HierVLS Hierarchical Docking Protocol for Virtual Ligand Screening of Large-Molecule Databases

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To provide practical means for rapidly scanning the extensive experimental combinatorial chemistry libraries now available for high-throughput screening (HTS), it is essential to establish computational virtual ligand screening (VLS) techniques to rapidly identify out of a large library all active compounds against a particular protein target. Toward this goal we developed HierVLS, a fast hierarchical docking approach that starts with a coarse grain conformational search over a large number of configurations filtered with a fast but crude energy function, followed by a succession of finer grain levels, using successively more accurate but more expensive descriptions of the ligand—protein—solvent interactions to filter successively fewer cases. The final step of this procedure optimizes one configuration of the ligand in the protein site using our most accurate energy expression and description of the solvent, which would be impractical for all conformations and sites sampled in the coarse level. HierVLS is based on the HierDock approach, but rather than allowing an hour or more to determine the best binding site and energy for each ligands (as in HierDock), we have adapted our procedure so that it can lead to reliable results while using only 4 min (866 MHz Pentium III processor) per ligand. To validate the accuracy for HierVLS to predict the experimentally observed binding conformation, we considered 37 cocrystal structures comprising 11 target proteins. We find that HierVLS identifies the correct binding mode for all 37 cocrystals. In addition, the calculated binding energies correlate well with available experimental binding constants. To validate how well HierVLS can identify the correct ligand in an extensive library of decoys, we considered a library of over 10 000 molecules. HierVLS identifies 26 out of the 37 cases in the top 2% ranked by binding affinity among the 10 037 molecules. The failures result from either metal-containing sites on the protein or water-mediated ligand—protein interactions, which we anticipate can be solved within the constraints of practical VLS. We then applied HierVLS to screen a 55000-compound virtual library against the target protein—tyrosine phosphatase 1B (ptp1b). The top 250 compounds by binding affinity included all six ptp1b cocrystal ligands added to the library plus three other experimentally confirmed binders. The best (top 1) binder is an experimentally confirmed positive. We conclude that HierVLS is useful for selecting leads for a particular target out of large combinatorial databases.

1. Introduction

The number of potential therapeutic target proteins is proliferating rapidly, making it increasingly important to develop techniques for rapidly discovering and optimizing novel therapeutic agents for these new targets. Experimental combinatorial chemistry has provided enormous libraries with millions of potential ligands quickly accessible for experimental tests to find positive lead compounds against specific target proteins. However, to achieve high-throughput screening (HTS), time is a critical limitation and it is often not practical to test experimentally all compounds against each target. This sets the stage for computational virtual ligand screening (VLS), where the task is to rapidly identify out of a large library all active compounds against a particular protein target. Here, it is essential to minimize elapsed computational time while ensuring that no active compound is missed. Thus, for a typical library of 500 000 compounds and a computer cluster of 200 processors all working on the same target site, the average time per ligand must be less than 4 min to finish the task within a week.

The two main VLS approaches are the following: (1) descriptor-based methods (e.g., QSAR) in which the screening is based on common structural features among known ligands to the particular target (this does not require the 3D structure of the target); (2) structure-based methods in which the 3D structure of the target is known and the affinity of a particular ligand for the target is estimated by how well its structure complements the target binding site (this does not require experimental data on various ligands to a particular target).

Our interest is in structure-based methods because they can be applied to novel targets for which there is little or no experimental binding data. Structure-based methods have successfully retrieved potential ligands from large databases, but it is necessary to reduce the costs and to increase the reliability of identifying in a large library a small subset containing all active compounds for subsequent synthesis and experimental testing.
This paper introduces the HierVLS protocol, a fast hierarchical docking approach that starts with a coarse grain conformational search over a large number of configurations filtered with a fast but crude energy function, followed by a succession of finer grain levels, using successively more accurate but more expensive descriptions of the ligand–protein–solvant interactions to filter successively fewer cases. The final step of HierVLS uses our most accurate energy expression and description of the solvent but would be impractical for all conformations and sites sampled in the coarse level.

HierVLS is a VLS adaptation of the HierDock ligand-binding protocol developed for predicting binding sites and energies for selected ligands.4–6 However, rather than allowing an hour or more to determine the best binding site and energy for each ligand (as in HierDock), we adapted the procedure to reduce the computational time so that we can obtain reliable results while using only 4 min (866 MHz Pentium III processor) per ligand.

We validate HierVLS in several ways. First, we consider 37 cocrystal structures comprising 11 target proteins and examine how well HierVLS predicts the binding mode found in the crystal. Here, we consider the performance both with crystallographic waters and without. We find that HierVLS identifies the correct binding mode for 37 out of the 37 cocrystals studied. In addition, the calculated binding energies correlate well with available experimental binding constants.

Then we considered a library of 10 037 molecules consisting of the 37 ligands plus 10 000 molecules. The VLS validation assesses how well we can predict the binding mode of known ligands and how efficiently we can select all high-affinity ligands among a large set of decoys. We find that for 26 out of the 37 cases, the current procedure identifies the correct ligand in the top 2% ranked by binding affinity among the 10 037 molecules. The average time for screening all 11 targets against the 10 037 compounds library was 4 min per ligand. We compare the result for HierVLS to the results of three other widely used docking methods (Dock4.0, FlexX, and ICM; Bursulaya et al., private communication), where we find HierVLS to represent a significant improvement.

As an example of the application of HierVLS, we used it to screen a 55000-compound proprietary virtual library against the target protein-tyrosine phosphatase 1B (ptp1b). The top 250 compounds by binding affinity included all six ptp1b cocrystal ligands added to the library plus three other experimentally confirmed binders. The best (top 1) binder is an experimentally confirmed positive.

The methods and other details are summarized in section 2. The results are discussed in section 3. In section 4 we summarize the results and improvements we are considering. Finally section 5 has the conclusions.

2. Methods

2.1. Overview. The three major elements of a docking algorithm are (1) a good representation of the molecular search space, (2) an efficient algorithm to search this conformational space, and (3) a scoring method that leads to accurate structures and interaction energies. Techniques available for docking ligands into receptor can be categorized as (1) matching methods, in which trial ligands are matched onto the ligand binding site on the receptor and (2) Monte Carlo methods that use statistical sampling techniques combined with simulated annealing.

Matching methods include Dock4.0, the docking protocol developed by Kuntz and co-workers.8,9 In Dock4.0 the potential ligand docking regions are represented by a set of overlapping spheres generated using the molecular surface of the target protein. A conformational search is performed using either rigid or flexible ligands for a fixed receptor conformation. Dock4.0 uses electrostatic and van der Waals interactions evaluated over a grid to calculate the binding energy of a docked conformation. Dock4.0 employs an efficient mapping of conformational space to be searched, but the conformational search may not be exhaustive.

Other docking methods in use involve evolutionary and heuristic algorithms10,11 and internal coordinate Monte Carlo.12,13 Rather than a single conformational search method using a single scoring function, a two-stage approach has been shown to be successful for docking.14,15 Bissantz and co-workers14 used three docking programs (Dock, FlexX, and Gold) and seven scoring functions (Chemscore, Dock, FlexX, Fresno, Gold, PMF, and Score) to assess the VLS performance of different combinations of docking/scoring methods applied to two targets: thymidine kinase (10 experimentally determined cocrystals structures) and estrogen receptor (2 known cocrystals structure). They found that the use of consensus lists using two or three scoring functions significantly improve hit rates and that accuracy in binding mode prediction does not necessarily translate to screening efficacy.

Methods combining docking and molecular dynamics (MD) simulations have been tested.16 The main drawback of these methods is that only one protein/ligand complex structure was kept from the coarse grain docking methods for MD simulations. This is risky considering that the coarse grain docking does not use an accurate scoring function including solvation.

Free energy perturbation methods lead to accurate energies of binding but are computationally intensive and not readily applicable to a wide variety of ligands.17 The HierVLS approach introduced here uses a hierarchy proceeding from a coarse grain conformational search through several levels of increasingly better descriptions of the protein–ligand interactions including solvation. This allows a practical procedure for examining a large number of protein–ligand configurations, progressively eliminating the less favorable ones while spending more time refining promising configurations. The sequence of steps is designed with the goal of retaining all active compounds while eliminating inactive compounds with the least possible computational effort.

2.2. Preparation of the Protein and Ligand Structures. The coordinates (in mol2 file format) for 11 protein targets and their 37 cocrystal ligands, plus an additional screening library of 10 000 molecules randomly selected from the ACD database, were kindly provided by B. D. Bursulaya and P. G. Schultz. This set was previously used in a comparative study of docking algorithms for virtual library screening.7 We assigned CHARMM22 charges for the proteins and Gasteiger14 charges for the ligands. The potential energy of each ligand was minimized (to an rms force of 0.5 kcal/(mol·Å)) in the gas phase using the Dreiding force field.18

The potential energy (using the Dreiding force field) of each protein was minimized in the gas phase to relax the structure, but this was done for just 100 conjugate gradient steps to ensure that no significant change would occur in the initial structure. This was necessary to guarantee that each target protein structure remains as close as possible to those used with other methods [Dock4.0, FlexX15, 1.8, AutoDock3.0, GOLD1.2, ICM11.2.8, and Dock1.0 (Daylight Inc.)] used in previous studies for cross comparison. Each ligand and amino acid used the formal charges appropriate to pH 7. All histidines were neutral except for His231 in thermolysin, which was assigned a +1 charge based on the nature of the ligand–protein interaction in the crystal structure.

The target proteins with their respective Protein Databank codes are listed in Table 1. There were at least two cocrystal
Table 1. The 11 Target Proteins and the PDB Codes for the 37 Cocrystal Considered in This Paper (Listed by PDB Code)\textsuperscript{a}.

<table>
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<th>Target Protein (Nickname)</th>
<th>Complex PDB Code</th>
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<td>1er, 1ets, 1ett</td>
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\textsuperscript{a} For each protein, there are at least two cocrystals; however, for docking multiple ligands, we used just the one protein conformation underlined. The exception is carbonic anhydrase II where we considered separately all three protein conformations.

structures for each target protein, but only one protein conformation was selected for each case and that conformation was kept frozen throughout the calculations. The only exception was carbonic anhydrase II where all three protein conformations were used to dock all three ligands. The PDB code corresponding to the reference conformation used in this study for each target is underlined in Table 1.

Water molecules that mediate hydrogen bonds (HB) between protein and ligand were included in the calculations only for those cases in which they were the only HB formed by that ligand or receptor atom (see Table 2). Hydrogen bonds were calculated using HBPLUS\textsuperscript{24} for the original PDB files (before force-field minimization). After addition of hydrogens to the ligands, the positions of crystallographic water molecules were optimized by minimizing the ligand and water with fixed protein. The waters were subsequently kept fixed during docking (level 0) but were allowed to relax with the ligands during optimization (level 1).

2.3. Hierarchical Virtual Ligand Screening (HierVLS) Protocol. The HierVLS protocol combines a coarse grain conformational search using Dock4.0 and its simple energy function, ligand conformation filtering using a combined criterion of buried surface area and energy score from Dock4.0, followed by minimization using an all-atom force field (FF) with solvation evaluated using the analytical volume generalized Born (AVGB) model. HierVLS consists of the following steps.

1. Definition of Docking Region. The docking region for each target protein was based on the superposition of the cocrystal structures available for that target. The coordinates of the superposed ligands were used as the sphere centers required by Dock\textsuperscript{4.0}.  

II. Protein Grid Calculation. The docking step uses an energy grid for the protein contribution to the interaction energy. This grid is calculated only once per target using the program Grid, which is part of the Dock4.0 package. This grid used a box constructed with a 10 Å margin around the sphere centers defining the docking region. Water molecules were considered as part of the target protein during conformational search, and thus, their energy contribution was included in the target energy grid.

III. Level 0: Coarse Grain Conformational Search. Generation of docked conformations of each ligand into the binding site of the target protein was performed using Dock\textsuperscript{9,22} 4.0. We use the options of flexible docking, Dock energy scoring, maximum of 8 bumps simplex minimization before scoring, and 300 maximum scored conformations. The Dock4.0 scoring function calculates Coulomb and van der Waals interaction energies for all atoms of the ligand. We used Gasteiger charges\textsuperscript{17} for the ligands and default van der Waals radii for Dock4.0. The van der Waals radii for the ligands (but not the protein or waters) were reduced by 25% to allow closer contacts to the target protein. We saved for subsequent steps the best 50 conformations per Dock scores for each ligand in the library. Crystal waters, if present, are considered as part of the protein and were kept fixed during docking.

IV. Buried Surface Filter. The percentage of ligand buried surface area was evaluated for the 50 conformations per ligand from level 0. Configurations with less than 30% of buried surface area were eliminated, and the remaining ligand configurations were scored by a double sorting of their percentage of buried surface (% BSA) and their Dock scores. The best five configurations per ligand were carried to the next step.

V. Level 1: Limited Minimization. For each of the five conformations per ligand that passed the buried-surface filter, we minimized the structure of the ligand with fixed protein (25 conjugate gradient steps) using the all-atom Dreiding FF for ligand and protein but no solvation. Any water molecules present in the binding site were allowed to relax during this minimization. The lowest energy configuration was selected for subsequent steps.

VI. Ligand Ranking. For the best conformation per ligand from level 1, we evaluated the solvation for both the protein–ligand complex and the free ligand using the AVGB\textsuperscript{23} continuum solvation approach. Binding affinities were calculated as the difference between the energy of the solvated complex and that of the free protein and free solvated ligand in their docked conformations:

\[
\text{BindE} = E_{\text{solvated \ complex}} - E_{\text{solvated \ free \ protein}} - E_{\text{solvated \ free \ ligand}} \tag{1}
\]

We denote this binding energy as the "vertical binding energy" to indicate that the conformations of the free protein and free ligand correspond to the ones in the optimized protein/ligand complex. The ligand list is then sorted by binding affinity.

2.4. Scoring the Experimental Cocrystal Conformations. We calculated the binding affinity for the cocrystal (reference) configurations using eq 1. The starting configuration of the complex was obtained by superposing the PDB files corresponding to different cocrystals of the same target protein and then placing the superposed ligands into the protein conformation chosen for docking (which was prepared as described in “Preparation of the Protein and Ligand Structures”). The reference cocrystal energies were obtained by minimizing each cocrystal ligand inside the chosen protein target conformation without allowing the protein conformation to relax (protein fixed minimization). Thus, for each fixed target conformation we find the best complex configuration matching that particular target conformation. Calculating the reference binding affinity in this way allows us to compare the HierVLS scores to the reference binding affinities corresponding to the same target conformation used for docking.
3. Results and Discussion

3.1. Binding Mode Accuracy for Known Cocrystal Structures.

The first test of the HierVLS protocol is the accuracy of predicting the crystallographic binding mode for the 37 cocrystal structures listed in Table 2. To do this, we performed steps I–VI of the HierVLS protocol as detailed in section 2. The best conformation for each ligand obtained from the HierVLS protocol was compared to the experimentally determined conformation for the cocrystal ligands. These results are shown in Table 3.

We found that 37 out of our 37 predicted cocrystals were within 2 Å CRMS (coordinate root mean square) deviation from the experimental crystal conformation (not the minimized structure), which we consider as the minimal agreement of a good match. We found that 21 out of 37 are within 1.0 Å CRMS to the crystal, which we consider as the desired accuracy for binding mode prediction. Thus, we consider that HierVLS performs this test satisfactorily.

In the above test, crystallographic waters found to mediate hydrogen bonds between ligand and protein were initially placed in their positions for the particular protein structure we used for docking (described more completely in the following sections). The position of such waters would be unknown for most applications. Hence, a better test is to remove all crystallographic waters before docking the ligands. In this case, HierVLS predicts 32 out of the 37 cocrystal structures within 2 Å. This can be compared to the performance of three other docking programs (Dock, FlexX, and ICM) as reported by Bursulaya et al. for the same set of 37 cocrystals presented here. These calculations did not include crystallographic waters. Thus, the number of cases in which the predicted ligand conformation is within 2 Å CRMS of the experimental crystal structure is as follows: HierVLS, 32 out of 37; Dock4.0, 11 out of 37; FlexX, 13 out of 37; ICM, 29 out of 37.

Binding mode accuracy has also been reported using Dock, Gold, and FlexX for 10 cocrystals of thymidine kinase. In that work, Dock and FlexX predicted 4 out of 10 cocrystal ligands within 2 Å CRMS, while Gold predicted 6 out of 10 within 2 Å CRMS. We believe that the better performance of the HierVLS protocol demonstrates the necessity of multiple-step hierarchical algorithms for VLS.

3.2. Database Screening Efficacy.

In addition to reproducing the correct binding modes, a useful VLS protocol must identify all high-affinity ligands as top rankers from a large and diverse ligand library. Here, we consider 2% as the minimal criterion for success. To provide a test for database screening efficiency, we used Table 2.

Table 2. Water-Mediated Hydrogen Bonds Found in the 37 Cocrystal Cases

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*a DA is the donor–acceptor distance in units of angstrom.
the 10,037 ligand library prepared as detailed in section 2 and performed steps I–VI of the HierVLS protocol on the 11 protein targets. This database screening was performed on a dual 866 MHz Pentium III processor Linux cluster with 80 nodes. Each processor performs one target/ligand combo at a time with the complete target/ligand list evenly distributed across all available processors.

When put to the 10,037 ligand library test, HierVLS found 23 out of the 37 cocrystal ligands in the top 2% of the library screening ranked by binding energy. These results are shown in Table 4. This can be compared to the results for the other methods as follows: HierVLS, 23 out of 37; Dock 4.0, 7 out of 37; FlexX, 11 out of 37; ICM, 20 out of 37.

If screening is done including crystal waters, we find the cocrystal ligands in the top 2% ranking for 26 out of the 37 cases (70% of true hits in the top 2% of the ranked database compared to 62% for no water cases).

Screening efficacy has also been evaluated for other VLS methods.14,24–26 Bissantz and co-workers14 docked (using Dock, FlexX, and Gold) and scored (using Chemscore, Dock, FlexX, Fresno, Gold, PMF, Score, and combinations of them) 10 experimentally active ligands plus a library of 990 decoy molecules to the targets thymidine kinase (TK) and estrogen receptor (ER). Out

<table>
<thead>
<tr>
<th>Protein</th>
<th>crms (Å) to xtal</th>
<th>Protein</th>
<th>crms (Å) to xtal</th>
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*The highlighting in yellow are cases in which the predicted cocrystal CRMS deviation is 2 Å or less. A single protein conformation was used for docking multiple ligands in all cases except carbonic anhydrase II. The PDB code of the complex from where the protein conformation was taken is underlined.
of the combinations tried, Dock docking with PMF scoring (Dock/PMF), FlexX/PMF, and Gold/Gold gave the best screening performances for TK, with Gold/Gold finding all 10 experimental hits in the top 10% of the ranked list, while the other two combinations find 7 of the 10 experimental positives in the top 10%. For the second target ER, Gold docking with Dock scores performed best. For both targets, the best hit rates were obtained when using two or more scoring functions in combination. In terms of how well the scoring functions correlate with measured binding, only Gold docking with gold scoring gave predicted binding energies with some statistical significance (correlation coefficient of 0.51) when applied to TK. As in some of our cases, the

<table>
<thead>
<tr>
<th></th>
<th>% better ranked molecules</th>
<th>% better ranked molecules</th>
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</tr>
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</tr>
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<tr>
<td></td>
<td></td>
<td>1pph</td>
</tr>
</tbody>
</table>

^a Column 2 shows the percentage of molecules with equal or higher score compared with that of the cocrystal ligand in column 1. Blue highlighting indicate those cases where the target ligand was in the top 2%. A single protein conformation was used for docking multiple ligands in all cases except carbonic anhydrase II. The underlined PDB code indicates the crystal complex from which the protein conformation was taken. ^b Poor discrimination of the cocrystal ligands appears to be associated with unaccounted water-mediated HBs for 1ett (inclusion of one water molecule improves the ranking of this ligand to the top 0.8%; see Table 8). ^c The trypsin cocrystals not ranked in the top 2% of the binding energy list are all low-affinity ligands, and their calculated binding energies may have been underestimated. Nonetheless, the correlation coefficient between experimental log $K_i$ and calculated binding energies for the trypsin complexes is 0.93 (see Table 7).
authors in that work attribute the poor screening efficacy and low binding energy correlation found for the target TK in part to unaccounted water-mediated interactions between ligands and protein.

A comparative study of the VLS performance of FlexX, FRED, and Glide as docking engines in combination with various scoring function for five protein targets (cyclooxygenase-2, estrogen receptor, p38 MAP kinase, gyrase B, α-thrombin, gelatinase A, and neuraminidase) has recently been published. In that work, the shape fitting docking method FRED performed better for cases where binding interactions are mainly lipophilic, while the incremental construction algorithm in FlexX worked better for cases where few hydrogen bonds could be used as anchor points. The particularly good performance of Glide for the most polar targets in the group, neuraminidase and gelatinase A, was attributed by the authors to the inclusion of repulsive electrostatic and van der Waals interactions in the force field filtering of docked configurations. The combination of docking/scoring methods FRED/ChemScore had the better overall performance in terms of screening efficacy across all seven targets. For neuraminidase, which is also in our target list, although we used a different set of coordinates, the FRED/ChemScore combination finds close to 20% (actual number not available in the paper) of the 51 experimental hits in the top 2% of the ranked lists. The best performance for neuraminidase though is achieved with the Glide/ScreenScore combination, which gives close to 80% of true hits in the top 2% of the ranked 7980 compounds database. However, the Glide/ScreenScore combination performed poorly for the other targets studied in that work.

We consider the performance of HierVLS in the screening efficacy test to be extremely encouraging.

3.3. Efficiency in the Generation of Docked Conformations. The conformational search performed at level 0 of the HierVLS protocol is far from an exhaustive search. Therefore, it is important to evaluate how efficient that step is in finding conformations that fall within a 2 Å RMSD deviation from the crystallographic structure of the known ligands. A larger number of correct binding mode conformations increases the chances of choosing a conformation that is both geometrically accurate and energetically favorable for screening. We also want to ensure that good conformations are not lost in the hierarchical process.

Table 5 reports the number of good conformations (i.e., having a CRMS deviation of 2 Å or less for heavy atoms when compared to the experimentally determined conformation) at each step of the HierVLS protocol. Here, we see that the chances of having high binding mode accuracy in the final best conformation increase with the number of configurations within 2 Å of the experimental crystals generated at level 0. This suggests improvements in which better energy evaluation is carried out in this stage.

Although the inclusion of crystallographic waters benefits the particular cocrystal ligand from which the waters were derived, they may interfere with the binding of other ligands to the same protein. For example, see the ret/1etr in Table 8. This suggests improvements in which waters necessary for hydrogen bonding to ligand and protein are placed during the conformational search step of the protocol.

3.4. Correlation between Scores and Experimental Binding Affinity. For the highest efficiency in database screening, it is important to have the correct binding mode and a good correlation between the calculated binding energies and the experimental binding constants (Ki). Accurate binding energies provide a difficult test for a VLS procedure because we do not allow relaxation of the full protein—ligand with solvation (which is required for the highest accuracy). Among our 11 protein targets, four cases have experimental Ki data for three or more of the ligands considered here and have been previously used in ligand screening studies: carboxypeptidase A (car) (three cocrystals); thermolysin (tmm) (three cocrystals); e-thrombin (ret) (three cocrystals); trypsin (trp) (seven cocrystals).

The calculated binding energies are compared to experimental binding constants in Table 6 and Figure 1. The binding energies for cocrystal structures were calculated using the multiple ligands/single protein conformation restriction, as described in "Methods". The experimental binding constants for ret, trp, car, and tmm were taken from Eldridge et al. That paper constructed five classes of protein/ligand complexes that the authors used as training sets to develop an empirical scoring function. They included only complexes involving small noncovalently bound ligands with known binding affinity and experimental structures deposited in the Protein Database (PDB). We grouped our targets into two (serine proteases and metalloproteases) of the five training set classes from Eldridge. Although other complexes in our set had reported binding affinities and belonged to one of the five classes in that paper, we only used the complexes that could be grouped into a class with at least six members.

The sets reported by Eldridge were also used in other studies evaluating performance of knowledge-based scoring functions. This allows us to compare our HierVLS scoring function performance to those functions. Muegge and Martin proposed and evaluated a potential of mean force (PMF) score calculated using protein—ligand atom-pair interaction potentials derived from the observed distance distribution of specific atom-type interactions found in the PDB. The performance of the PMF scores for 77 cocrystals with known experimental binding affinities was compared to two other scoring functions, the empirical LUDI and the statistical SMOG. Goehke et al. developed a scoring function ("DrugScore") based on structural information for protein/ligand complexes from the Relibase. DrugScore includes distance-dependent atom pair and solvent-accessible surface-dependent potentials.

The reported correlation coefficients between scores and log K, are shown in Table 7. The performance of the various methods for the serine protease class is comparable to that of HierVLS, all having significant linear correlation between scores and log K, but the correlation coefficient between calculated HierVLS scores and experimental binding constants for trypsin (trp) and e-thrombin (ret) (which are together in class "serine proteases") is 0.94 for cocrystal conformations scored with the HierVLS scoring function and is 0.93 for the conformations obtained by docking the cocrystal ligands...
using HierVLS. We consider these correlations as excellent because (1) cocrystal and HierVLS predicted scores were both calculated for multiple ligands using a single protein conformation and (2) our scoring function is based on first principles and not calibrated to fit experimental values.

All the scoring functions in Table 7 (including HierVLS) seem to have trouble describing the binding affinities for the metalloprotease complexes. The best correlation for this class was obtained by the empirical scoring function described by Eldridge et al., but a good correlation is expected because the complexes were in the training set used to derive the equation.

The correlation coefficients $R^2$ for linear fitting of calculated versus experimental binding affinities define the proportion of variance in common between two variables. The value of $R^2$ that is statistically significant depends on the number of variable pairs and the probability of correlation by chance for that number of pairs. For the serine protease set (10 components), an $R^2$ higher than 0.55 can be considered significant, while for the metalloproteases set (six components) $R^2$ needs to be higher than 0.73 to be significant.

The metalloprotease class has proteins with metals playing a role in ligand recognition in the binding site. Carboxypeptidase A (car) and thermolysin (tmn) are two such targets in this test set. The correlation coefficient for these systems is 0.50 for cocrystal configurations and 0.68 for the predicted HierVLS configurations. The low correlation for cocrystal cases is probably because we

Table 5. Efficiency in Generating Docked Conformation at Each Level of the HierVLS Protocol

<table>
<thead>
<tr>
<th>Case</th>
<th>No water</th>
<th>With water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final crms (Å)</td>
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<tr>
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</tr>
<tr>
<td>let</td>
<td>0.80</td>
<td>1.30</td>
</tr>
</tbody>
</table>

a The table shows at each step the number of cocrystal ligand configurations having (heavy atom) a CRMS deviation of 2 Å or less from the experimental cocrystal. The total number of configurations considered in each step is shown in the first row. The CRMS deviation for the final selected conformation is in the second column. Both water and no-water cases used a single protein conformation per target, with the water molecules coming from the underlined structure. When present, the water molecules were kept fixed during docking but were allowed to relax along with the ligand at level 1 (protein fixed minimization) of the HierVLS protocol. Highlighted cases indicate no configuration generated at level 0 (black shade), final configuration within 2 Å CRMS (grey shade), and one configuration below 2 Å that was subsequently lost (yellow shade).
The vast majority of the known structures of bond interactions requiring training sets of experimental data, and can be used in screening large databases, is not limited by other approaches. Since HierVLS is fast enough to be used a fixed protein conformation to score different ligands. In addition, the Dreiding FF used here was not developed to describe interactions with metals. To improve this performance, we plan to optimize the Dreiding force field for describing the interaction of protein-bound metals with small organic molecules.

The scoring functions in Table 7 were from knowledge-based (PMF, SMOG, DrugScore; based on experimental structural data), empirical (LUDI, Eldridge; based on experimental binding data), or first principles (HierVLS; based on theoretical description of atomic level interactions). These comparisons show that the first-principles scoring function of HierVLS performs comparably to the other approaches. Since HierVLS is fast enough to be used in screening large databases, is not limited by requiring training sets of experimental data, and can be improved systematically by improving the accuracy of describing the fundamental interactions between protein and ligands, we consider that HierVLS is very promising.

### 3.5. Importance of Water-Mediated Hydrogen Bonds
The vast majority of the known structures of protein/ligand complexes have all the buried polar hydrogen bond donors/acceptors satisfied by hydrogen atoms in the binding site mediate hydrogen bonds between ligand and protein. These water-mediated HBs stabilize bound conformations that would be less favorable otherwise. However, within the context of VLS it is difficult to include the crystallographic water molecules needed to form these HBs in the binding site. This is because any particular water molecule important for one ligand may be unimportant or detrimental for another ligand bound to the same protein. Thus, protein/ligand conformations that should be stabilized through HBs to water molecules in the binding site will lack that extra contribution to their energy, making it more likely that they will score more poorly than ligands that do not depend on such interactions.

The importance of such water-mediated hydrogen bonds for correctly predicting binding mode and affinity is shown in Table 8. There, we compare binding mode and ranking with and without water molecules for 25 cocrystal ligands. Crystallographic waters found to mediate hydrogen bonds between ligand and receptor were included as part of the receptor whenever the ligand or receptor atom involved in these HBs could not make any HBs without this water. The PDB files where the water-mediated HBs were found and the water number are also in Table 8. The waters were kept fixed along with the receptor for level 0 (configuration generation) but were allowed to move along with the ligands at level 1 of the HierVLS procedure. Not all waters included as part of the receptor were conserved across the cocrystals of a particular receptor, indicating that caution should be taken when evaluating the predicted binding mode for cocrystal ligands other than the ones from which the included waters were taken. For instance, the water molecule added to -thrombin/1etr improves the HierVLS score for 1etr and for 1ett but clashes with the ligand in 1ets. Thus, the protein/water configuration for 1ett cannot be used for 1et.

For five cases that were not within 2 Å CRMS when the docking was done without water, the accuracy of the binding mode improves to less than 2 Å upon inclusion of crystal waters. Two of these cases (cah/1okl and tmn/6tmn) involve proteins that have a metal ion interacting with the ligands, and three are sugars bound to -arabinose binding protein (ara/1abe, ara/1abf, ara/5abp). To correctly predict the binding mode for carbonic anhydrase II/1okl also required choosing an adequate conformation of the target protein as discussed below.

Introducing the water molecules that mediate protein–ligand hydrogen bonds in the database screening also achieves much better efficacy. To assess this improvement in binding affinity ranking, we docked and scored all 10 036 decoy ligands against targets with added cocrystal waters. We found that including the waters promoted three cocrystal ligands (cah/1cll, cah/1cnx, and tmn/5tmn) to the top 2% of the decoy list. In addition, including these waters consistently improved the score of the ligands corresponding to the cocrystal from which they were derived (marked in bold in Table 8). Level 0 sampling also benefited from adding these waters. For penicillopepsin (pep), -thrombin (ret), and trypsin (trp), including water did not change the binding mode and affinity outcome, which were already good.

Similar results were found for predicting the binding mode in the case of ligand dhbt (no structure or name provided in the reference) virtually docked to thymidine.

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**Table 6. Calculated Binding Scores Compared to Experimental Association Inhibition Constants (K)ab**

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<th>target</th>
<th>PDB code</th>
<th>exptl log Ki, 27,44</th>
<th>score for crystal conformation</th>
<th>score for HierVLS conformation</th>
<th>CRMS (Å) for HierVLS conformation to crystal conformation</th>
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* Underlined is the PDB code of the complex from where the protein conformation was taken. b Scores for all crystal conformations, trp HierVLS, and ref HierVLS are for no-water cases; HierVLS scores for car and tmn are from water cases.
The rms deviation from the crystal structure was 3.65 Å without waters and went down to 0.70 Å when the active site was filled with waters during docking. However, the effect in screening efficacy was minimal (ranking down from 24 to 18 when filling the site with waters) for that particular ligand. The inclusion of crystallographic waters and use of different protein conformation, on the other hand, was found to improve considerably the correlation between binding energies and scores.

For the L-arabinose binding protein (ara) bound to L-arabinose (1abe), D-fucose (1abf), and D-galactose (5abp), including a single conserved water [310 (1abe)] changes the binding mode prediction from none (without water) to all (with water) ligands having a CRMS lower than 2 Å. It has been suggested that the crystallographic water molecules (309, 310, and 311) in these structures contribute to the specificity of L-arabinose binding protein toward those three ligands. The groups common to the three sugars are positioned identically in the site, and all three ligands form the same protein/ligand HBs. However, the water-mediated HBs and the positioning of the water molecules in the site differ in each case, contributing to the observed differences in specificity. Even though the predicted HierVLS conformations without water are not within 2 Å from the cocrystals, they still represent the affinity of the site for sugars because all three cocrystal ligands rank in the top 1% of the 10 037 list. The no-water HierVLS conformations differ from the cocrystal ones by rotations of the sugar around an axis perpendicular to the ring. When ranking is performed with the inclusion of water

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**Figure 1.** Comparison of predicted binding scores against experimental binding affinities for the experimentally determined cocrystal conformation and the conformation predicted by HierVLS for (A) serine proteases (trypsin and α-thrombin) and (B) metalloproteinases (carboxypeptidase A and thermolysin). The A and B grouping and experimental values were based on Eldridge et al., 1997. The cocrystal HierVLS scores were calculated for a single protein conformation per target (the same one used in the HierVLS procedure), with the different ligands placed into the binding site by superposing the PDB files and minimizing the ligand with fixed protein. Therefore, the HierVLS scores do not correspond exactly to scoring the cocrystal (PDB) conformation except for the cases from where the protein conformation was taken. The scores reported in the literature are for the protein–ligand configuration in the original PDB files. This difference should be taken into account when comparing the correlation coefficients. Comparative scores were taken directly from published tables in Eldridge et al. in 1997 or estimated from published graphics in Muegge and Martin in 1999 for the same cocrystals reported in the present work. The cocrystal used for the protein conformation is underlined in the figures.
Table 7. Correlation between Calculated and Observed Binding Affinities for Cocrystals

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<th>scoring function</th>
<th>serine proteases (trp and ret) no. complex</th>
<th>R²</th>
<th>metalloproteases (tmn and car) no. complex</th>
<th>R²</th>
<th>predicted ligand configurations</th>
<th>serine proteases (trp and ret) no. complex</th>
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<th>metalloproteases (tmn and car) no. complex</th>
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<td>15</td>
<td>0.58</td>
<td>(0.57)</td>
<td>DrugScore29</td>
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<td>0.86</td>
<td>0.77</td>
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<tr>
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<td>15</td>
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<td>0.70</td>
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</table>

a The cocrystal HierVLS scores were calculated for a single protein conformation per target (the same one used in the HierVLS procedure), with the different ligands placed into the binding site by superposing the PDB files and minimizing the ligand with fixed protein. Consequently, the HierVLS scores do not correspond exactly to results from scoring the cocrystal (PDB) conformation except for the cases from which the protein conformation was taken. The scores reported in the literature are for the protein–ligand configuration in the original PDB files. This difference should be taken into account when comparing the correlation coefficients. We also report the correlation coefficients for the predicted (docked using HierVLS) complexes. Our set did not include structures with PDB codes 1bra, 1pcc, 1tmn, 1tnh, tm1. c Our set did not include structures with PDB codes 1mnc, 1tlp, 1tmn, 2tmn, 4tln, 4tnm, 5tmn, 7cpa, 8cpa. d The number in parentheses is the correlation coefficient for the same set of structures as in the MSC HierVLS. The scores used here were either taken directly from tables (Eldridge’s case) or estimated from published graphics (Muegge and Martin’s case).

310 (1abe), the binding affinities of 1abe and 5abp increase while 1abf loses a bit of affinity but still ranks in the top 2% of the decoy list.

The same effect of bound water can be seen for the thermolysin/6tmn complex. In this case, the water-mediated hydrogen bonds involving water molecules 5 and 362 in the cocrystal are believed to contribute to mediated hydrogen bonds.38 The native conformation of thermolysin/6tmn complex. In this case, the water-improved the scores of all three thermolysin cocrystal ligands and promoted 5tln to the top 2% of the ranked list. However, 6tmn scored worse than 2% of the decoy list even with the inclusion of waters, which indicates that we are still missing important interactions for this ligand.

4.6. Metals, Water Coordination, and Water Displacement. Four of our 11 target proteins (cytochrome P450cam (cyt), carbonic anhydrase II (cah), carboxypeptidase A (car), and thermolysin (tmn)) have metal groups that participate in ligand binding. The structure of cytochrome P450cam with no ligand has the heme ferric cation coordinated to a cysteine residue and a water molecule in the binding site.38 Binding the substrate displaces the coordinated water (as well as other conserved water molecules present in the active site) and induces a transition from a hexacoordinate low-spin ferric state to a pentacoordinate high-spin ferric state.38,39

Carboxypeptidase A (car), thermolysin (tmn), and carbonic anhydrase II (cah) have Zn²⁺ in their active sites. For carboxypeptidase A and thermolysin without the ligand, the Zn²⁺ is coordinated to two histidine groups, one glutamate group, and one water molecule. Substrate binding displaces the coordinated water from the active site,36,40 and it has been suggested that further stabilization of the complexes is achieved through water-mediated hydrogen bonds.38 The native conformation of thermolysin remains essentially unchanged upon binding numerous inhibitors.36 Of the three thermolysin complexes studied here, 3tmn has a water molecule coordinating the zinc (even with the ligand in place) and 6tmn has multiple water-mediated HBs. The experimentally determined structures of carbonic anhydrase II complexes show that some ligands (e.g., PDB codes 1cnx and 1cnw) form water-mediated hydrogen bonds to the protein.

In all of these targets, binding of the ligand displaces a metal-coordinated water molecule. There are three reasonable options for predicting binding affinities in such cases: (1) assume that the reference state for the unbound protein has a free coordination site (no water in the active site); (2) assume that the reference state of the protein has a water molecule coordinated to the metal and that binding of the ligand displaces the water to a different position still in the active site; (3) assume that the reference state of the protein has a water molecule coordinated to the metal but that binding of the ligand ejects the water from the active site.

For options 1 and 3, the reference state for the unbound protein is the same for all ligands and there is no possibility of stabilization of each complex by the displaced water. Thus, these options lead to the same ranking as that in a ligand list by affinity. However, the values for the binding energies and hence their correlation to experimental affinities when considered among a set of unrelated targets depend on the choice of the unbound protein reference state.

Option 3 is more computationally challenging, since it is necessary to determine the best configuration for the water molecule with the ligand in the binding site. This coupled docking problem will lead to a different configuration for the water with each ligand conformation. Consequently, including the displaced water in the complex may change the outcome for both the binding mode and the affinity ranking. In addition, we must consider extra stabilization through water-mediated HBs between the ligands and the binding site involving other buried waters in the site. The stabilization arising from including one water molecule in the binding site may range up to 2 kcal/mol (the number of hydrogen bonds made by a water buried in a protein may be up to three, with each one stabilizing the cavity by 0.6 kcal/mol).41 This explains why 5 out of 12 cocrystal ligands...
of metal-bound targets ranked worse than 2% of the decoy list, even after some of the water-mediated hydrogen bonds found in the cocrystal structures and the receptor bound conformation were included.

These results suggest improving HierVLS to include the favorable interactions due to water-mediated coupling of the ligand with the protein by always adding to the ligand/protein binding cavity waters needed to

<table>
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<tr>
<th>Target</th>
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<th>With water</th>
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</tr>
<tr>
<td>1ap</td>
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<tr>
<td>ara</td>
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<td>1.07</td>
</tr>
</tbody>
</table>

Table 8. Comparison of HierVLS Results with and without Inclusion of the Crystallographic Waters Found To Mediate Target–Ligand Hydrogen Bonds

a Except for cash with water, all the other cases used a single protein conformation (PDB code underlined) per target. The coordinates for the water molecules were extracted from the same PDB file as the protein. Water molecules were kept fixed during docking but were allowed to relax along with the ligand at level 1 (protein fixed minimization) of the HierVLS protocol. Yellow highlighting indicate CRMS below 2 Å. Blue highlighting indicates cases where the target ligand was in the top 2%.

b The cases with no water used 1cil protein conformation for all ligands, while the water cases had the ligands docked to their respective protein and water conformations. All the other targets used a single protein conformation.
were the ligand was bound to its proper receptor and binding affinity ranking always comes from the case overall performance in terms of binding mode prediction any of the receptor conformations. Of course, the best correctly predicted just by adding cocrystal waters to the 1cnx receptor, all the other binding modes were drase II are shown in Table 9. Except for 1cil bound to but different target conformations for carbonic anhydrase II complexes of cah. These two structural features imply that the conformation of the native protein remains rather saturated the hydrogen bond network. These waters would have to be modified appropriately during ligand conformational sampling.

Certainly the increased errors for ligand binding to metalloproteins is caused by using a force field that was never optimized for metals (Zn and Fe). We can improve our description of the metalloprotein complexes by optimizing the force field for such metals binding to organic ligands.

### Table 9. Effect of Protein Conformation on Binding Mode Prediction for Carbonic Anhydrase II (cah)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>cah/1cil, H2O</th>
<th>cah/1okl, H2O</th>
<th>cah/1enx, H2O</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>crms</td>
<td>HierVLS score</td>
<td>% better ranked</td>
</tr>
<tr>
<td>1cil</td>
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<tr>
<td>1okl</td>
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<tr>
<td>1enx</td>
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<td>-39.2368</td>
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</table>

* Each column corresponds to a different crystal structure of cah. The three cocrystal ligands were docked to each one of the protein conformations using HierVLS. The best overall performance in terms of binding mode prediction and binding affinity ranking (in bold font) consistently comes from the case where the ligand was bound to its proper receptor conformation. The binding mode accuracy is below 1 Å in all three cases.

| 3.7. Changes in the Protein Conformation Induced by the Ligand. | The experimental structures of carbonic anhydrase II complexed with three structurally related inhibitors (1cnx, 1cnw, and 1cn) reveal that the conformation of the native protein remains rather unchanged upon binding. However, another set of three experimentally determined carbonic anhydrase II complexes show that in the binding of a specific inhibitor (1cil), the side chain attached to the 4-amino group is in a conformation that forces His-64 to occupy a non-native position and causes the bound water to be absent. This change in conformation was used to explain why 1cil binds more tightly than the other two structurally related inhibitors also studied in that paper (PDB codes 1cin and 1cm). The structure of the 1okl complex shows that a conformational change of Leu-198 is required to accommodate that ligand, compared to other complexes of cah. Two such structural features imply that the conformation of this protein can change upon binding and may be very specific for a particular ligand. Without including any water molecules and using the same protein conformation (1cil) for all three ligands, HirVLS fails to predict the correct binding mode for 1okl. Using the proper target conformation for 1okl leads to only a marginal improvement in the predicted binding mode (CRMS error decreases from 3.73 to 2.72 Å). However, including the water molecules that mediate receptor–ligand hydrogen bonds in the crystal has a stunning effect. The CRMS deviations to the cocrystal conformations obtained using the same water molecules but different target conformations for carbonic anhydrase II are shown in Table 9. Except for 1cil bound to the 1cnx receptor, all the other binding modes were correctly predicted just by adding cocrystal waters to any of the receptor conformations. Of course, the best overall performance in terms of binding mode prediction and binding affinity ranking always comes from the case were the ligand was bound to its proper receptor conformation. This is consistent with a mechanism of ligand binding in which the ligand first adopts the proper position and then induces a conformational change to improve affinity.

3.8. Predicting Binding Affinities for Low-Affinity Ligands. The correlation coefficient between the seven trypsin complexes with experimental log K_i and the predicted binding energies is 0.93, indicating that our procedure leads to accurate relative affinities. On the other hand, five of the eight trypsin cocrystal ligands led to 11% or more of the decoy molecules having binding affinities ranking better than the correct ligands. We found that the ligands with low experimental affinity to the site (1tng, 1tni, 1tnj, 1tnk, 1tnl; K_i ranging from −9.6 to −16.7 kJ/mol) were exactly the ones that ranked poorly (11−52%) in the HierVLS scoring. This suggests that the problem results from a poor description of the solvation for loosely packed ligands.

3.9. Timing. We timed each step of the HierVLS protocol using an 866 MHz Pentium III processors running Linux. These results are shown in Table 10. Times are in seconds except for last column, which is in minutes. The times reported are user plus system times. For the level 0, filter, and level 1 steps, these times are the average over all 10 037 ligands against each target, while the AVGB times are reported for a single protein/ligand case. Row “average” lists the average times calculated over all 11 targets. The average run time for no-water cases was 4 min per ligand. The run time for water cases ranged from 4.6 to 6.7 min per ligand.

As can be seen in Table 10, the most time-consuming step of the protocol is level 1, which consists of energy minimization of 50 docked configurations of each ligand with fixed protein coordinates. The time spent at level 1 greatly depends on the number of atoms of the target protein, with bigger proteins requiring the largest amount of CPU time. Run time at level 1 also depends on the size and rigidity of the ligands and on the fitting quality of the bound configurations. In our particular case, the same ligand library was docked to each target, and therefore, the differences in CPU time at level 1 come from protein size and binding site fitting. Large or shallow binding sites that can physically accommodate most ligands in the database without very many bad contacts will require, on average, fewer steps of minimization (less CPU time) at level 1. Ligands that
It predicts the correct binding mode (within 2 Å CRMS) protocol is sufficient for use in virtual ligand screening.

4. Assessment of Performance

We conclude that the performance current HierVLS protocol is sufficient for use in virtual ligand screening. It predicts the correct binding mode (within 2 Å CRMS) with 100% success. In addition, HierVLS successfully selects the active ligands in the top 2% for 21 of the 37 cases out of a large database. HierVLS is based on a generic all-atom force field to calculate the binding energies. Thus, it should be applicable to a broad range of biosystems, including proteins, DNA, glycosilated systems, and lipids.

The failures in the current level of HierVLS result for either metal-containing sites on the protein or water-mediated ligand–protein interactions. We expect that the metal-containing systems can be handled by extending the FF straightforwardly while water-mediated interactions may be taken into account by saturating the hydrogen bonds within the binding cavity during conformational search. However, we believe that the current HierVLS is already useful for many interesting applications in screening ligands, particularly for proteins that do not contain metals at the active site and for cases that do not rely on water-mediated HBs for optimal binding.

Structural features in the target receptor were found to be very important for binding mode accuracy. Special care should be taken with disulfide bridges and protonated states of His residues. For proteins known to change conformation upon binding of different ligands, screening should be performed against multiple protein conformation and/or allowing the protein to change conformation as an additional last step of docking.

The current generation of HierVLS uses the program Dock4.0 to generate docked conformations in level 0.

5. Suggested Improvements

The studies reported here suggest several improvements in HierVLS that we are implementing. However, they have not yet been applied to full scale tests.

Recent improvements in the coarse grain Docking step of HierVLS are (1) energy scoring using the Dreiding force field, (2) continuum solvent scoring based on the analytical volume generalized Born (AVGB) continuum solvation approach, (3) automatic selection of the size of the space to be sampled in the coarse grain docking step by using diversity saturation criteria.

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Table 10. HierVLS Times

<table>
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<tr>
<th>target</th>
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<th>level 0 (50 conf/lig)</th>
<th>filter (50 conf/lig)</th>
<th>level 1 (5 conf/lig)</th>
<th>AVGB typing (1 conf/lig)</th>
<th>AVGB complex (1 conf/lig)</th>
<th>AVGB ligand (1 conf/lig)</th>
<th>AVGB protein + H2O (1 conf/lig)</th>
<th>time per ligand min/ligand</th>
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* Reported times are from a single 866 MHz Pentium III processor running Linux.
combined with enrichment of energetically favorable orientation families, and (4) alternative strategies of conformation sampling. We expect that these improvements will greatly increase the efficiency of level 0, increasing both binding mode accuracy and screening efficacy while decreasing the average run time.

We are also implementing side chain optimization in the last stage of HierVLS to account for changes in the binding site conformation to accommodate the best ligands. We intend to also include water-mediated interactions.

To decrease the time spent in screening a VLS library, it would be useful to prescreen the library using a pharmaphore model derived from known experimental complexes of a particular target. This prescreening option is also being implemented and tested.

6. Conclusions

On the basis of the validation against known co-crystal structures, the success in identifying the active ligands from a database, and the application to the protein—tyrosine phosphatase 1B (ptp1b), we believe that the current generation of HierVLS is useful and reliable for targets with known co-crystal structures and no metal atoms in the binding site. The most problematic cases would be the ones for which the protein conformation changes considerably upon binding and/or the bound conformation involves water-mediated hydrogen bonds. We consider that a number of improvements are possible that could further improve the performance while maintaining or decreasing the computational costs.

Acknowledgment. This project started as a challenge from Prof. Peter Schultz of Scripps Research Institute. We thank Mr. Sheng Ding, Dr. B. D. Bursula, and Dr. Schultz for helpful discussions. We thank Prof. Schultz and the GNF for use of their computer facilities for carrying out many of the calculations. In addition, we thank Dr. B. D. Bursula and Prof. Charles Brooks for permission to report some of their results prior to publication. This research was supported partially by Grants NIH-BRSG01-GM62523, NIH-R29AI40567, and NIH-HD36385, and the computational facilities were provided by an SUR grant from IBM and a DURIP grant from AR0. The facilities of the Materials and Process Simulation Center are also supported by DURIP-ONR, DOE (ASCI ASAP), NSF, MURI-ARO, MURI-ONR, General Motors, ChevronTexaco, Selko-Epson, Beckman Institute, and Asahi Kasei.

References


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