3DPL Case Study: Identification of Inhibitors of the Calcineurin / FKBP-12 Complex

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Background

3DPL™ (3-D Protein-Ligand Map) technology is an ultra-fast database searching system developed by ChemNavigator in San Diego, California. The technology is a protein-ligand extension of the Directed Tweak[1] technique. The basic algorithm has been patented[2], and represents a successful and efficient method to promote new lead discovery.

There are other computer programs designed to dock small molecules into a receptor. These programs were created with the goal of either reproducing the binding configuration of known ligands, or accurately estimating the binding energy of those interactions. They are generally not well suited to searching databases of millions of compounds because of their computationally intense nature. In addition, most require specific indication of the binding site, which is only available for those receptors that have 3D structures determined by X-ray crystallography with a co-crystallized ligand. For structures without a co-crystallized ligand, or produced using other methods such as NMR and homology modeling, a predetermined active site may not be available.

3DPL technology, on the other hand, was designed specifically to search databases of millions of compounds for those most likely to show activity in biological screens. 3DPL can search structures at a rate of up to 30 compounds per second, which allows many millions of structures to be investigated in the course of several days on a single CPU.

ChemNavigator's objective is to allow our partners to identify a target-focused set of chemistry and move to bioassay and lead identification as quickly and efficiently as possible. 3DPL has four major advantages that allow for rapid identification of potential active molecules:

- Knowledge of active site not required: 3DPL includes technology for automated identification of all sites on the protein surface of appropriate size for binding with a therapeutic molecule. This Convex Hull technology identifies all potential binding sites that can be used in flexible screening, and it eliminates the need for an identified biding site as input for the screening experiment.
- Entire protein surface is considered: Each small molecule is compared against all potential binding sites on the 3-D protein surface to look for potential binding interactions. This approach offers the opportunity to identify ligands that would have

[1] Hurst, T. "Flexible 3D Searching: The Directed Tweak Technique", *J. Chem. Inf. Comput. Sci*. **34** (1994), 190.

[2] US patent application 10/222,452 allowed. US patent application 10/192,763 pending.

been overlooked by virtual screening technologies that focus on only one or more pre-defined active sites on the protein surface.

- Automatic conformational analysis: Each small molecule ligand is flexed, in an energetically directed approach, and re-oriented thousands or millions of times in the search for potential matches between the ligand and the protein surface.
- Speed enables *in silico* screening of millions of small molecules: 3DPL Employs a unique and patented derivative field grid to direct small molecules to favorable binding conformations. The use of these grids significantly reduces computational time. Running on a single 1.4GHz server, 3DPL is able to evaluate over 8 million chemical structures for binding across the entire protein surface in less than 4 days – a 600-fold increase over the fastest technology available today. Much larger structure sets can be run, which eliminates the need to filter out large numbers of potentially valuable samples before the virtual screening experiment.

Case Study: Calcineurin/FKBP-12

3DPL has been validated for a number of targets by selecting known active molecules from among a general set of molecules. This paper details the results of identification of previously unknown calcineurin inhibitors using 3DPL technology coupled with biological screening.

Calcineurin is an enzyme that is modulated by calmodulin and catalyzes dephosphorylation of NFAT and other substrates. It is implicated in transcription regulation and apoptosis, and it is a therapeutic target for immunosuppression. Calcineurin consists of two domains. Griffith et al.[3] describe the inhibition mechanism of calcineurin by the complex of the protein FKBP12 and the Immunosuppressant drug FK506 (Tacrolimus). FK506 first binds to FKBP12. This complex then binds tightly to calcineurin, and provides a steric barrier to entry of the substrate (Figure 1). Not all compounds that bind tightly to FKBP12 provide inhibition of calcineurin activity. For example, rapamycin binds to FKBP12, but that complex does not bind tightly to calcineurin.

[3] Griffith JP, Kim JL, Kim EE, Sintchak MD, Thomson JA, Fitzgibbon MJ, Fleming MA, Caron PR, Hsiao K, Navia MA., Cell. 1995, **82**(3), p507-22.

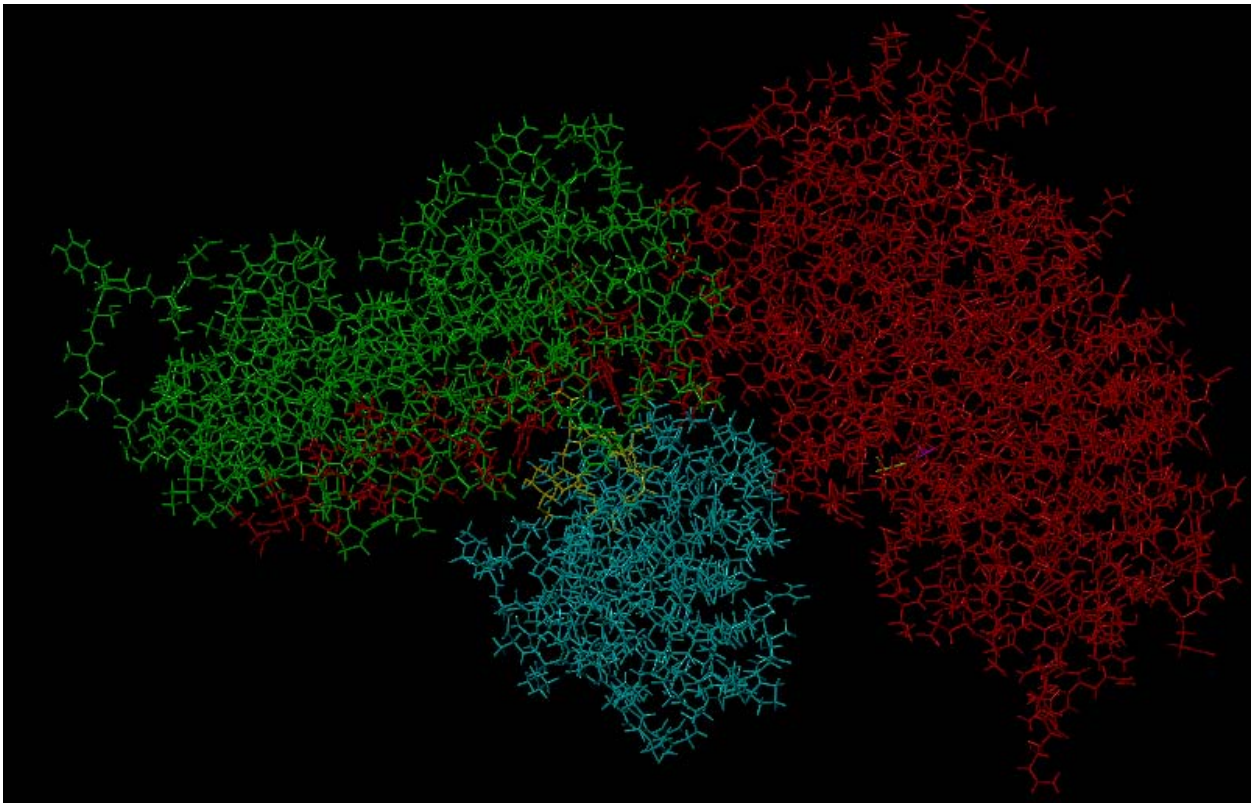

Figure 1. Calcineurin/FKBP12/FK506 complex. The two calcineurin domains are colored red and green. The FKBP12 protein is light blue, and the immunosuppressant drug FK506 is colored yellow. In addition, the phosphate binding site is colored yellow.

3DPL Virtual Screen

3DPL was used to search the structures of ChemNavigator's iResearch Library. This library contains over 15 million commercially available compounds, representing over 8 million unique structures and is available under license from ChemNavigator. The iResearch Library was first filtered to exclude non-drug-like compounds using the ChemNavigator standard rule set. This rule set only allows compounds that have cLogP values between 0 and 7, and that have molecular weights between 100 and 800. In addition, the compounds can have no more than 6 Chlorine or Bromine atoms and no more than 12 Fluorine atoms. Compounds that pass these rules must also not contain unacceptable functional groups, including nitro groups, acid chlorides, epoxides, and several others.

After filtering for the ChemNavigator Standard drug like rules, there were approximately 3.4 million unique structures to be used in the 3DPL virtual screening experiment. The 3D coordinates of the 3.4 million structures were generated using ChemNavigator's coordinate generator.

For the initial work, 3DPL technology was used to search for compounds that dock to the FKBP12 protein. The structure for FKBP was taken from the PDB file "1fkj" – the complex of FKBP12 and FK506. The co-crystallized ligand (FK506) was removed, as were the water molecules. Hydrogen atoms were added to all atoms to fill the proper valences.

The 3DPL system was run with the following configuration:

1) Because an identified binding site was available, the virtual screening was focused on potential binding sites that are near the general position of the known ligand. This limits the search to a volume within 8 angstroms of the atoms of the co-crystallized ligand.
2) All potential binding sites were investigated within that cavity
3) The site of the known ligand was used as a potential binding site in addition to the sites auto-generated using 3DPL technology. In total, 11 sites were generated.
4) Automatic adjustment of protonation states for physiological pH was enabled for both the protein and the small molecule structures.
5) Full flexibility of the small molecule structures was enabled.
6) Leonard-Jones 2-12 potentials were used for van der Waals interactions
7) Hydrogen bonding contributions were calculated using 2-12 potentials.
8) Electrostatic interactions were not enabled.
9) Hydrophobic interactions were enabled with a reward factor of 1.5 for the vdW interactions between a hydrophobic atom in the ligand and one in the receptor.
10) The penalty for solvent-exposed hydrophobic groups was set to 3.0 kcal/mol for each hydrophobic atom that is 5 or more angstroms from the nearest atom.
11) Site-focusing was enabled and checked after every 5000 small molecule structures were screened. This feature removes the site points for potential binding sites that have not been used as starting positions for any of the hits so far. After 10,000 compounds, the potential binding sites had been reduced from 11 to 2 sites, which showed small molecule binding.
12) Maximum allowed number of hits was set to 200. This causes the system to find the best 200 structures across all selected binding sites.
13) The initial maximum energy was set to –310 kcal/mol such that any structure with a binding energy lower than this was accepted. This number is artificially low because of the use of Leonard Jones 2-12 potentials. The actual cutoff was adjusted automatically as 3DPL picked the lowest scoring 200 compounds.
14) 5 initial (starting) conformations were tried for each small molecule in the database. 3DPL flexes these initial conformations while searching, thereby investigating thousands of conformations for each structure.

The 3DPL search ran for approximately 4.5 days. The resultant 200 lowest scoring compounds were examined for availability using the ChemNavigator's Chemistry Procurement tools, and of those readily available, the top 25 were purchased.

Biological Screening

These 25 samples were sent to an independent lab for testing in a standard calcineurin screen[4]. Of the 25 tested, 1 displayed activity in the 100 nM range, and 3 others in the 1 μM range. These 4 compounds were tested for general cytotoxicity. The 100nM compound was found to be cytotoxic; the other three were not cytotoxic.

The structures of the remaining 3 compounds are not revealed in this paper. They are all variants of a common core compound. We are considering testing of further analogs of these compounds for improved efficacy.

[4] Con-A stimulated lymphoproliferative assay

Further Database Results
Since binding to FKBP12 alone is not expected to predict fully the overall binding of that complex to calcineurin, it is thought using 3DPL to search for structures that fit the calcineurin-FKBP12 complex, rather than just FKBP12, might be even more predictive than using just the FKBP12 structure. Such a structure is found in the PDB entry "1tco". This is the complex of the known ligand FK506 with FKBP12 and calcineurin. The known ligand was removed from the 1tco structure, and the structure was prepared for use in the search query as described above.

We reran the 3DPL search, using the calcineurin/FKBP12 structure, of those structures from the iResearch Library that pass the ChemNavigator Standard drug like rules and identified the top scoring 200 structures.

The 25 structures originally assayed after virtual screening against FKBP12 alone were also docked to the 1tco calcineruin/FKBP12 structure. The 4 active compounds from this set of 25 all fell within the half of the structures with the best-predicted binding energy from the calcineurin/FKBP12 experiment. This suggests that searching for structures that fit the overall complex might in deed be a better predictor for activity than the FKBP12 structure alone.

Additional samples out of the top scoring 200 structures from this second experiment will be selected for procurement and testing.

Conclusion (to date)
3DPL technology proved to be remarkably efficient in finding new active compounds, as 4 samples out of 25 assayed showed activity, with only 1 showing general cytotoxic activity. This represents a 12% hit rate based on a very limited population of only 25 compounds tested. Compared to the standard hit rate of 0.5% for randomly selected compounds from a diverse library, 3DPL offered a significant improvement. The need for purchase and storage of a larger library was eliminated and the cost to assay thousands or more samples was eliminated. In addition, a significant amount of time was eliminated due to the small set of samples assayed and the small amount of data to be analyzed.

In addition, the active compounds identified are not related structurally to any known calcineurin inhibitors, and they represent a new class of inhibitors.