Pushing the Limits of Detection of Weak Binding Using Fragment-Based Drug Discovery: Identification of New Cyclophilin Binders

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Abstract

Fragment-based drug discovery is an increasingly popular method to identify novel small-molecule drug candidates. One of the limitations of the approach is the difficulty of accurately characterizing weak binding events. This work reports a combination of X-ray diffraction, surface plasmon resonance experiments and molecular dynamics simulations for the characterization of binders to different isoforms of the cyclophilin (Cyp) protein family. Although several Cyp inhibitors have been reported in the literature, it has proven challenging to achieve high binding selectivity for different isoforms of this protein family. The present studies have led to the identification of several structurally novel fragments that bind to diverse Cyp isoforms in distinct pockets with low millimolar dissociation constants. A detailed comparison of the merits and drawbacks of the experimental and computational techniques is presented, and emerging strategies for designing ligands with enhanced isoform specificity are described.

Introduction

Fragment-based drug discovery (FBDD) is frequently used to identify small organic molecules (fragments) as starting points for further structure-based drug design (SBDD) programs that aim to deliver drug-like molecules suitable for clinical studies. Fragments can be described using the "rule of 3" [1–3]. According to this rule, a fragment is typically an organic molecule with molecular weight (Mw) of $\leq$300 Da, number of H-bond acceptors of $\leq$3, number of H-bond donors of $\leq$3 and clogP (computed partition coefficient) of $\leq$3 [1]. Fragments typically exhibit a dissociation constant in the micromolar to low millimolar range, and the success of FBDD can be linked to steady improvements in robust biophysical characterization of weak binding [2,4].

A number of problems are associated with the effective screening of fragment libraries. It is often difficult to solubilize fragments at concentrations required to saturate a protein target. In addition, the presence of aggregates, impurities and/or reactive intermediates can also lead to false positives or negatives. These issues are exacerbated for particularly small fragments ($\leq$150 Da) that are likely to exhibit at best mM dissociation constants, and there is a need for pushing the limits of detection of weak binding to broaden the scope of FBDD.

The past decade has seen rapid developments in the application of molecular simulations to SBDD [5]. Molecular simulations and free energy calculations are now being used to complement experimental approaches for a wide range of protein–ligand complexes, including estimation of binding energies of drug-like molecules [6,7], and fragments [8]. This report focuses on the combination of biophysical measurements and molecular simulation methods to characterize weak cyclophilins (Cyps) binders present within a library of small fragments.

Cyps are a family of peptidyl–prolyl isomerases (PPIases) that catalyze the isomerization of proline residues, promoting and facilitating protein folding [9]. The human Cyp family counts 17 members with the prototype and most abundant being cyclophilin A (CypA) [10]. Cyp orthologs can also be found in most plants, parasites and animals [11,12]. The amino

acid sequences of all human Cyps are well established, as well as their secondary and tertiary structures [10]. All Cyps share a highly conserved PPIases domain. Cyps are also members of the immunophilins class of proteins [13–15], and many Cyps are inhibited by the natural immunosuppressant cyclosporin A (CsA) [10,15]. CsA is used in organ transplantation to prevent immune response and organ rejection. Cyps are also involved in (mis)regulation of several biological signaling pathways including damage-induced cell death [16], RNA splicing [17] and different types of cancer. Cyps are also known to be involved in the life cycle of different viruses such as human immunodeficiency virus 1 (HIV-1) and hepatitis C virus (HCV) [18,19].

Because of their diverse biological roles, Cyps are recognized as potential biological targets for the treatment of HCV [19–21], HIV [22–24], cancer [25–27] and neurodegenerative diseases such as Parkinson's and Alzheimer's [28–30]. Originally, efforts for the identification of Cyp inhibitors were focused on cyclic peptides, analogs of CsA [20,21,31,32]. In recent years, a number of small-molecule Cyp inhibitors have been reported in the literature [33–41]. A major unsolved challenge is to achieve robust binding affinity and specificity to distinct Cyp isoforms. Difficulties in achieving strong binding affinities arise from the shallow, solvent-exposed nature of the active site of Cyps. Furthermore, the high degree of structural similarity between isoforms makes it challenging to achieve high binding specificity. Nevertheless, this is widely thought to be necessary to produce chemical probes able to elucidate the biological roles of different Cyp isoforms, and to pave the way for next-generation Cyp drugs with reduced side effects in comparison with CsA analogs.

The present work used surface plasmon resonance (SPR), X-ray diffraction and molecular dynamics (MD) simulations to screen a focused library of small fragments against the most common Cyp isoforms—CypA, CypB and CypD. This combination of multiple methodologies leads to the characterization of novel Cyp fragments that are suitable starting points for further optimization into more potent and isoform-specific lead molecules.

Results

A focused fragment library to interrogate ligandability of the Abu pocket in Cyps

The starting point for this focused library design was 2,3-diaminopyridine, for which SPR and X-ray data had been generated previously, demonstrating the stoichiometric binding of this fragment to the Abu pocket of CypA (Fig. 1) [42]. Comparative analysis of primary protein sequences has suggested that the

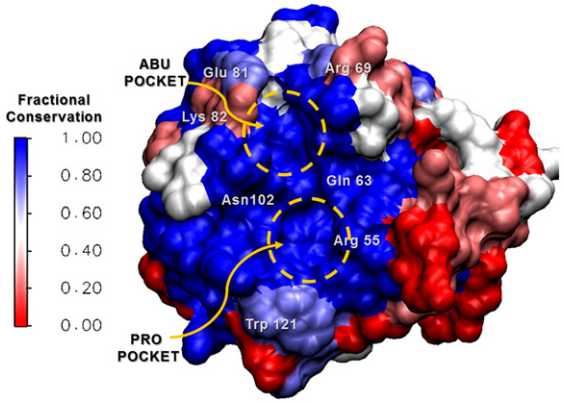

Fig. 1. 3D surface structure of CypA color coded according to degree of residue conservation across isoforms A, B, C, D and F (blue, strictly conserved; red, different in every isoform). Abu and Pro pockets and key active side residues are also highlighted.

Abu pocket in Cyps may be utilized to engineer isoform-selective ligands owing to small variations in amino acids that line up the edge of the pocket between the common isoforms, Cyp A–D. This contrasts with the nearby Pro pocket that is more conserved across the Cyp family. Thus, small fragment analogs of 2,3-diaminopyridine were selected to study the chemical diversity that would be tolerated by the Abu pocket. A total of 100 small fragments, structurally distinct from those previously tested, were chosen based on chemical similarity and commercial availability. Analogs include substituted aromatic rings such as pyridines, pyrazines and pyrimidines, as well as nonaromatic rings. A full list is provided in Supplementary Table 1. The relatively low average molecular weight (ca. 150 g mol^{-1}) of the library members is dictated by the small size of the Abu pocket; hence, any binders are expected to exhibit at best high micromolar to low millimolar binding constants.

SPR suggests that several of the fragments may bind weakly to diverse Cyp isoforms

All the fragments were initially screened by SPR at 1 mM, using conditions described previously [42], on a high-density (to account for the mass ratio of the fragments to His-CypA, His-CypB and His-CypD) surface of 3200, 3000 and 3000 RU covalently stabilized His-CypA, His-CypB and His-CypD, respectively. Apparent Cyp-specific hits were further analyzed with a 2-fold concentration series from 0.015 to 1 mM.

This exercise provided evidence for specific binding in the range of 0.5–20 mM against at least one isoform for approximately 15 fragments. However, it was not possible to derive reliable K_d estimates due to limitations in compounds solubility and difficulties in rigorously removing potential