Published in final edited form as:

Bioorg Med Chem Lett. 2017 September 01; 27(17): 4133–4139. doi:10.1016/j.bmcl.2017.07.035.

Pocket detection and interaction-weighted ligand-similarity search yields novel high-affinity binders for Myocilin-OLF, a protein implicated in glaucoma

Bharath Srinivasan^{a,b}, Sam Tonddast-Navaei^{a,b}, and Jeffrey Skolnick^{a,c}

^aCenter for the Study of Systems Biology, School of Biology, Georgia Institute of Technology, 950, Atlantic Drive, Atlanta, Georgia 30332, United States

Abstract

Traditional structure and ligand based virtual screening approaches rely on the availability of structural and ligand binding information. To overcome this limitation, hybrid approaches were developed that relied on extraction of ligand binding information from proteins sharing similar folds and hence, evolutionarily relationship. However, they cannot target a chosen pocket in a protein. To address this, a pocket centric virtual ligand screening approach is required. Here, we employ a new, iterative implementation of a pocket and ligand-similarity based approach to virtual ligand screening to predict small molecule binders for the olfactomedin domain of human myocilin implicated in glaucoma. Small-molecule binders of the protein might prevent the aggregation of the protein, commonly seen during glaucoma. First round experimental assessment of the predictions using differential scanning fluorimetry with myoc-OLF yielded 7 hits with a success rate of 12.7%; the best hit had an apparent dissociation constant of 99 nM. By matching to the key functional groups of the best ligand that were likely involved in binding, the affinity of the best hit was improved by almost 10,000 fold from the high nanomolar to the low picomolar range. Thus, this study provides preliminary validation of the methodology on a medically important glaucoma associated protein.

Graphical abstract

^cCorresponding author, skolnick@gatech.edu, Tel: (404) 407-8975, Fax: (404) 385-7478.

^bBoth authors contributed equally to this study

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Keywords

Binding; Myocilin; Glaucoma; drug discovery; virtual ligand screening; thermal shift assay

The art of drug discovery relies on identifying small molecules that bind to and modulate the behavior of a target protein. Attempts have been made to achieve this objective using both experimental high throughput screening (HTS) and computational virtual ligand screening (VLS) approaches¹. Though identifying small-molecules employing experimental HTS approaches would be ideal, its cost and time-consuming nature can be greatly reduced by efficient VLS approaches that narrow down the set of candidate small molecule ligands that will be experimentally screened. Traditional VLS exclusively relies on structure-based or ligand-based approaches with their associated advantages and disadvantages as delineated in²⁻⁸. Structure-based VLS require no *a priori* knowledge of binding ligands and could target a specific pocket of interest⁹, but most variants require a high-resolution structure and even then, their success rate for a novel protein target is quite low^{3-5, 7, 8, 10}. In contrast, ligand-based VLS approaches require at least one known bioactive molecule that serves as a seed to fish out database molecules with similar chemotypes and thus cannot explore new regions of chemical space. In practice, many high value therapeutically important protein targets lack either structural and/or significant ligand binding information. For these targets, ligand copying from evolutionarily related homologous proteins is a quite effective alternative which has higher success rates than traditional approaches even when the crystal structure is unknown^{3-5, 7, 8, 10}. However, these methods lack the ability to target a specific protein pocket and were limited to the identification of ligands similar to that in the template protein. To address the former issue, we previously demonstrated that since the number of distinct small molecule ligand binding pockets in proteins is small, copying ligands from pockets similar to the target pocket performs fairly well¹¹. Here, we apply a generalization that enables both pocket selection and exploration of a broader range of ligand space to a medically relevant protein myocilin for which only very poor quality binders were previously known¹² (Fig 1).

In a previous study, we implemented a pocket based VLS approach called *PoLi* to determine small-molecule binders for *Escherichia coli* dihydrofolate reductase, DHFR, with an 18.4% success rate¹¹. Here, success is defined as identifying small molecules with μM or better binding affinities. DHFR is a well-studied protein with a lot of structural and small-molecule binding information available making it a relatively easy case. However, this methodology has not been implemented on less well-documented proteins that lack extensive structural or small-molecule binding information, nor does it allow for the exploration of a broader range of ligand space. In this study, we address these issues and demonstrate the successful application of our improved pocket based VLS approach to myocilin-OLF. Olfactomedin or oligosaccharide-lipid flippase (OLF) domains have been implicated in several disorders in humans ranging from inflammatory bowel disorders, cancers to glaucoma¹³. Mutant variants of human myocilin are implicated in causing primary open-angle glaucoma (POAG)¹². There has been a paucity of information on the small-molecules that show binding to myoc-OLF as a possible ameliorative tool to prevent its aggregation. A recent study reported 14 compounds that bind to the protein, with two, GW5074 and apigenin,

capable of inhibiting myoc-OLF amyloid formation *in vitro*¹⁴. However, the binding affinities of GW5074 and apigenin for myoc-OLF, as determined by SPR, were 33.5 ± 20.9 and 41.6 ± 39.6 μ M. Of greater concern, apigenin gave a somewhat incomprehensible negative T_m of -0.3 °C, possibly indicative of very weak binding, while GW5074 gave a minuscule T_m of 0.2 °C. Thus, these molecules are quite weak hits. Here, we use a hybrid pocket- and ligand-based virtual screening algorithm to predict novel ligands that can bind to myoc-OLF, see Fig 1. Furthermore, we employ a newly developed iterative ligand seeding approach, instead of traditional quantitative structure-activity relationship studies (QSAR), to predict small molecules with tighter binding potential (Fig 1). This search based on iterative geometric and chemical similarity represents a promising approach towards identifying the active pharmacophore and is an alternative to traditional QSAR.

Following the protocol schematically outlined in Fig. 1, pocket detection was carried out for the olfactomedin domain of myocilin as follows: The experimentally solved crystal structure of Myocilin Olfactomecin Domain (PDB_ID: 4WXQ¹⁵) was employed to detect potential small molecule binding pockets. In the first step, Concavity with default parameters¹⁶ identified cavities on Myocilin's surface and the associated pocket residues were identified. Subsequently, we used APoc¹⁷ to calculate the pocket-pocket similarity between myocilin and the holo pockets of the holo-protein database, PDB-holo (having more than 50,000 entries), used previously¹⁸ in order to copy ligands using pocket similarity. A ligand is copied only if the pocket similarity score has a statistically significant p-value (less than 0.01). Two different methods were utilized to find similar ligands to the seed ligand (the ligand copied from the template pocket). A) If a small and localized fragment of a seed ligand was responsible for enthalpic interactions (e.g. hydrogen bonds) given by PoseView¹⁹, and the region of interaction was aligned to the pocket of Myocilin-OLF, the fragment was used as a template for pharmacophore search. Given a pharmacophore of interest, the OpenBabel²⁰ cheminformatics tool was used to generate the SMARTS patterns. Next, the pattern was used to screen against the ligand library (NCI diversity set). B) If multiple non-localized regions (separated by more than one rotatable bond) of the seed ligand participate in an interaction with the template's pocket, then LIGSIFT²¹ was used to calculate the 3D shape and chemical similarities of the seed molecule with the ligand library.

Myocilin's structure was scanned for potential ligand binding pockets and similar holo-structures as described above. This resulted in a total of two potential binding pockets, PKT1 (green) and PKT2 (blue) shown in Fig 2. Each pocket was then used to scan PDB-holo for similar pockets. Initially, we found 40 small molecules with their pocket's having significant similarities to either PKT1 or PKT2. This list was then assessed manually and reduced to 26 molecules that had "drug-like" properties²² (Table S1). Finally, three seed molecules from PKT1 and two from PKT2 were chosen to scan against the NCI diversity set (Table 1).

Global structure similarity to holo-proteins also provided alternative binding pockets (Figure 2) of PKT3 (yellow) and PKT4 (magenta). Ligands were also copied based on the global similarity of the proteins using TM-align (a widely used protein structural alignment algorithm)²³ to identify holo-proteins with high structural similarity (TM-score > 0.5) whose ligands will then be copied to the myocilin pocket of interest. The structure of myocilin was compared to PDB-homo. A ligand is copied from a holo template protein if

more than two thirds of its holo pocket residues are aligned (distance $< 3 \text{ \AA}$) to those in myocilin. Since the templates of these two pockets provided us with seed ligands (Table 1), we simply used them to scan the NCI set and didn't use PKT3 and PKT4 to search for similar pockets. Scanning the NCI diversity set using the set of 8 seed molecules from PKT1–PKT4 gave 55 molecules (Table S2).

To experimentally assess the validity of the predictions, high-throughput thermal shift assays were carried out following established guidelines employing differential scanning fluorimetry (DSF)^{24, 25}. Briefly, samples in 96-well PCR plates were heated from 25 °C to 74 °C using a 1 °C/min heating ramp in a RealPlex quantitative PCR instrument with 5 X Sypro orange as the reporter fluorescent dye. A single data point was acquired for each degree increment. The reaction was carried out in a volume of 20 μL , with 100 mM HEPES pH 7.3, 150 mM NaCl and 5 μM of the respective protein^{8, 11, 26, 27}. The experiments were performed with biological replicates and the mean value was considered for further analysis. Curves showing a single sigmoidal thermal transition were selected and normalized for further analysis. The curves were fit to Boltzmann's equation (Eq. 1) to obtain the melting temperature, T_m , from the observed fluorescence intensity, I by:

$$I = I_{min} + \frac{[I_{max} - I_{min}]}{1 + e^{\left(\frac{T_m - T}{a}\right)}} \quad (1)$$

where I_{min} and I_{max} are the minimum and maximum intensities; a denotes the slope of the curve at the unfolding transition midpoint temperature, T_m . To estimate the approximate ligand-binding affinity at T_m , Eq. (2) was used; C_p is ignored.

$$K_L(T_m) = \exp\{-\Delta H/R(1/T_m - 1/T_o)\}/L \quad (2)$$

$K_L(T_m)$ is the ligand association constant and $[L]$ is the free ligand concentration at T_m ($[L]_{T_m} \sim [L]_{total}$, when $[L]_{total} \gg$ the total concentration of protein. K_D is the inverse of $K_L(T_m)$. ΔH is the enthalpy change and R is the gas constant. ΔH was estimated employing both van't Hoff and Gibbs-Helmholtz analyses. This methodology has been described previously for apparent K_D computation in⁸.

A total of 55 small molecule predictions belonging to approximately the top 1 % of the ligand library were selected to be assessed with DSF. As stated above, these hits were derived from 4 pockets and 8 seed molecules [0G1, 0OE, FUA, RQQ, TYC, 2ET, VH1, VH2]. All 55 molecules yielded interpretable curves, and seven (Fig 3A) showed positive shifts with the magnitude of the shifts indicated in Fig 3B and Table 2. This indicates a 12.7% success rate in the screening of the top 55 molecules, reflecting a 50% success in seed selection. Fig 3B shows the resulting experimental curves. The best hit was NSC37433, with a thermal shift (ΔT_m) of 16.4 °C with respect to the protein alone control. It should be noted that the reported T_m for NSC37433 is an approximate estimate given that the shape of the curve deviates substantially from a typical two-state unfolding curve and has a small

plateau making the fit have broad confidence intervals. This T_m corresponds to an approximate dissociation constant of 99 nM. Note that the pocket similarity of the template's small-molecule (ligand ID: RQQ from PKT1) binding site with the myoc-OLF domain's NSC37433 binding site has a P-value of 0.008. This site has two amino acids (Ile477 and Asn428) that partly overlap with Ca^{2+} binding site of myoc-OLF comprised of five amino acids (Asp380, Asn428, Ala429, Asn428 and Ile477). This indicates the likely functional significance of the predicted and experimentally assessed binding of NSC37433. In this initial round of screening, there were 6 additional hits that showed high μM or better affinities for myoc-OLF domain (Table 2).

Next, we took the strongest binder (NSC37433), which was predicted to bind to PKT1 based on pocket similarity and used it as a seed for the second round of screening with the hope of increasing binding affinity. For this purpose, we used NSC37433's scaffold (two benzene rings with hydrogen bond donor or acceptors groups, connected by three or four linear linkers) to search for similar molecules in the NCI diversity set. The second round of screening provided us with 35 (Table S3) new predictions.

Very few VLS studies have explored the power of iterative screening whereby predictions are experimentally assessed and hits from the first round are used to identify additional and better ligands using ligand-similarity metrics^{28–30}. Here, in contrast, a total of 35 small molecule predictions were tested with DSF. Analysis indicated that 7 curves were uninterpretable and 25 curves showed either no shift or negative shifts vis-à-vis the protein alone control indicating that there was no potential binding. However, we identified an additional set of 3 new ligands as potential binders of Myoc-OLF. Three small molecules (Fig 4A) showed positive shifts with the magnitude of the shifts as indicated in Fig 4B and Table 3. However, it would have to be once again pointed out that the reported T_m for the three top hits are gross approximations given that the shape of the curves deviates substantially from a typical two-state unfolding curve with NSC80735 and NSC201634 showing biphasic transitions possibly indicative of multiple unfolding intermediates. Further, most of the curves have minimal plateau regions with the resultant fits having broad confidence intervals. Nevertheless, despite these concerns, the results indicate an 11% (3/28) success rate. While two of the three hits did not yield better apparent binding affinities than the parent NSC37433, all three hits from this round of screening had affinities less than $\sim 10 \mu\text{M}$ with the best hit showing an approximate pM affinity for the protein (Table 3). However, these affinities have to be treated with caution given the biphasic nature of the curves and the lack of a proper plateau that results in an increased error. The best hit in this seeded round of screening was NSC201634, with an approximate thermal shift (T_m) of 32°C with respect to the protein alone control. This indicates an approximate dissociation constant of 8 pM. Furthermore, this represents a $\sim 10,000$ -fold increase in the affinity of the best hit compared to the best hit from the initial round of screening i.e. NSC37433.

The olfactomedin domain of human myocilin has been implicated in POAG. Small-molecule probes binding to this protein target might have potential applications in alleviating conditions caused by aberrant form of this protein. Mutations on Myocilin inhibit the proteolytic processing of the full length protein which occurs in the linker (Arg226-Ile227) between amino-terminal domain and the C-terminal OLF domain³¹. Thus, compounds

stabilizing the protein's native conformation could potentially restore this proteolytic cleavage, thus reducing the secretion of the aggregation prone protein. With this goal, we employed our pocket based VLS in combination with high-throughput experimental screening to find novel small molecule binders of this protein. As shown in Fig 5, the application of iterative pocket-based VLS on myoc-OLF demonstrates that it is not only able to predict novel small molecules with better binding affinities for a target of interest than were previously known, but on iteration it can yield better binding ligands. On the other hand, ligand identification based on global fold similarity alone was much less successful, with only a single 2 μ M hit, NSC79688, found. Thus, this study paves the way for future efforts employing this approach to predict better binding ligands for hitherto intractable but therapeutically important targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This project was funded by R35GM118039 of the Division of General Medical Sciences of the NIH. The authors wish to thank Prof. Raquel Lieberman, Georgia Institute of Technology, for providing purified human myocilin olfactomedin domain. We would also like to thank the Developmental Therapeutics Program of the National Cancer Institute and the Medicines for Malaria Venture (MMV) for providing the small molecules used in this study.

Abbreviations

OLF	eukaryotic oligosaccharide-lipid flippase superfamily
POAG	primary open-angle glaucoma
MYOC	myocilin

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Fig 1.

Schematic representation of pocket-based VLS approach. Given a target protein (red), seed ligands (orange and magenta) from the holo proteins are copied based on either global (blue template) or local (green template) pocket similarities reported by TM-align and APoc respectively. Next, the ligand library is screened against the seeds using shape or fragment similarities. The results are then validated experimentally using thermal shift assays. Finally, the “hits” are used as new seeds for additional iterative rounds of VLS.

Fig 2. Structure of Myocilin Olfactomecin Domain (white). The binding sites (PKT1: green, PKT2: blue, PKT3: yellow, PKT4: magenta) and their corresponding seed molecules are highlighted and color-coded. Dashed lines around fragments of a ligand indicate the pharmacophore region used for finding similar molecules. LIGSIFT was used to find similar small molecules to the seed ligands marked with asterisk. The identification of each pocket's amino acids is found in Table S4.

Fig 3.

Initial round of screening with Myoc-OLF (A) Structure of small molecules showing binding to Myoc-OLF as assessed by thermal shift assay methodology. The SDF files for the small molecules were downloaded from Pubchem (<http://pubchem.ncbi.nlm.nih.gov>) and the figure was generated using ChemBioDraw 15.1. (B) Thermal unfolding curves for Myoc-OLF in the presence of the various small-molecules that were screened from *PoLi* VLS predictions and yielded positive shifts.

Fig 4.

Second round of screening with Myoc-OLF predictions done using seeding and ligand similarity algorithm LIGSIFT (A) Structure of small molecules showing binding to Myoc-OLF as assessed by thermal shift assay methodology. The SDF files for the small molecules were downloaded from Pubchem (<http://pubchem.ncbi.nlm.nih.gov>) and the figure was generated using ChemBioDraw 15.1. (B) Thermal unfolding curves for Myoc-OLF in the presence of the various small-molecules that were screened from *PoLi* VLS predictions after seeding and that yielded positive shifts.

Fig 5.

Application of iterative pocket-based VLS to myoc-OLF. Ligand RQQ was copied based on a statistically significant pocket similarity (P-value = 0.008) of a pocket in template protein 4BDK (purple) to myoc-OLF (white). The first round of screening provided a nM binder (NSC37433) and the second round increased the affinity into pM (NSC201634) regime.

Table 1

List of seed molecules and their template copied using global or local similarities.

Pocket	PDB ID	Ligand ID	N _{pkt}	N _{aligned}	TM-Score	P-value (Pocket)	N _{pred}	N _{hit}
PKT1	3V5L	OG1	15	15	0.27	0.001	5	1
	4BDK	RQQ	12	12	0.25	0.008	10	3
	1QCA	FUA	12	11	0.33	0.003	8	0
PKT2	4YKA	TYC	12	11	0.36	0.002	11	0
	4I93	OOE	10	10	0.30	0.0003	10	2
PKT3	2XZG	VH1	13	12	0.61	N/A	5	0
	4G55	VH2	12	11	0.61	N/A	3	0
PKT4	4MSL	2ET	15	11	0.50	N/A	4	1

N_{pkt}: Number of the template PDB amino acids in contact with its ligand.

N_{aligned}: Number of the amino acids that are aligned to Myocilin's structure.

N_{pred}: Number of predictions from the NCI diversity set.

N_{hit}: Number of cases showing a positive thermal shift.

Summary of virtual ligand screening, thermal shift assay and binding parameters for the initial hits obtained on myoc-OLF.

Table 2

Molecules	Seed	Pocket	T_m (°C)	T_m	K_D (approx.)(M)
Myoc-OLF	NA	NA	49.83	NA	NA
NSC37433	RQQ	PKT1	66.27	16.4	9.9×10^{-8}
NSC643148	OGI	PKT1	51.85	2.02	1.7×10^{-4}
NSC408734	OOE	PKT2	51.61	1.78	1.9×10^{-4}
NSC93033	OOE	PKT2	51.49	1.96	2.0×10^{-4}
NSC79688	2ET	PKT4	51.43	1.60	2.1×10^{-4}
NSC11437	RQQ	PKT1	51.33	1.50	2.2×10^{-4}
NSC16722	RQQ	PKT1	50.74	0.91	3.0×10^{-4}

NA, not applicable

Table 3

Summary of virtual ligand screening, thermal shift assay and binding parameters for the hits obtained after initial seeding with known binders of myoc-OLF from the previous round of virtual screening.

Molecules	T_m (°C)	T_m	K_D (approx.)(M)
NSC201634	83.79	~32 *	8.0×10^{-12}
NSC80735	62.86	11.2	6.5×10^{-7}
NSC46492	57.96	6.3	1.1×10^{-5}

S. No., serial number; NA, not applicable,

* The confidence interval of the curve was too wide and hence the estimate represents an approximate fit.