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Investigating Combinatorial Approaches in Virtual Screening on PFKFB3: a Case Study for Small Molecule Kinases

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Abstract

Fruitful efforts toward improving the predictiveness in tier-based approaches to virtual screening (VS) have mainly focused on protein kinases. Despite their significance as drug targets, small molecule kinases have been rarely tested with these approaches. In this paper, we investigate the efficacy of a pharmacophore screening-combined structure-based docking approach on the human inducible 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase, an emerging target for cancer chemotherapy.

Six out of a total 1,364 compounds from NCI's Diversity Set II were selected as true actives via throughput screening. Using a database constructed from these compounds, five programs were tested for structure-based docking (SBD) performance, of which, MOE showed the highest enrichments and second highest screening rates. Separately, using the same database, pharmacophore screening was performed, reducing 1,364 compounds to 287 with no loss in true actives, yielding an enrichment of 4.75. When SBD was retested with the pharmacophore filtered database, 4 of the 5 SBD programs showed significant improvements to enrichment rates at only 2.5% of the database, with a 7-fold decrease in an average VS time. Our results altogether suggest that combinatorial approaches of VS technologies are easily applicable to small molecule kinases and, moreover, that such methods can decrease the variability associated with single-method SBD approaches.

Keywords

Drug Discovery; Computational Biology; software evaluation molecular-docking; enrichment factors; 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase; small molecule kinases; glycolysis; PFKFB3; cancer; Warburg; Fructose-2,6-bisphosphate

Introduction

Compared to the traditional, costly methods of high throughput screening (HTS), the search for the potential drugs with computational technologies, also called virtual screening (VS), continues to grow in popularity.^[1-3] Unlike HTS, which requires constant human intervention, even with the usage of robots, VS needs only a good computational resource and, more attractively, can be performed in a 'fire-and-forget' manner with only minimal human input once properly started. Although each has its own distinct advantages,

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these methods share a common goal in that they both aim to identify a small number of true biological positives amidst a vast amount of biological negatives.^[4, 5]

A computational drug search, or VS, is carried out to find biologically positive compounds from compound databases, which typically contain molecular information on compounds that range in number from thousands to millions. Depending on the screening factors, VS involves diverse strategies: molecular features of the known ligand characteristics, pharmacophore screening (PhS); ligand alignments based on both structural and physical characteristics of ligands, ligand similarity analysis (LSA); and the interactional relationship between ligands and their target receptors, structure-based docking (SBD).^[6] Depending on the availability and capacity of computational resources, an overall strategy of VS can be varied to employ either one, or combinations, of these technologies to appropriately meet the user's needs.

Assuming that data handling is performed in an organized manner, PhS can be carried out in a very short amount of time using even a personal desk computer. SBD, on the other hand, requires an extensive amount of calculations of Gibbs free energy changes involved in the various ligand receptor interactions.^[6] Consequently, a high capacity computational resource, such as a processor cluster, is usually necessary for moderate-to-large SBD projects. However, even with such a facility, the amount of required computation time is often still very considerable, a circumstance owing to the fact that the vast majority of SBD time is consumed performing extensive calculations on outright negatives simply because of low hit rates associated with non-enriched databases. To improve the cost efficiency of SBD, efforts have been made to combine SBD with other less-time consuming technologies.^[7] As a result, tiered screening strategies, in which ATP was considered as single ligand, have been successful in protein kinases (PK), one of the most popular drug targets up to date.^[8-10]

Despite their significance as drug targets, studies for strategic VS of small molecule kinases (SMK) are extremely rare compared to those of PK.^[8, 11] To explore how well the concepts of tiered screening translate to SMK, we sought to design a dual-step screening protocol that could accurately and efficiently identify potent inhibitors through a combination of pharmacophore screening (PhS) and structure-based docking (SBD). Both PK and SMK have a second substrate pocket in addition to the widely conserved ATP pocket. Compared to PK, SMKs can have one, or possibly two, ligands that can bind the non-ATP binding site, most of which are known from biochemical/structural characterizations. We speculated that those known ligands may serve as a good resource of pharmacophore and may serve to add more target specificity. Thus, our study was targeted at the second substrate binding site, the pocket for the acceptor of the phosphoryl group from ATP.

For this project, the human inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3), a newly emerging drug target for potential cancer chemotherapeutics, was taken as the receptor.^[12] The kinase domains of the four PFKFB isoforms catalyze the synthesis of fructose-2,6-bisphosphate (F-2,6-P₂), which is the most potent allosteric stimulator of glycolysis, using adenosine triphosphate (ATP) as the phosphoryl donor and fructose-6-phosphate (F-6-P) as the acceptor.^[13, 14] Predominantly expressed in neoplastic cells by the action of HIF-1 among the four isoforms, PFKFB3, with its kinase activity at least 10× that of the second most active isoform, rapidly increases the level of Fructose-2,6-bisphosphate (F-2,6-P₂).^[15, 16] As a result, glycolysis in neoplastic cells such as cancer is very active, which has been long known as the Warburg effect.^[17, 18] Recent studies have suggested that PFKFB3, whose expression is induced during cancer growth, is one of the most causative molecules of the Warburg effect.^[15, 19] To explore the possibility of PFKFB3 as a new cancer therapeutic target, we determined the crystal structure of PFKFB3 to 2.1 Å and

elucidated the catalytic mechanism for F-2,6-P₂ synthesis at the molecular level.^[20] The resulting molecular structure/function information has been used as the foundation of this study.

In this report, we investigate the efficacy of combinatorial screening of PFKFB3 targeting for the F-6-P site. To evaluate the efficacy of our tiered approach, we developed our own database of active/non-active molecules from the National Cancer Institute's (NCI) Diversity Set II through a biochemical throughput study, since no non-ATP datasets of competitive inhibitors for the second substrate. Using the actives as reference compounds, we explored applications from the two most common approaches in computational drug discovery: pharmacophore screening and structure based docking. Herein, we present the results from our combinatorial approach, demonstrating the enrichment of a 1,364 compound database, containing six true actives 'T-actives', to a resultant database of 287 compounds, still containing six 'T-actives', while using only one seventh of the computational resources required for standard docking procedures.

Materials and Methods

Biochemical throughput screening

The recombinant human PFKFB3 was expressed and purified as described elsewhere.^[20] The 1,364 individual compounds of NCI's Diversity Set II were acquired from the NCI.

The 2-kinase activity assay for throughput screening was performed using a F-2,6-P₂ assay modified for 96-well plates from the conventional method.^[21] This assay consisted of two sequential steps: F-2,6-P₂ production by PFKFB3 and allosteric activation of PFK-1 by produced F-2,6-P₂. The first reaction, F-2,6-P₂ synthesis by PFKFB3, was started by adding 130 nM PFKFB3 to mixtures containing 20 mM pH 8.0 TES, 1 mM DTT, 2 mM MgCl₂, 50 μ M F-6-P, 50 μ M ATP, 0.5% Tween, and 10 μ M of each inhibitor. This reaction was allowed to run for 10 minutes at 25°C and then stopped by the addition of 0.1 M KOH. Aliquots of 1-4 μ L of the first reaction were transferred, after pH neutralization, to the reactions of the second step, which consisted of 50 mM pH 8.0 Tris-HCl, 0.2 mM NADH, 5 mM DTT, 1 mM F-6-P, 2 mM MgCl₂, 0.70 units/mL Aldolase, 0.45 units/mL GDH, 0.60 units/mL TIM, and 10 mU PPi:PFK. The reactions of second step were started by adding 0.5 mM sodium pyrophosphate (PPi) and were then measured for changes in absorbance at 340 nm over a period of 30 minutes.

For molecules showing strong inhibition in the throughput assay, a study of the steady-state inhibition kinetics was carried out using a method in which the concentrations of F-6-P, ATP, and/or inhibitors were varied according to experimental purposes.

Ligand Library Design

The virtual ligands of NCI's Diversity Set II were obtained from NCI's Developmental therapeutics program (DTP). The ligands were acquired in SMILES format and standardized using a template to ensure that each was minimized, pH adjusted, and devoid of salts and other non-ligand contaminants. Additional adjustments, such as explicit hydrogens and force fields were added in a program-dependent fashion depending on manufacturer recommendations. Using the standardized ligand database, an additional conformer database was generated for pharmacophore filtering. For this, MOE was used to generate a conformer database based on default settings with a maximum of 250 conformers per ligand and no post-generation refinement.

Structure-Based Virtual Docking

Virtual docking was carried out using 5 different programs, DOCK, VINA, FlexX, MOE, and GOLD in an effort to determine the suitability of each for PFKFB3 screening.^[22-26] For this, the protein structure '2AXN' was used as the receptor macromolecule for docking.^[20]

Pharmacophore Screening

MOE was used to generate pharmacophores from three molecules known to bind to the F-6-P pocket of the PFKFB3: Fructose-6-Phosphate (F-6-P), Ethylenediaminetetraacetic acid (EDTA), and Phosphoenol Pyruvate (PEP). The conformations for these molecules were obtained from the structural data (*PDB ID*: 2AXN, 2DWO, 2I1V, 2DWP). By superimposing the conformers, property features were extracted and merged and tolerance values were adjusted in accordance with results through retro-fitting. After refinement, eight features were chosen to be included in the final pharmacophore map; however, only 5 features are required to be met at any one time for a compound to pass the filter. Additionally, inclusion and exclusion spheres were added and constraint allowances were adjusted for preference. All pharmacophore searches were carried out within MOE.

Results

Biochemical Throughput Screening

To generate a framework of actives and non-actives for VS, a throughput study of 1,364 NCI compounds was carried out. The inhibition extent of 10 μ M of each compound, in substrate saturation conditions, was quantified and the results of the top 50 compounds are shown in Fig. 1. An arbitrary cutoff was chosen at 75% inhibition to describe compounds that were to be considered 'potential' actives. Based on this cutoff, 10 compounds were identified from the original 1,364.

To select the true positives, the 10 potential actives were subsequently tested for specificity for the F-6-P site, because the VS was targeted for the F-6-P site. Using conventional steady state inhibition kinetics, 6 compounds were selected as the 'true actives (T-actives)' and listed in Fig. 2. All T-actives exhibit competitive inhibition against F-6-P and uncompetitive against ATP, as a representative example, NSC278631, is shown in Fig. 3. The K_i 's for each compound was determined to be at or below 20 μ M.

Pharmacophore Screening

Using ligands already known to bind to the F-6-P site from crystallographic evidence, namely, F-6-P^[27], F-2,6-P₂^[27], EDTA^[20], and PEP^[27], a pharmacophore model was built and used to screen the NCI diversity set via MOE's pharmacophore screening module (Fig. 4). Overall, from this filtering process, the database size was reduced from 1364 to 287 ligands while retaining 6 out of 6 'T-actives'. The results of this procedure demonstrate a significant reduction in non-actives and no reduction in actives. The total screening time was 206 seconds on a 2GHz processor with a conformer database creation time of 9911 seconds.

Performance comparisons of docking programs

Because it has been demonstrated in numerous studies that the efficacy of a SBD program directly ties to the target protein, we chose to test the individual performances of several SBD programs. Using PFKFB3, a full database evaluation was conducted to compare the enrichment factors of five popular SBD technologies (Fig. 5). The results revealed that each of the tested SBD technologies significantly enriched the NCI diversity set II database. However, as seen in other studies, the enrichment rates varied significantly according to the SBD technology.^[28-30] For comparison purposes, we investigated the enrichment at two

database sizes, 2.5% and 10%. MOE performed best, showing higher enrichments at all database sizes. The other SBD programs were more varied in their performances with VINA having the second highest enrichment rates at 2.5% and GOLD at 10%.

Combinatorial Screening Efficacies

To measure the efficacy of the combinatorial screening protocol, the pharmacophore filtering results were subsequently docked using each of the SBD technologies. For this, the PhS enriched database, consisting of the 287 hit molecules with all actives present, was docked and the enrichment rates were evaluated at 2.5% and 10% database sizes (Fig. 6). The results demonstrate improved enrichment rates for four of the five SBG technologies at 2.5% database size and five of five at 10% database size compared with docking-only methods. Additionally, it was determined that the application of the combined protocol, greatly reduced the variability of the incorporated SBD technologies, changing the enrichment differences between the highest and lowest scoring technologies from 13 to 6.5 and 4.9 to 3.2 at 2.5% and 10% database sizes, respectively. Reductions in the overall time were also witnessed, showing nearly a 7-fold decrease in the average total time for a complete database screening using the tiered approach.

Discussion

In this work, the enrichment capability of a commonly employed, tier-based virtual screening approach was evaluated using the small molecule kinase 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase (PFKFB3). For this, biochemical throughput data was generated using the National Cancer Institute's Diversity Set II compound library, serving as a metric upon which enrichment rates were calculated (Fig. 1). The aim was to evaluate the efficacy of the combined pharmacophore-docking protocol in relation to docking-only methods using a representative protein from the small molecule kinase family. Additionally, an investigation into the predictiveness of the major docking technologies was conducted; seeking to identify which offered best enrichments for PFKFB3.

The results of our investigation revealed that tiered screening, in the case of PFKFB3, in which, pharmacophore filtering precedes structure based docking, is clearly favorable to any individual screening method, both in efficacy and efficiency (Fig. 6). An analysis of our results can be evaluated in terms of three factors: speed, accuracy, and consistency.

The accuracy of in silico predictions is always a critical factor of virtual screening. Hence, our investigation into tiered approaches of virtual screening initially focused on differences in enrichment rates between single SBD and combined PhS-SBD approaches. The results from our testing revealed that, in the case of PFKFB3, the enrichment factors of the combinatorial strategy was always comparable to, or in excess of, the values demonstrated from the single-run structure based simulations. Such results were not entirely unexpected when considering the results of previous studies demonstrating similar findings for single-ligand proteins and protein kinases.^[31, 32] However, since no similar, large-scale, studies of small molecule kinases are yet published, it was not known if such trends would extend to small molecule kinases. The findings presented here should serve to lessen our knowledge gap involving the enrichment efficacy of tiered approaches to virtual screening for small molecule kinases.

Another finding reported here involves the changes to SBD consistency when incorporated into tiered-based approaches. From our single-method docking results, it was seen that vast differences exist between the enrichment rates of differing technologies. However, upon analysis of the PhS-SBD tiered approach, it was clear that most of the differences in enrichment among the SBD technologies efficacy had significantly lessened. Such findings

are promising, in that they help to lessen the need to test multiple SBD technologies for any given project. Because it is well known that different SBD technologies offer differing performances depending on the target macromolecule, such findings suggest that the need to test many SBD technologies can be significantly lessened by using tiered approaches.

Lastly, and most apparent among our findings, was the obvious advantage of the tiered-based approach in reducing the computational time necessary to screen the Diversity Set II database. Despite having higher enrichment rates, the tiered screening was, on average, 7-fold faster than its single-run counterparts. The principle behind such an increase hinges on the role of pre-filtering. By using a loose, lightly-selective filter early on, many of the more egregious ligands can be removed prior to the computationally intensive step of docking, thus significantly reducing the time necessary for any virtual screening project.

The results altogether indicates that a combinatorial approach for VS is far more efficient than a blind run of time- and resource-consuming simulated docking. However, it has to be admitted that there are some shortfalls in combinatorial approaches. First, it requires at least one ligand that is already known to bind to a target receptor and, practically, the more the better. In reality, this problem is almost ignorable for most of the active drug target proteins. It is because only a few proteins whose functions are not known are selected as drug targets. For most cases, when a protein was selected as a drug target, its function is clearly defined and, accordingly, at least one or two ligands are already known.

Second, as apparent from the results of our studies, there is no missing 'T-actives' from the pharmacophore screening. And while this is not necessarily a problem, and was desired as part of our ultimate aim, such a favorable 'T-active' recovery from pharmacophore screening is dependent upon the experience of the modeler. For this project, the creator set out with the intention of designing a loosely selective map rather than one maximizing enrichment at the expensive of recovered 'T-actives'. Such a choice was made upon the basis of the size of the database to be screened, however, we do not anticipate that alternative selections based on enrichment preferences, would dramatically influence the benefits of combinatorial screening.

In this paper, we demonstrate that the combined use of ligand- and structure-based screening for small molecule kinases can decrease screening times and increase enrichment rates as compared with single step screening approaches.

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Abbreviations

VS	virtual screening
SBD	structure-based docking
PhS	pharmacophore screening
LSA	ligand similarity analysis
PFKFB3	Human inducible 6-Phosphofructo-2-kinase/Fructose-2,6-bisphosphatase
HTS	high-throughput screening
PK	protein kinase
SMK	small molecule kinase
F-6-P	fructose-6-phosphate
F-2,6-P₂	fructose-2,6-bisphosphate
T-actives	true actives
PEP	phosphoenol pyruvate
NCI	national cancer institute

Fig.1. Identification of potent PFKFB3 inhibitors via a single-dose (10 μ M) primary screening assay

The top 50 PFKFB3 inhibitors of NCI's Diversity set II are shown in relation to four experimental controls. *Inh1* and *inh2* are in-house inhibitors that have been tested and shown to target the PFKFB3 kinase domain. The controls, *Pos* and *Neg*, depict the uninhibited presence and complete absence of PFKFB3, respectively, and thus were used to represent the theoretical maximum and minimum inhibition values by which all screening compounds were compared.

Fig. 2. Inhibition for the PFKFB3 2-kinase by NSC278631

A double-reciprocal plot shows the competitive inhibition for NSC278631 against F-6-P. The lines represent varying inhibitor concentrations and were generated by data fitting using the program GraphPad Prism.^[33]

Fig. 3.
The selected actives from the throughput screening of the NCI Diversity Set II.

Fig. 4. Pharmacophore map used in PFKFB3 virtual screening

(a) A ribbon diagram of PFKFB3 in complex with three ligands, ADP, EDTA, and F-6-P (*PDB ID: 2AXN*). The boxed ligand, EDTA, is occupying the F-6-P site of the kinase, which is the target site for our screening protocol. (b) A magnified view of the F-6-P site including a pharmacophore feature map. The feature map consists of 8 spheres of varying sizes and chemical properties, with at least 5 being needed to be met for a ligand to pass the filter. *Pharmacophore features: Red (AccP/AccS), Light-Blue (HydS/AccP), Magenta (ML&(AccS/AccP)), Green (HydP/HydS), Dark Red (Ani&(AccS/AccP)), Gray (ML).*

Fig. 5. Enrichment Comparison of Popular SBD Technologies on PFKFB3

Full Database enrichment rates were calculated for comparison between VINA (red), MOE (blue), FlexX (green), DOCK (purple), GOLD (gray), and random (dotted).

Fig. 6. Performance comparison between sequential and non-sequential virtual screening protocols

(a and b). Calculated enrichment rates at differing database sizes for Docking (D) and Pharmacophore+Docking (P+D) screening protocols. Shown on the x-axis are the five docking programs, each of which was evaluated individually, and, in conjunction with, a pharmacophore filter as previously described. (c) Measured screening times for Docking (D) and Pharmacophore+Docking (P+D) screening protocols. Shown on the x-axis are the five docking programs, each of which was evaluated individually, and, in conjunction with, a pharmacophore filter as previously described.