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Guide to Virtual Screening: Application to the Akt Phosphatase PHLPP

William Sinko, Emma Sierecki, César A.F. de Oliveira, J. Andrew McCammon

Abstract

We present an example-based description of virtual screening (VS) techniques used to identify new regulators of the Akt phosphatase PHLPP (PH domain Leucine-rich repeat Protein Phosphatase). This enzyme opposes the effects of two kinases, Akt and PKC, which play a major role in cell growth and survival. Therefore, PHLPP is a potential therapeutic target in pathophysiologies where these pathways are either repressed, such as in diabetes and cardiovascular diseases, or over-activated as in cancer. To the best of our knowledge, no PHLPP inhibitors have been reported so far in the literature. In this study, we used a combination of chemical and virtual screening techniques that led to the identification of a number of inhibiting compounds with diverse scaffolds. These compounds bind PHLPP and inhibit cell death when tested in cellular assays. We employed GLIDE docking software to screen a library of more than 40,000 compounds selected from the NCI open depository (250,000 compounds) by similarity searches. We compare the efficiency at which we determined binding compounds from the chemical screen, and compare enrichment factors of the virtually discovered compounds over chemical screening.

Keywords

Docking; Virtual screening; PHLPP; Akt phosphatase; Drug discovery; Computer aided drug design

1. Introduction

In the past few decades, the role of computation in drug discovery efforts has increased dramatically. As of 2004, approximately 50 compounds that had been discovered with computational approaches had entered human clinical trials and some are FDA approved (1). With the constant increase in computational power and improvements in methodology, computers are aiding drug discovery more so than ever. Computational methods can be employed to effectively design experiments more likely to succeed (2). In this example-based chapter, we present a workflow from recent research in which experimental and computational scientists worked closely to improve the efficiency of searching for drug-like compounds of a new target, the Akt phosphatase PHLPP. PHLPP is a newly discovered phosphatase that dephosphorylates Akt and PKC (3), leading to the inactivation of the former and downregulation of the latter (4). Since PHLPP is a negative regulator of two major survival pathways, this enzyme is increasingly found to play a major role in cancer as

a tumor suppressor. It is located on chromosomes often lost in colon (18q21, PHLPP1) and breast (16q22, PHLPP2) cancer; loss of PHLPP has been reported in different cancers at both the mRNA (5, 6) and the protein level (7-9). On the other hand, studies indicate that activation of Akt may positively affect those suffering from myocardial infarction or diabetes mellitus (10, 11). Thus, development of chemical tools for the modulation of activity of this enzyme is critical. The phosphatase domain of PHLPP belongs to the PP2C family of enzymes for which no general inhibitors have been described (12).

Prior to the virtual screening procedures, we initially performed an experimental screen using about 2,000 compounds (13) from the NCI Diversity Set. The results were used to optimize the PHLPP homology model and select libraries of compounds for use in high-throughput virtual screening (HTVS) (14). Experimental high-throughput screening (HTS) has been a well-established process for drug discovery (15). It requires an assay that can distinguish between compounds that bind the protein target and often inhibit or activate its function. This assay is used to test a large and diverse library of compounds, and find compounds with the desired activity. As the chemical libraries increased in size, the number of compounds tested per day increased (10,000–100,000 per day for HTS, >100,000 per day for uHTS) and automation and robotics became necessary. A campaign of HTS is therefore highly expensive and often requires large amount of reagents. Often HTS is limited to large pharmaceutical companies and national agencies due to the cost and specialized equipment requirements (15, 16). Virtual screening (VS) using docking software has been instituted in an attempt to enrich libraries of compounds prior to the expensive experimental screens. Docking calculations are simple and quick to use and can guide the experimental screening toward “focused” libraries to reduce reagent use and labor.

By integrating knowledge of recently discovered inhibitors with docking studies, in this study, we showed a nearly tenfold increase in the ratio between the number of hits found using VS and the number of compounds tested (enrichment factor). An enrichment factor of 10 was observed when we applied a structural similarity search, based on known binders, to build libraries of compounds in order to be used in the VS calculations. Interestingly, without any previous knowledge of inhibiting compounds (VS of the entire diversity set), the enrichment factor only decreases to 6.4 (see Table 1). It should be noted that the virtual screening described here was performed on a desktop computer and over 50,000 compounds were selected from a library of a quarter million compounds. Compounds that inhibited PHLPP were found to have IC_{50} s in the range of 4–100 μ M.

2. Materials

This section is meant to briefly illustrate the methodology used in the experimental in vitro compound screening. Experimental screens and IC_{50} determination assays are necessary to determine how well docking results correlate with the experimentally determined IC_{50} s and to verify virtual hits.

2.1. Experimental Screen of a Focused Library

In a 96-well plate, compounds diluted in DMSO are tested in duplicate at the desired concentration (50 μ M) alongside 12 controls (DMSO) and 4 background controls (without

enzyme). Detailed reaction conditions are described elsewhere (14). The dephosphorylation of the substrate, *para*-nitrophenol phosphate (pNPP) is monitored by spectrophotometry as the increase in absorbance at 405 nm. The activity of the protein (determined by the slope of OD vs. time) is compared to the averaged activity of the controls. Compounds which decrease the activity of the protein below a defined cut-off (0.5) are subjected to the next step, the determination of IC₅₀.

2.2. Determination of IC₅₀

This assay is also performed in a 96-well plate. An 8-points range of dilutions in DMSO (0.1–100 µM) is carried out for each compound of interest. An activity control (DMSO) is included for each range. The assay is performed as previously described and the inhibition is calculated as a percentage of the averaged activity of the controls. This assay is performed in triplicate for better accuracy. The data are fitted against a decreasing exponential. The IC₅₀ value is determined as the concentration resulting in a 50% inhibition of the enzymatic activity.

3. Methods

In this section we describe how to build and set up the homology model of PHLPP2 and perform the docking calculations. We describe how to use GLIDE docking software to screen thousands of compounds on a desktop computer (see Note 1; all notes are compiled in Subheading 4). A workflow diagram summarizes the steps in this section (see Fig. 1). Maestro software from the Schrödinger Suite provides a graphical user interface from which many modeling programs can be run (17).

3.1. Selecting a Structure

All docking software requires at least one structural model of the target, which can be crystallographic, NMR, homology, or Molecular Dynamics (MD) derived structures. The protein data bank is an excellent resource for biomolecule structures (18). For PHLPP, since there is no X-ray crystallographic structure deposited to date, we built our protein model via homology modeling (see Note 2).

3.2. Homology Modeling

When a crystal structure is not available for a given target, such as for PHLPP, homology modeling can be used to create a structural model of the target. The program MODELLER

¹There are many docking programs available, including GLIDE (28), Autodock (29, 30), GOLD (31), ehits (32), and Surflex (33), just to name a few, and it is difficult to determine which one is best to use. For this article, we used GLIDE inside the Schrödinger Suite of molecular modeling software. This software has a simple to use Graphical User Interface, and a funneling scheme for docking, which removes low scoring compounds rapidly, then scores the remainder with higher accuracy. This allows for a great number of compounds to be evaluated in a short amount of time, on a desktop computer.

²Crystal structures are often preferred because of the high resolution that they are resolved at. As described, homology models can be extremely useful structures for screening purposes (14). Recently, molecular dynamics MD simulations have been shown to be very useful for providing structural models. Through MD techniques, receptor flexibility can be taken into account in virtual screening procedures by using ensemble-based screening or the relaxed complex scheme (34–36). Additionally, enhanced sampling techniques, such as Accelerated MD simulations, can be used to identify new biologically relevant conformational states of the receptor (37–43). These states, which may represent low populated states in the MD trajectory, are usually not captured by ensemble-based methods and can be extremely important in the search for drug candidates. A comprehensive review of ensemble-based screening is available here (44).

(19) was used with standard settings to produce the homology model of the PP2C phosphatase domain (residues 745–1102) of PHLPP2 (an isoform of PHLPP (4)) from the crystal structure of PP2Ca (PDB id: 1A6Q) (20, 21), and ClustalW was used to align the sequences with default parameters (22) (see Note 3).

3.3. Preparing the Structure

The program Maestro inside the Schrödinger Suite was used to assign bond orders, partial charges and atom types, according to the OPLS 2001 force field (17). Hydrogens were added to the protein based on the standard pK_a of each residue. Ligands not believed to be important in compound binding should be removed from the structure. All these steps can be performed using the “Protein Preparation Wizard” under the Workflows dropdown menu (23). There is evidence of the presence of metal ions in the PHLPP active site. Since our homology model does not include the metals, we modeled 1, 2, or 3 manganese ions into the structure and relaxed the protein structure using MacroModel from the Schrödinger Suite (24) (see Note 4).

3.4. Receptor Grid Generation

The first step in any docking calculation consists of the generation of a three dimensional grid from the receptor structure file. This grid is used to dock the compounds and estimate their poses and free energy of binding. The grid center is usually defined around the region of interest to dock the ligands. The size of the grid depends mostly on the size of the ligands present in the library of compounds. In this study, the center of mass of the three Manganese ions was selected as the center of the box. In GLIDE, two boxes are defined: a small one inside which the center of mass of the docked compound must lie, (we used a cubic box with an edge of 14 Å) and a larger one defining the outer edges of the grid in which the entire molecule must sit (we used a cubic box with an edge of 44 Å).

3.5. Compound Library Selection

When looking for a compound library there are many characteristics to evaluate. Among these are its size, cost per compound, and intent of use (see Note 5). When screening against PHLPP, we first used the NCI Diversity Set (~2,000 compounds), which was screened in vitro, and next looked for new compounds in the NCI Open Depository (~250k compounds). The Diversity Set (13) and Open Depository (25) are available to download free of charge.

³. Programs such as MODELLER are available to download, and there are free web servers available like SWISS-MODEL (45). All that is needed is the sequence of the protein or partial sequence such as a given domain. The program will search for similar proteins, for which there is a crystal structure available, to base the structural model on and produce a structure file, which can then be used in the docking program. Generally an X-ray crystal structure is desired to begin a docking study, however success is possible using homology models.

⁴. PHLPP is from the Protein Phosphatase Magnesium/Manganese dependent (PPM) family of phosphatases (46, 47). For example, PP2Ca, our alignment model, possesses two manganese ions in its active site (20). In this study we developed a number of models with different numbers of Mn^{2+} ions because we were unsure of how many Mn^{2+} ions were present and where they would be located in the structure. These structures were evaluated for their ability to determine true binding compounds as determined by the experimental studies from decoy compounds, and the best performer was chosen for further study.

⁵. In most of the cases, the size of the compound library is often not an issue in the virtual screening. However, some compound libraries, such as ZINC with over 13 million compounds (48), are so large that virtual screening would take a very long time. The cost per compound is also a major issue. While many libraries can be obtained *in silico* for free, commercial libraries charge a considerable fee for any compounds purchased for experimental testing. The National Cancer Institute (NCI) has a few targeted libraries and their compounds can be obtained free of charge. Some distributors of compounds make tailored libraries that may consist of fragments, or libraries tailored to kinases or other proteins of interest.

3.6. Selecting from the Compound Library

In order to eliminate excess screening and analysis, it may be helpful to sort through the compound library prior to virtual screening. If there is information concerning known binders, a similarity search may be performed. For this study, we performed a similarity search based on the 11 families of compounds identified via in vitro screening and the seven most potent ones. Inside Accelrys Discovery Studio (26), we used the “Find Similar Molecules by Fingerprints” protocol with long range functional class fingerprint description 6 keys (FCFP 6 keys), and a Tanimoto distance coefficient to calculate the similarity score. We kept the 33,000 compounds that were similar to compounds from 11 structurally similar families determined to inhibit PHLPP. We also kept 10,000 compounds that were similar to the seven most potent known inhibitors resulting in 43,000 compounds to screen (14).

3.7. Docking Calculations

With the two necessary components for VS, the structural model of the receptor and a compound library, the user can now perform docking to evaluate the compound poses in the active site (see Note 6). The Virtual Screening Workflow in Maestro was used to run the screens. The LigPrep (Ligand Preparation) program was used to parameterize the ligands before the docking (27). In this step, partial charges, tautomers, stereoisomers, and protonation states are defined and included in the calculations. Default parameters were used for the VS workflow except that after the first stage (HTVS) only the top 20% of compounds were selected for next stage, standard precision (SP), and again the top 20% of these were docked with extra precision (XP) (see Note 7).

3.8. Analyzing the Results

Analysis of the results can be as simple as choosing compounds based on their ranked order, which is based on the estimated free energy of binding (docking score), given by the docking program. However, it is important during the selection process to use discretion (see Notes 8 and 9). From our virtual results, we suggested 80 compounds to be experimentally

⁶In general, docking programs generate poses of the compound in the specified region of the receptor and perform a complete 3-dimensional search, creating numerous poses of the ligand on the receptor. The next step is to evaluate these poses with a scoring function and determine the best pose. GLIDE uses a modified Chemscore (49) function to evaluate the poses (28, 50) and produce an estimation of the free energy of binding. Compounds are commonly ranked by this free energy of binding, but may also be ranked by ligand efficiency, which takes into account the size of the molecules in relation to their free energy of binding.

⁷HTVS (performs rigid ligand docking) is for screening large libraries of compounds and is not as accurate as SP or XP (both perform flexible ligand docking). Standard precision (SP) has softer potentials and is more forgiving than extra precision (XP), which has harder potentials and is the most accurate and therefore time consuming (28, 50). HTVS and SP are used as preliminary steps to funnel only good scoring compounds into GLIDE XP. XP is meant to remove false positives, and was used to generate the final docking scores in this project. XP and SP are quite similar however they have different intended uses. XP may be used to get the highest enrichment factor in VS or it may be used in lead optimization but may eliminate some true binding compounds. SP is intended to forgive small imperfections in poses and should have a lower enrichment factor, but has less of a propensity to eliminate true positives. GLIDE software uses a funneling scheme to go through these three docking levels so that the number of compounds docked in the XP stage that use the most computer resources are minimized (28) (see Fig. 1). Cutoffs to make it through the three stages of docking should be determined based on library size, and computer resources available. High cutoffs will increase computational time, while low cutoffs may eliminate more hits early on.

⁸Often, it is necessary to eliminate compounds that do not appear to be bound within the active site, or compounds that are very similar to each other. When initially searching for compounds with diverse backbones, it can be wasteful to test many similar compounds before it is known which ones work well. However, after confirmation of compound binding (inhibition), similar compounds can be proposed in the searching for higher affinity compounds. Structure-activity relationship studies are also a good alternative. Before screening any compounds in vitro first examine the poses. Sometimes the docking algorithm will score compounds in unreasonable poses. Be aware of the limitations involved in all docking programs. The first thing to check is if the majority of the molecule is in the binding pocket. It is important to visually evaluate high scoring poses before the results are accepted. In the case of

tested for PHLPP inhibition. These compounds were selected because of low free energy of binding scores (less than -10 kcal/mol), poses deemed plausible by visual inspection, diversity, and availability. See Fig. 2 for two examples of the poses generated by GLIDE compounds that were strong hits in the VS and confirmed PHLPP binders experimentally.

3.9. Testing Compounds Experimentally

As described in Subheading 2, the compounds were tested for IC_{50} s. Of the compounds tested, 20 of the 80 showed an IC_{50} below $100\text{ }\mu\text{M}$. This represented a 25% hit rate for our VS overall. The docking software greatly improved the efficiency of screening but still 3 out of 4 compounds did not inhibit PHLPP (see Note 10).

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PHLPP, we were able to determine the mode of inhibition (competitive or noncompetitive) by the pose generated by the docking software. Most compounds we found virtually were noncompetitive and bound primarily to a hydrophobic cleft adjacent to the metal ions.

⁹Often, docking programs may score large compounds more favorably than small compounds. Docking of very large flexible molecules is usually challenging because of the large number of rotatable bonds and hence should be analyzed carefully. Ligand efficiency, which is equal to the free energy of binding over the weight of the heavy atoms, will help indicate how strong the interactions between the compounds and the target are while taking into account its size. Small molecules are a good start for drug design campaigns because functional groups may be added to optimize the physicochemical properties without compromising binding affinity or potency, during medicinal chemistry development.

¹⁰Again, owing to the limitations involved in all docking software, it is vital to test the compounds experimentally for IC_{50} s before further work is invested to develop better inhibitors. Numerous factors can result in inaccurate results in docking calculations such as force field inaccuracies, not accounting for protein flexibility properly, poor description of solvation, and entropic effects. On the other hand, the selection of compounds to be tested experimentally may be hindered by physical properties such as compound impurity, insolubility, aggregation, or poor cellular entry. However, docking is one of the most rapid and cost-effective ways to find compounds that bind biomolecules despite the high rate of false-positives and -negatives (36). If the docking software ranks certain molecules well, it is likely that similar molecules will also be ranked accurately. It is advisable to screen similar molecules to known binders to find more inhibitors or higher affinity inhibitors.

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Fig. 1.

Docking scheme: A 3-dimensional grid file and a compound library are necessary for docking. The grid file is derived from a PDB file or from a homology model. The homology model is then modified to add or remove ions or ligands in the active site, and fill in missing residues. The compound library is narrowed via similarity searches. Then GLIDE using the funneling scheme is employed.

Fig. 2. PHLPP2 phosphatase domain model structure. **(a)** The entire phosphatase domain homology model with the approximate area which formed the docking grid for PHLPP2 *lightly shaded*, the rest of the phosphatase domain is *darkly shaded*, the two Mn²⁺ ions are represented as *dark orbs* in the center of the image, and the *dashed lines* represent the region enlarged in subsequent panels. **(b, c)** Docked poses of two hit molecules, which were verified as low μ M inhibitors of PHLPP2, compound 45586 **(b)**, and compound 134145 **(c)**, both in stick representation (a full color image is available online or from the authors).

Table 1

Efficiency and enrichment factors of various methods used

Method used	Efficiency (%)	Enrichment factor
Chemical screen	2.5	1
Virtual screen (VS)	16	6.4
Structural search $\Rightarrow$ VS	25	10

Efficiency is the percentage of compounds tested experimentally that were confirmed to inhibit at 100 μ M or less. Enrichment factor compares the efficiency of the virtual screening method over the baseline efficiency of chemically screening the NCI diversity set