNCO-type spectrum, uniquely identifying the amide resonance of Val115.

Five test compounds were mixed into a compound cocktail. One compound (BVT.1960) had been identified as a FABP-4 binder in a ligand-based ($T_{1\rho}$-relaxation filter) NMR screen of FABP-4. Figure 3 shows spectral expansions of spectra recorded on selectively labeled FABP-4 in different mixtures. The heteronuclear single quantum coherence (HSQC) cross-peak belonging to Val115 experiences a chemical shift-change upon addition of the test cocktail (Fig. 3c). Omitting BVT.1960 from the cocktail yields a spectrum similar (Fig. 3b) to that observed in the absence of the compound cocktail (Fig. 3a). The X-ray crystallographic structure of FABP-4 in complex with BVT.1960 subsequently confirmed that the compound binds in the Val114–Val115 epitope (Fig. 4).

The site-selective screening approach provides the means to find binders to a selected site identified from either X-ray or NMR structures or protein homology models. For example, one can screen for ligands that bind to a site that confers specificity

for one target within a protein family. The approach also makes it possible to confirm that ligands identified by other methods bind to the desired site of the target.

Scaffold selection

Once hits have been identified, appropriate scaffolds need to be selected for further development. To establish a crude SAR the relative binding strengths of the hits must be determined [42]. Several techniques are available for this purpose. Fluorescence spectroscopy (requires appropriate fluorescence tracers), microcalorimetry and surface plasmon resonance represent accurate and well-established methods. They are, however, not always suited for the detection of weak binders and could require careful preparation of samples and/or reagents.

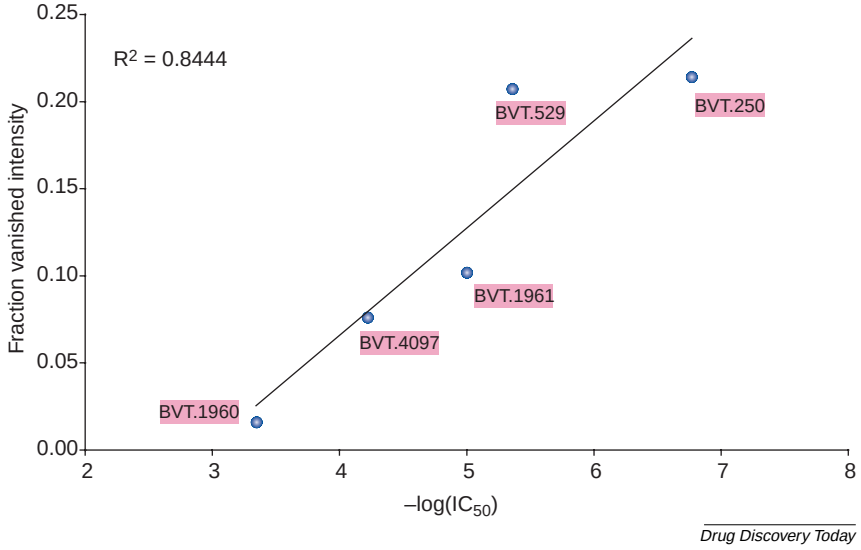

Figure 5. Nuclear magnetic resonance (NMR) relaxation filter experiments for affinity ranking of NMR hits applied to FABP-4 in combination with five different ligands. A clear correlation between the fraction residual intensity in $T_{1\rho}$-filter experiments and binding affinity was observed. The concentration of FABP-4 is 10 μM with 100 μM concentration of the ligand. The IC_{50} values were determined from a fluorescence polarization competition assay using a fluorescent fatty acid analog for the detection.

Several NMR-based techniques are also available [3,25,43–48]. A simple NMR-based approach is to measure the line-widths of the compound signals in the absence and presence of target protein. If fast exchange kinetics is assumed and possible exchange contributions to the line-widths are small or negligible, it is simple to estimate the binding affinity [3,43]. Because the NMR screening is often performed on cocktails of compounds, spectral overlap could render accurate line-shape analysis difficult. In our laboratory we, therefore, take a less rigorous approach and simply rank the NMR screening hits based on the fraction residual-signal intensity observed in relaxation filter experiments. Figure 5 shows the results from relaxation filter experiments for the ranking of five different ligands for FABP-4. For these ligands there is a clear correlation between the fraction residual intensity in the $T_{1\rho}$-relaxation filter experiments and binding affinity (compared with IC_{50} values from a competitive binding assay developed for FABP-4 using a fatty acid labeled with a fluorescent tracer) for the aromatic resonances (van Dongen, M. *et al.*, unpublished data). The method is fast and allows the data to be collected without the need to prepare additional samples or to carefully adjust acquisition parameters. However, the underlying assumption of fast exchange kinetics should always be kept in mind. If the exchange between free and bound states is in the intermediate time regime, resonance lines could experience significant exchange broadening, leading to an overestimation of the binding strength.

By combining the affinity data with an analysis of structural features of the ligands and preferably the target, a crude SAR is formulated. The selected analogs are tested and ranked in the NMR-binding assay. The process is repeated iteratively and as analogs with higher-binding affinity are identified, the probability of a boost to the process, in the form of high-resolution (X-ray or NMR) structures of the target-hit complexes, increases (Fig. 1).

Structure-based optimization of NMR hits

Using structural information there are two major routes for the optimization of hits generated in the NMR screening: decoration or fusion (Fig. 6). The application of linking hits from NMR for the application in drug discovery was introduced by Fesik and co-workers, in that by tethering weak-binding ligands to two adjacent sites they were able to achieve nanomolar affinities for the final product. This approach was applied to FK506 [5] and stromelysin [49]. In both cases, the structures of the protein complexes with the ligands were determined by NMR spectroscopy to design linkers of appropriate lengths. The site-selective screening approach (as described) could be useful in this context, because it enables the identification of scaffolds that bind to defined sites in the target protein.

Another approach to find ligands that bind to targeted sites on proteins using an intermediary disulfide tether was recently introduced [50].

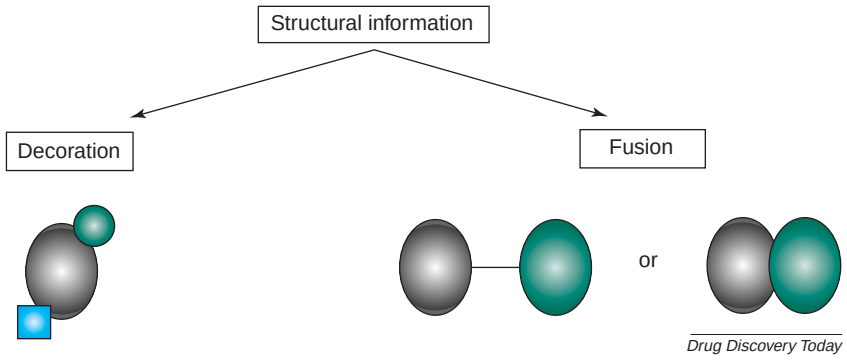

Figure 6. The optimization of hits based on the structural information can be done in two ways: decoration, in which the analogs are identified or synthesized, and fusion, in which fragments binding to adjacent sites in the target are linked together.

Decoration of hits involves the search for analogs that would occupy unused space in the binding pocket and potentially introduce additional and favorable interactions. We applied this approach to find ligands for FABP-4 with improved binding characteristics compared with the initial NMR hits (van Dongen, M. *et al.*, unpublished data). The NMR screening of FABP-4 resulted in a total of 52 hits. Based on crude SAR data from the screen, four compounds were selected for further structural elucidation. X-ray crystal structures of complexes were obtained for two of these hits. Figure 4 shows the crystal structure of the binding site of FABP-4 in complex with BVT.1960. It revealed interactions between the carboxylate group of the NMR hit and a conserved tyrosine side-chain that was similar to the interaction between FABP-4 and fatty acids [51]. In addition, a novel interaction (as compared to the binding of fatty acids) was observed for the distal hydroxyl group, which forms a hydrogen bond to an aspartic acid side-chain. In parallel to the NMR screening efforts, a competition assay based on fluorescence polarization was developed using a fatty acid analog with a fluorescent group for detection. By comparing the binding affinity of BVT.1960 for FABP-4 and FABP-3 it was shown that BVT.1960 had a selectivity of at least 25-fold for FABP-4 (IC_{50} = 590 μM) over FABP-3 (IC_{50} >15 mM). From a comparison of the two protein sequences it was concluded that this selectivity is probably a result of the effect of V115L and C117L substitutions in FABP-3. This scaffold was, therefore, selected as the starting point for the generation of analogs.

It was inferred from the structural information that one flank of the ligand was pointing towards an unoccupied portion of the binding pocket. To identify analogs, a similarity search on commercial and in-house compounds was performed. Eleven compounds were tested and it was found that substitutions at the ortho and meta positions could improve binding affinity with retained selectivity. Based on these findings, 12 analogs were synthesized and binding studies clearly showed a beneficial effect of substitutions at the meta position. The analog BVT.1961, with an isopropyl group at this position, resulted in a binding affinity of 10 μM with retained selectivity for FABP-4. The X-ray crystal structure of BVT.1961 in complex with FABP-4 showed that the binding mode was similar to that of BVT.1960, with the added isopropyl group occupying the previously unused region in the binding pocket (Fig. 4).

In many cases HTS will also be performed, and by the time of its completion the available knowledge collected on small molecule–target interactions will enable a head start in developing the HTS hits. In cases in which the lead identification after the HTS proves difficult, structure-based screening techniques can also have a strong impact. The HTS hits can be dissected into fragments and NMR screening techniques applied to analyze the fragments and analogs thereof. Potentially new and improved fragments can then be identified and incorporated into the original scaffold. This approach was applied to adenosine kinase inhibitors to improve their binding affinity and physicochemical properties [52].

Conclusions

The structure-based screening approach, as described in this review, should be considered as an important complement to traditional HTS in the drug discovery process. The NMR-binding assay represents a generic screening method that can be applied to any soluble target as soon as protein is available. This could prove particularly useful in the context of the large number of new potential targets from genomics-based target identification in which the knowledge of the target is limited, and could in such cases quickly provide chemical tools (in the form of compounds) and assessment of drug-ability (small-molecule binding propensity).

The ligand-based screening approach is not limited by the molecular weight of the target and has recently even been used to detect the binding of small molecules to membrane-bound targets [33]. This opens up new and exciting applications for NMR-based screening. The site-selective screening method using ^{13}C-^{15}N amino acid pairs has the potential to increase the molecular weight limit of target-based screening with retained information on the site of interaction.

A key component for the successful application of structure-based screening is the ability to establish a crude SAR to enable the selection of promising scaffolds. Structural information (from modeling, NMR or X-ray) on the binding of hits to the target is essential to drive the optimization process. We have been able to obtain high-resolution structures even for compounds that bind weakly to the target ($K_d \approx 1$ mM). This is most probably because the compounds are polar and highly soluble, thereby increasing the chances of obtaining co-crystals.

In conclusion, structure-based screening could provide leads with improved physicochemical properties compared with HTS, thus improving the chances of success in the later stages of drug discovery projects.

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