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Advantages of crystallographic fragment screening: Functional and mechanistic insights from a powerful platform for efficient drug discovery

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ABSTRACT

X-ray crystallography has been an under-appreciated screening tool for fragment-based drug discovery due to the perception of low throughput and technical difficulty. Investigators in industry and academia have overcome these challenges by taking advantage of key factors that contribute to a successful crystallographic screening campaign. Efficient cocktail design and soaking methodologies have evolved to maximize throughput while minimizing false positives/negatives. In addition, technical improvements at synchrotron beamlines have dramatically increased data collection rates thus enabling screening on a timescale comparable to other techniques. The combination of available resources and efficient experimental design has resulted in many successful crystallographic screening campaigns. The three-dimensional crystal structure of the bound fragment complexed to its target, a direct result of the screening effort, enables structure-based drug design while revealing insights regarding protein dynamics and function not readily obtained through other experimental approaches. Furthermore, this "chemical interrogation" of the target protein crystals can lead to the identification of useful reagents for improving diffraction resolution or compound solubility.

1. Introduction

Fragment-based drug discovery (FBDD) has gained popularity as an effective and efficient approach for identifying pharmacophores for lead development. FBDD entails screening of small-molecule libraries against a target protein to identify weakly potent, bioactive molecules. The smaller, less complex nature of fragments increases the probability of binding to a target protein, resulting in higher hit rates and efficient search of diverse chemical space (Hadjuk et al., 2007; Hesterkamp et al., 2008; Erlanson et al., 2004). Hits identified from fragment screening do not require deconstruction and can be efficiently developed for specificity and inhibitory activity.

Today, an array of biophysical methods has been developed to rapidly identify weakly binding fragments to a target protein. Nuclear magnetic resonance (NMR) and X-ray crystallography were among the first applied and, more recently, surface plasmon resonance (SPR) and differential scanning fluorimetry (thermal shift) have become popular as primary screening methods. X-ray crystallography provides structural information that enables rapid and efficient assessment of hits with respect to synthetic tractability for structure-based drug design. With respect to FBDD, high-resolution X-ray crystal structures provide a three-dimensional perspective of the binding landscape for potential fragment expansion, merging, or linking. Compared with other screening methods, X-ray crystallographic fragment screening is unmatched in terms of the range of ligand binding affinity (sub-nanomolar to millimolar, limited only by solubility of the ligand) and the size of the target macromolecule (up to megadaltons, limited only by crystal robustness and resolution limit). However, application of X-ray crystallography as a primary screening method has been under-appreciated and often considered to be impractical due to its relatively low throughput and highly resource-intensive nature.

Recent advances in crystal transport, robotic crystal mounting, powerful detectors, and automated data collection software have

Abbreviations: BpGCDH, *B. pseudomallei* glutaryl-CoA dehydrogenase; DHNA, 7,8-dihydroneopterin aldolase; DMSO, dimethylsulfoxide; FBDD, fragment-based drug discovery; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NMR, nuclear magnetic resonance spectroscopy; NNRTI, non-nucleoside reverse transcriptase inhibitor; PA_N, N-terminal domain of the PA protein; PR, protease; RT, reverse transcriptase; TMAO, trimethylamine-N-oxide; TS, thymidylate synthase.

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significantly improved the throughput and reduced the amount of labor associated with this approach. The technical improvements combined with an efficient strategy for data collection and rapid hit identification makes X-ray crystallography an attractive approach for primary screening, in that it not only identifies fragment binding but also provides a three-dimensional structure that can enable and facilitate rapid structure-based optimization.

2. Historical perspective

Mattos and Ringe (1996) were among the first to demonstrate the potential for systematic exploration of potential small molecule binding sites by soaking organic solvents into crystals that diffract X-rays to high resolution. This approach, referred to as multiple solvent crystallographic structures (MSCS), uses organic solvents as probes for locating and characterizing potential binding sites for ligand design (Allen et al., 1996; Mattos and Ringe, 1996; English et al., 1999). Superimposing crystal structures from soaking with different organic solvents can provide insight regarding the conformational landscape, chemical complementarity, plasticity, and surface hydration for a binding site of interest. In addition, the structural information, with the aid of molecular modeling, can be used to develop pharmacophoric models or improve binding affinity and specificity for lead optimization (Mattos et al., 2006; Behnen et al., 2012). However, difficulties in reliably predicting optimal binding modes and overcoming exquisitely demanding stereochemical requirements for linked molecules have limited the application of MSCS for drug discovery.

In 1998, Stout et al. developed a deconstruction approach using *Escherichia coli* thymidylate synthase (TS) and its natural substrate, deoxyuridine monophosphate (dUMP), to understand ligand binding. Structural analysis of small molecule fragments of dUMP in complex with TS revealed the relative contributions of each individual chemical component to the overall binding of dUMP. This modular approach to deconvolution of a ligand hinted that the opposite methodology where compatible fragments can be merged in an additive manner might be promising.

Christophe Verlinde and Wim Hol were among the first to utilize X-ray crystallography to identify small molecule binding from a fragment library of 384 compounds to triose phosphate isomerase from *Trypanosoma brucei* (Verlinde et al., 1997). For high-throughput purposes, crystallographic screening was conducted by soaking pre-formed crystals into three mixtures, commonly referred to as cocktails, of 128 compounds (Verlinde et al., 2009). Electron density from one cocktail soak indicated fragment binding; however, compound identification was not readily possible due to a limited resolution of 2.8 Å. Subsequent deconvolution indicated that the observed electron density was likely from a product of a chemical reaction between fragments within the cocktail. Based on the experimental results, it was clear that X-ray crystallography in combination with cocktails could be used for fragment hit identification. However, design of these cocktails should take into consideration chemical reactivity, solubility, and shape diversity.

Initial studies conducted by both academic and industrial groups pioneered the use of X-ray crystallography as a primary screening method for fragment-based drug discovery. Along the way, several important experimental factors were found to be critical for a successful crystallographic screening campaign. This review will describe some of the experimental challenges and approaches associated with X-ray crystallographic fragment screening. In addition, interesting biological and technical insights gained through crystallographic fragment screening campaigns will also be highlighted.

3. Experimental considerations

3.1. Cocktail design

The approach to cocktail crystallography devised by Nienaber et al. (2000) at Abbott Laboratories led to the development of the CrystaLEADS system. To enable rapid hit identification, cocktails were designed to maximize the structural diversity of the fragments. Urokinase was used to demonstrate the applicability of CrystaLEADS for hit identification. Nine crystals of urokinase were used to screen a fragment library of 61 compounds grouped into cocktails of 6–8 fragments. Screening identified five promising fragments hits, of which 8-hydroxy-2-aminoquinoline was found to be the most potent. With the availability of a crystal structure combined with previously established SAR, a single modification of the fragment hit allowed for extension into an adjacent pocket leading to a 151-fold improvement in potency. More commonly, the CrystaLEADS approach entails grouping a 10,000 compound library into 100 fragments per cocktail. The application of this cocktail design was demonstrated by crystallographic screen of 7,8-dihydroneopterin aldolase (DHNA). Screening identified two fragment hits, which were subsequently developed into lead compounds with μM activity against DHNA (Sanders et al., 2004).

Astex Pharmecuticals pioneered a successful approach for cocktail crystallography as part of their proprietary Pyramid platform. Cocktail design limited the number of fragments per cocktail to four and strongly emphasized chemical diversity within a cocktail (Hartshorn et al., 2005). This allowed for efficient deconvolution and reduced the likelihood of multiple fragment binding. In contrast, Johnson and Johnson's approach to cocktail design focused on designing cocktails of five compounds with similar shape (Spurlino, 2011). This approach took advantage of multiple-fragment binding within one cocktail to strengthen the electron density for a fragment hit.

The Biomolecular Structure Center at University of Washington as part of the Medical Structural Genomics for Protozoan Parasites Consortium (MSGPPC) reported using a computational approach for cocktail design. A 680 compound library was designed from an initial library of 9486 compounds using a series of filters to limit the size, number of heteroatoms, hydrophobicity, and number of rotatable bonds as well as to remove compounds that were chemically reactive and expensive. A shape fingerprint analysis was conducted to design 68 cocktails of 10 structurally diverse compounds. This not only takes advantage of shape diversity to facilitate deconvolution but also allows for rapid screening.

3.2. Protein crystallization

Verlinde et al. (2009) described the importance of protein crystallization towards the success of a fragment screening campaign. The MSGPPC screened a total of 26 protein targets, of which 19 were found to be impervious to fragment binding. The high percentage of nonproductive targets was due to poor resolution (lower than 2.8 Å), reduced crystal stability or robustness in the soaking conditions, or simply no observed fragment binding. The number of cocktails that could be successfully screened was limited by either poor resolution or crystal robustness for certain protein targets. For instance, only 42 useful datasets were obtained from soaking 66 cocktails into 147 crystals of *Leishmania major* coproporphyrinogen III oxidase. Similarly, only 42 of the 68 cocktails screened against *Leishmania naiffi* uracil-DNA glycosylase produced datasets useful for fragment identification.

Thus, the success and throughput of crystallographic fragment screening is heavily dependent on resolution and robustness of the protein crystal. Ideally, protein crystals that diffract X-rays to high