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Virtual Screening to Enrich Hit Lists From High-Throughput Screening: A Case Study on Small-Molecule Inhibitors of Angiogenin

Jeremy L. Jenkins,[1] Richard Y. T. Kao,[1] and Robert Shapiro[1,2]*
[1]*Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Cambridge, Massachusetts*
[2]*Department of Pathology, Harvard Medical School, Boston, Massachusetts*

ABSTRACT **"Hit lists" generated by high-throughput screening (HTS) typically contain a large percentage of false positives, making follow-up assays necessary to distinguish active from inactive substances. Here we present a method for improving the accuracy of HTS hit lists by computationally based virtual screening (VS) of the corresponding chemical libraries and selecting hits by HTS/VS consensus. This approach was applied in a case study on the target-enzyme angiogenin, a potent inducer of angiogenesis. In conjunction with HTS of the National Cancer Institute Diversity Set and ChemBridge DIVERSet E (~18,000 compounds total), VS was performed with two flexible library docking/scoring methods, DockVision/Ludi and GOLD. Analysis of the results reveals that dramatic enrichment of the HTS hit rate can be achieved by selecting compounds in consensus with one or both of the VS functions. For example, HTS hits ranked in the top 2% by GOLD included 42% of the true hits, but only 8% of the false positives; this represents a sixfold enrichment over the HTS hit rate. Notably, the HTS/VS method was effective in selecting out inhibitors with midmicromolar dissociation constants typical of leads commonly obtained in primary screens. Proteins 2003;50:81–93.**

Key words: virtual screening; ligand docking; high-throughput screening; angiogenin; consensus scoring; false positives; enrichment; enzyme inhibitor; ribonuclease

INTRODUCTION

High-throughput screening (HTS) is one of the most powerful approaches available to identify new compound leads for the steadily growing catalog of validated drug targets.[1] To obtain a sufficient number of high-quality leads for drug development by this method, it is desirable to screen with as many molecules as possible; consequently, the compound collections used in HTS are expanding and diversifying dramatically,[2] and assay systems that are capable of evaluating >125,000 compounds in a single day have been developed.[3,4] At the same time, the enormous quantity of hits obtained from HTS presents at least two difficult challenges: (i) how to "mine" the large volume of data to select those compound hits with the greatest potential as leads and (ii) how to efficiently weed out those hits that represent false positives. Several studies have now focused on the first of these issues, using statistical approaches to setting hit cutoffs[5,6] or partitioning and clustering HTS hits according to similarity, based on pharmacophore information or quantitative structure-activity-relationship models such as comparative molecular field analysis (CoMFA).[7–14] In contrast, the second problem has received relatively scant attention, although it is well established that HTS hit lists contain numerous (in many cases a majority) false positives.[15–18] The follow-up hit confirmation assays required are laborious and costly, whether they involve simple retests in the same assay system (a weak, although common, form of verification that only corrects machine errors), control assays, or measurements in a completely independent system. Thus, it is critical to develop new and efficient strategies to distinguish active from inactive substances at the primary screening stage. Here, we have explored a fast and inexpensive method for improving HTS hit rates by computationally based library docking.

Ligand-docking programs have been used in recent years for high-throughput docking, or virtual screening (VS), of chemical databases,[19,20] often as an alternative to HTS. VS involves rapid fitting of chemical library members into the active sites of three-dimensional protein structures. (The term "virtual screening" can be used more widely to mean any in silico technique for screening databases of molecules; here, we are using it more narrowly to mean high-throughput docking.) Several studies have shown that docking programs can reliably select out known high-affinity (nanomolar K_D) inhibitors added to a library of random or inactive compounds.[21–25] Accurate selection of ligands that exhibit micromolar binding constants more typical of the leads present in actual libraries

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R.Y.T. Kao's present address is Department of Microbiology, The University of Hong Kong, Hong Kong.

*Correspondence to: Robert Shapiro, Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, One Kendall Square, Building 600, 3rd Floor, Cambridge, Massachusetts 02139. E-mail: Robert_Shapiro@hms.harvard.edu

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remains difficult,[22,26] although there have now been some notable successes in identifying novel lead compounds by VS (for reviews, see Refs. 27 and 28). Significant improvements in VS have been made by consensus scoring of multiple scoring functions[22,24,25,29–31,34] and by clustering docking positions, or "poses," from multiple docking tools before scoring.[35] VS is particularly valuable for screening the vast number of compounds not present in HTS libraries and for screening against targets for which no high-throughput assay is available.

HTS and VS are not necessarily mutually exclusive techniques; by integrating the two technologies, there is the potential for powerful synergy.[36–39] In the present study, we have explored an integrated approach, where docking is used as an enrichment tool to eliminate false positives from HTS hit lists and as a means to pare down HTS libraries before screening. The target protein, angiogenin (ANG), is a potent inducer of new blood vessel growth in vivo[40] whose expression has been shown to be elevated in many human cancers (e.g., see Refs. 41–45). The available ANG antagonists—monoclonal antibodies, antisense oligonucleotides, and the ANG-binding protein actin—are extremely effective in preventing the establishment and/or metastatic spread of multiple types of human tumors in athymic mice.[46–48] Although some of these agents may have use as drugs in humans, small-molecule inhibitors would clearly be much more advantageous. Efforts to develop small antagonists have focused largely on an unusual aspect of ANG: its homology to bovine pancreatic RNase A. ANG is 33% identical to RNase A in sequence,[49] and its crystal structure reveals a similar fold, as well as some striking differences.[50,51] Importantly, ANG exhibits a characteristic ribonucleolytic activity that is critical for its biological action,[52–54] suggesting that compounds directed at the active site may have anticancer activity.

Initial efforts to identify ANG inhibitors focused only on nucleotides and related compounds. Although many of these molecules have relatively high affinity for RNase A (high nanomolar to low micromolar K_D), the lowest K_D value measured with ANG was only in the upper micromolar range at physiological pH[55,56] (J.L.J. and R.S., unpublished data.) Recently, a fluorescence-based assay for ANG that is much more sensitive than any method available previously was reported,[57] and we adapted this for use in HTS, allowing exploration of a much wider range and larger number of small-molecules for more potent antagonists. Screening was performed with two libraries (18,310 compounds altogether) and yielded a hit rate of 1%. Examination of these primary hits in more rigorous secondary and tertiary assays revealed that only a small fraction were active inhibitors ("true hits"); all of the true hits bind at least 4-fold more tightly than the best nucleotide inhibitors and one is 13-fold more effective.[58]

The high percentage of HTS false positives observed prompted the present study to create a method for enriching HTS hit lists by using VS only; in principle, the types of errors that generate most false positives in HTS (e.g., optical absorbance, insolubility, and delivery error) are unlikely to influence ligand-receptor docking scores in VS. It is important that this is a "real-world" case study conducted with the same libraries used for HTS; these libraries contain compounds with no better than midmicromolar K_D values. VS of the complete chemical databases for the HTS libraries was conducted with two docking and scoring programs, and different VS scoring thresholds were evaluated for their ability to improve the HTS hit list. We found that in the optimal scoring scheme with one of the VS programs, the hit rate (the percentage of true hits in the hit list) was increased by nearly sixfold. Alternative consensus VS/HTS schemes using either one or both of the docking methods have also been developed, which take into account the relative importance of minimizing the number of true hits discarded versus maximizing the elimination of false positives in any specific case.

MATERIALS AND METHODS

The HTS Data Set

The National Cancer Institute (NCI) Diversity Set (1990 compounds) and the ChemBridge DIVERSet (16,320 compounds) were screened for inhibitory activity against ANG in an HTS assay.[58] The HTS assay measured ANG-catalyzed cleavage of the fluorogenic substrate 5′-FAM-$(mA)_2$rC$(mA)_2$~Dabcyl-3′ (Integrated DNA Technologies), where FAM is 6-carboxyfluorescein, rC is ribocytidine, mA is 2′-O-methyl-riboadenosine, and Dabcyl is 4(4-dimethylaminophenylazo)benzoic acid. In the intact substrate, the fluorescence emission of FAM is largely quenched by the Dabcyl group. Cleavage by ANG relieves the quenching and increases the fluorescence emission by >25-fold. The assay was conducted in 384-well plates, under conditions where 7–10% of the substrate is cleaved in the absence of inhibitor. The median fluorescence value on each plate was normalized to 1.0 FI (arbitrary unit) for analysis; this FI value is similar to that measured when no library compound is added. The fluorescence in the absence of ANG was 0.2 FI, yielding a signal to background ratio of 5:1. The Z-factor[5] for the HTS assay was 0.6, indicating that a reasonable separation of background and sample signal variability was achieved. Compounds designated as HTS hits reduced fluorescence by 33% of the median value for each plate, the equivalent of 1.7 SDs (σ) from the population average. For the NCI set, which was assayed at a final concentration of 25 μM (i.e., a 400-fold dilution from 10 mM stocks), this FI cutoff theoretically translates into an upper limit of 50 μM for K_D. For the ChemBridge set, which was assayed at 20–70 μM (a 400-fold dilution of 5 mg/mL stocks), the predicted limit on K_D is 40–140 μM, depending on molecular weight (58 μM for compounds of median molecular weight). These cutoffs are relatively conservative and were not expected to result in a large number of false positives by themselves. Indeed, five of the true hits ultimately identified had FI values only slightly below the cutoff. As discussed below, 199 of the 18,310 library compounds were problematic for VS because they contained atom types not recognized by one or both docking programs (e.g., As, Cu, Pd, and Sb). These were discarded from the libraries used for VS/HTS consensus

analysis, reducing the number of compounds in our data set to 18,111.

Hit Validation and K_D Value Determinations

HTS hits were tested by additional methods to distinguish true actives from false positives.[58] The reaction buffer (20 mM Hepes, pH 7.0, 0.1 M NaCl) was the same as that used for HTS. First, all of the hits were assayed for their capacity to inhibit ANG-catalyzed mRNA degradation; compounds that still appeared to be active were then subjected to detailed kinetic analysis with a highly accurate HPLC-based assay. In the first assay, luciferase mRNA was incubated with ANG in the presence or absence of test compound and then added to an in vitro translation system. The luciferase produced was quantified by adding luciferase substrate and measuring light output in a luminometer. The ANG concentration used, 60 nM, was sufficient to reduce luminescence by ~70% in the absence of inhibitor. The compounds were tested at a concentration of 50 μM; those that restored luminescence to a level greater than that observed with 30 nM ANG in the absence of compound were regarded as hits. For the purposes of the present VS study, it was important to establish that this cutoff would eliminate few, if any, true hits. This was achieved by performing the HPLC assay on several compounds that produced luminescence values slightly lower than the cutoff: in all cases, the K_D values measured were well above 200 μM, suggesting that the luciferase assay is in fact a somewhat more sensitive indicator of ANG inhibition than is the HTS assay. In addition, we note that false negatives due to inhibition of luciferase translation or enzymatic activity are unlikely to occur because the compounds were diluted appreciably (10-fold and 200-fold, respectively) during the translation and light-producing reactions.

The HPLC assay for final hit confirmation monitored ANG-catalyzed cleavage of an octanucleotide substrate at a concentration well below K_m. Reaction mixtures with and without test compounds were incubated with 5 μM ANG for 2 h at 37°C and chromatographed on a Mono Q anion-exchange column. Remaining substrate and a hexanucleotide product resolve fully from each other and from all test compounds in this system and were quantified by peak area at 254 nm. This information was used to calculate k_{cat}/K_m values, and K_D values were obtained from fits to an equation for simple inhibition:

$$(k_{cat}/K_m)_i = (k_{cat}/K_m)_0 / (1 + [I]/K_D), \quad (1)$$

where $(k_{cat}/K_m)_i$ and $(k_{cat}/K_m)_0$ are values in the presence and absence of inhibitor, respectively, and [I] is inhibitor concentration. Compounds were tested initially at 25–70 μM. At least three concentrations (25–100 μM) were used ultimately for all true hits.

HTS Control Screen

The fluorescent substrate (100 nM) was cleaved completely by incubation with 10 nM pancreatic ribonuclease A for several minutes in the HTS assay buffer. This mixture was then aliquoted onto 384-well plates (Multidrop 384 dispenser, Thermo Labsystems) followed by automated injection of the compounds (final concentration 25 μM). Fluorescence measurements and data analysis were then performed as for HTS.

Preparation of Databases for Docking

The NCI Diversity Set database containing 1990 compound structures was obtained as a three-dimensional .sd file from the NCI Developmental Therapeutics Program (http://www.dtp.nci.nih.gov). The ChemBridge DIVERSet E (ChemBridge Corporation, San Diego, CA) of 16,320 structures were obtained as a two-dimensional .sd file and converted to three-dimensional coordinates by using Converter from Insight II (Accelrys Inc., San Diego, CA). A Silicon Graphics Indigo2 Maximum Impact 10000 workstation (195 MHz, 256 MB memory) was used for all computational procedures. For DockVision docking, concatenated .pdb files were generated by using the program dbconvert from DockVision, and hydrogen atoms were added in Babel v.1.6.[59] Two hundred of the library members, all from the NCI set, contained metal or other atoms not recognized by at least one of the docking programs, or were in fact compound mixtures. These were eliminated from both the HTS and VS data sets for analysis, with the exception of NCI 146443, an HTS hit that comprised two compounds. In this case, the inhibitory activity measured was determined to derive from only one of the compounds, and this molecule was used for VS. No other filters were applied to the libraries.

Preparation of Angiogenin for Docking

Crystal structures have been determined for free ANG (1.8 Å resolution[51]) and for complexes of ANG with phosphate and pyrophosphate (2.0 Å resolution[60]). We opted to use the free ANG structure (PDB entry: 1B1I) for docking because of its slightly higher resolution and to avoid any bias in favor of compounds containing phosphates or similar anionic groups. All water molecules were removed and hydrogen atoms were added in Insight II. Ligand docking was conducted in a sphere of 12 Å radius whose origin corresponds to the position occupied by the phosphorus atom in the superimposed coordinates of free ANG and the ANG-phosphate complex (PDB entry: 1H52[60]) (Fig. 1). This sphere encompasses all residues known to be involved in RNA substrate binding and specificity.[50,51]

DockVision/Ludi Virtual Screen

For one VS study, library docking was conducted in DockVision 1.0[61] (University of Alberta, Canada) by using the RSDB algorithm. RSDB uses a grid-based function for steric complementarity and a hydrogen-bonding function to compute distance and angles between potential donors and acceptors in the ligand and protein, generating an energy-like score on the basis of deviation from ideal values. Molecular charges were assigned automatically, and ligand flexibility was simulated by docking 100 randomly generated conformers of each compound. Docking was performed by an initial 100 trials of Monte Carlo-simulated annealing (with 200 steps), followed by a single

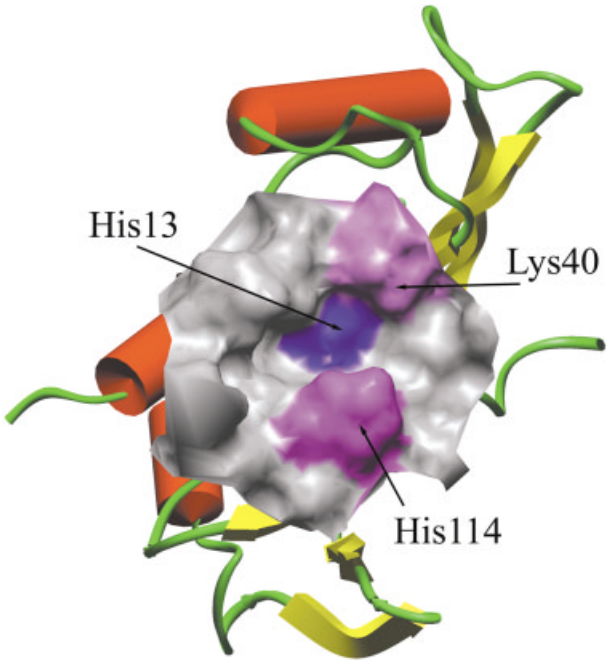

Fig. 1. Schematic representation of the three-dimensional structure of ANG showing the surfaces of residues encompassed in the area defined for docking. The catalytic residues involved in substrate phosphodiester cleavage are labeled and colored differently for visual distinction.

positional refinement (1000 steps), giving a total of 21,000 energy calculations per database member. The average CPU time for docking a single ligand in this manner was 11.4 s.

Docking poses were then scored with the Ludi empirical energy scoring function.[62] In this program, the estimated free energy of binding for a given ligand represents the sum of individual free energy contributions from several variables: loss of overall translational and rotational degrees of freedom, hydrogen bonds, ionic interactions, lipophilic interactions, and a rotatable bond penalty. The ligand scores and variable parameter values were imported into Sigma Plot 2000 (SPSS Science) for ranking and statistical evaluation. For analysis of the relationship between Ludi parameters and Ludi score, correlation coefficients (Microsoft Excel) were derived from the covariance of the Ludi scores and variable parameter values. Correlation coefficients range from −1.0 to 1.0, denoting complete negative and positive correlation, respectively.

GOLD 1.2 Virtual Screen

A second VS study was performed by using GOLD v.1.2[63] (Cambridge Crystallographic Data Centre). All atom types and charges were assigned in GOLD. To generate the full range of ligand conformational sampling and partial protein flexibility, each angle about a rotatable bond is encoded in bytes within a binary string. A genetic algorithm (GA) is then used to "evolve" the optimal ligand geometry and binding position in the active site. Five independent docking runs were performed for each ligand, and the top scoring poses were used for ranking in the library set. All parameters were taken from the default settings for library screening. Early termination of ligand docking was permitted when the top three solutions were within 1.5 Å root-mean-square deviation (RMSD) of one another. Approximately 15 s of CPU time were devoted to each ligand for docking. The GOLD force field-based fitness score was used in conjunction with GOLD dockings; this approach has been shown to perform strongly relative to other docking/scoring combinations.[24,35]

Previous VS studies have shown that scoring functions are often more important than the docking programs themselves for accurate ranking of ligands.[25] Most, if not all, scoring methods exhibit some bias in favor of ligands with specific chemical components (e.g., aromatic rings or hydrogen bond donors and acceptors) or properties (e.g., larger molecular size), often deriving from the protein-ligand complexes used for calibration.[64] Ludi and GOLD would seem to be appropriate choices for the present study on ANG. Ludi has a well-developed treatment of hydrogen bond scoring,[62,65] and GOLD is known to favor hydrophilic ligands.[63] The interaction of ANG with RNA substrates is expected to be largely dominated by hydrogen bonds of active site residues with nucleotide phosphates and bases,[51] and it is likely that many of these same types of interactions will be used by small-molecule inhibitors for binding.

Evaluation of HTS/VS Scoring

For any scoring scheme, the "% actives" is defined as $(A_H/A) \times 100\%$, where A_H is the number of true hits in the hit list and A is the number of true hits in the whole library database. The "% yield" is defined as $(A_H/T_H) \times 100\%$, where T_H is the total number of compounds in the hit list. "Hit enrichment" is then defined as $\{(A_H/T_H)/(A/T)\}$, where T is the total number of library compounds under consideration. A "goodness-of-hit" (GH) metric was used to penalize the loss of actives during enrichment.[66] GH is defined as:

$$GH = \{A_H\,(3A + T_H) / 4T_HA\} \times \{1 - ((T_H - A_H) / (T - A))\} \quad (2)$$

For the HTS hit list, GH = 0 by definition (because $T_H = T$ and $A_H = A$). The GH metric adds carefully calibrated weights to two parameters essential for evaluating hit lists: the percentage of actives in the hit list (% yield) and the percentage of the total actives retrieved (% actives); in addition, it contains a coefficient to penalize excessive hit list sizes. GH values range from 0 to 1, where a value of 1.0 signifies a perfect hit list (containing all of the actives and only actives).

For VS consensus schemes applied post-HTS, enrichment is presented relative to the HTS hit list (i.e., T is the number of compounds in the HTS hit list alone). For VS consensus schemes applied pre-HTS, enrichment is presented relative to the total library. Overall, three consensus strategies were examined. (i) The "intersection" score includes HTS hits that are ranked in the top *N*% of *both* VS functions (see Refs. 22,24,25, and 33). (ii) The "inclusive" score selects HTS hits that are ranked in the top *N*% of *either* VS function. (iii) The "mean-rank" score selects

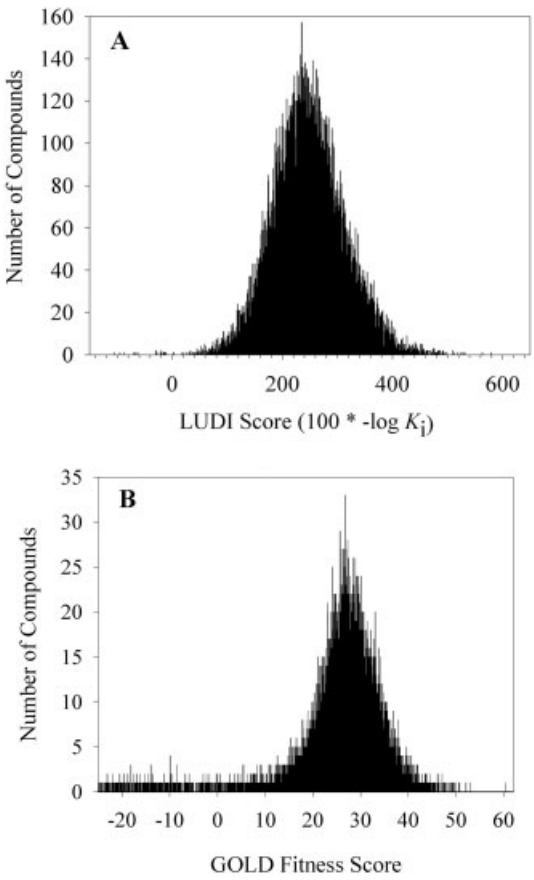

Fig. 2. Frequency of virtual screening ligand scores. **A:** The distribution of scores for the combined NCI and ChemBridge libraries docked with DockVision and scored with Ludi. The Ludi score is equivalent to $(-\log K_D) \times 100$. **B:** The distribution of scores for the combined NCI and ChemBridge libraries docked and scored in Gold v.1.2. The reduced frequency of compounds at any given score [Fig. 2(B) vs 2(A)] reflects an additional decimal place in Gold fitness scores.

compounds in the top *N*% on the basis of their averaged rank from both VS methods.[33]

RESULTS AND DISCUSSION

HTS Hit List and Identification of True Hits

The NCI Diversity Set and the ChemBridge DIVERset E (hereafter combined for analysis) were screened by HTS for compounds that appear to inhibit ANG-catalyzed oligonucleotide cleavage in a fluorescence-based assay.[58] From the combined library set of 18,111 compounds, 178 compounds met the designated criterion for an HTS hit. (The accession numbers for these compounds are provided in the Supplementary Materials.) HTS hits were then tested in an independent assay system that measures ANG-catalyzed degradation of luciferase mRNA. Only 19 compounds were also hits in this assay. These were investigated further in a rigorous HPLC-based oligonucleotide cleavage assay, from which K_D values were determined. We had originally intended to use a K_D value of 100 μM as the cutoff for a true hit, and 10 compounds satisfied this criterion (K_D values 41–85 μM). However, we found that two compounds had only slightly higher K_D values (107 and 131 μM) and that there was a large gap in affinity between these inhibitors and the next best compounds (K_D >200 μM). Therefore, it seemed reasonable to also include them among the true hits for our analysis. Thus, the final HTS hit confirmation rate was 6.7% (12 of 178). (The numbers of HTS hits and true hits given here differ somewhat from those cited in our HTS report[58] because we are now using a slightly higher K_D cutoff and, as discussed under Materials and Methods, are excluding compounds with problematic structures.)

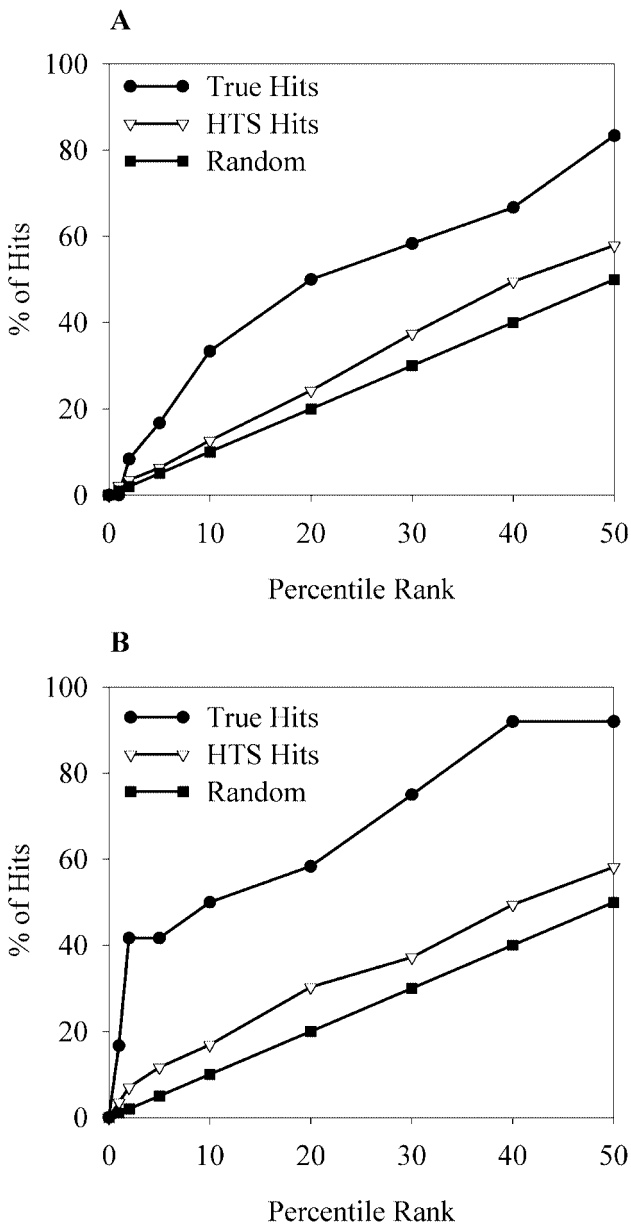

Fig. 3. Predictivity of the docking/scoring functions. The percentages of primary HTS hits and true hits within a given percentile rank of docking scores are shown. Only the top 50th percentile of all docked ligands are displayed. The percentages for random selection are shown for comparison. **A:** DockVision/Ludi virtual screen. **B:** Gold virtual screen.

The follow-up assays used to characterize the HTS hits effectively ensure that all of the true hits identified

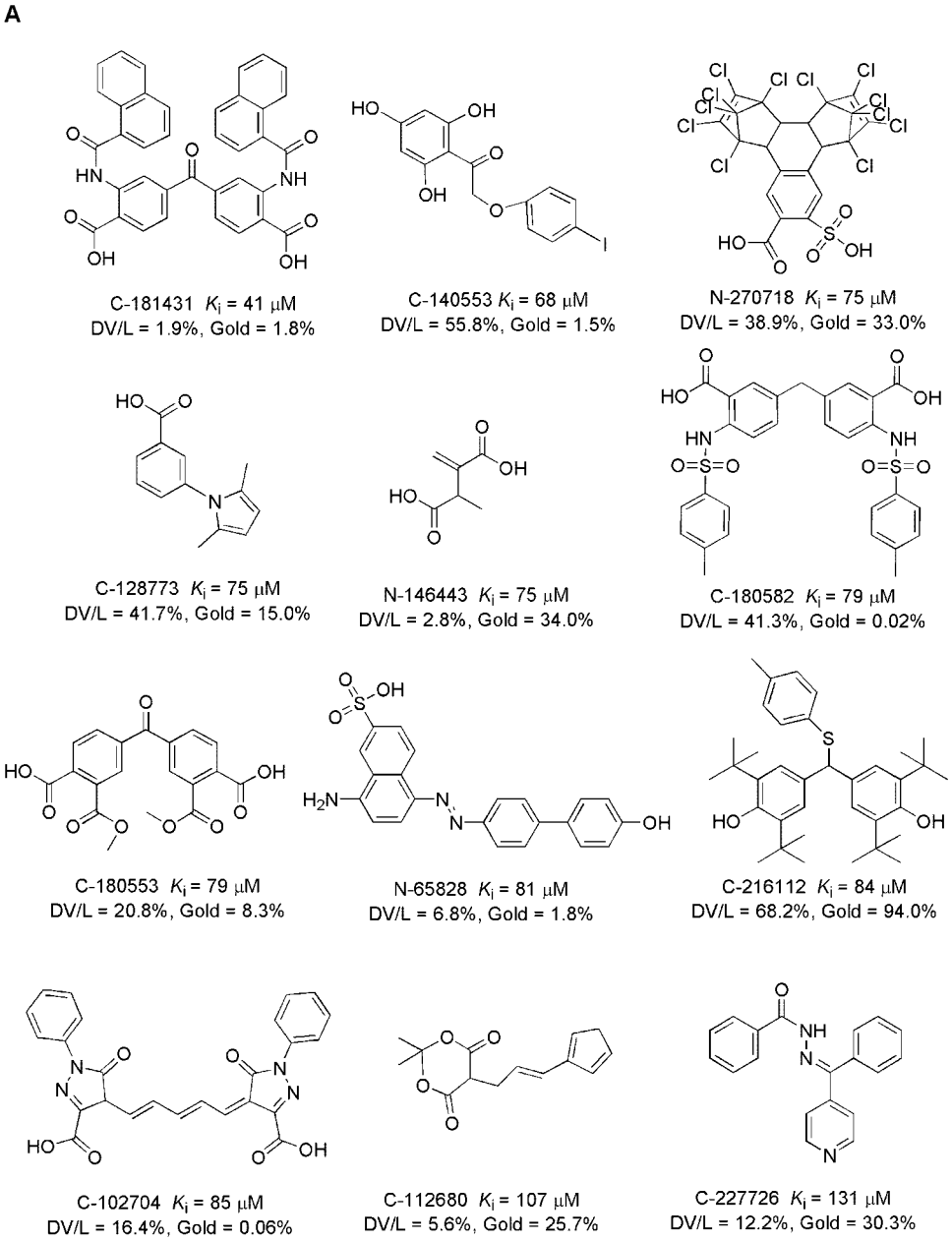

Fig. 4. NCI (N) and ChemBridge (C) library compounds from the HTS hit list and their percentile ranks from DockVision/Ludi and Gold. **A:** All 12 true hits. The mean and median molecular weights of the active compounds are 426 and 403, respectively. **B:** Some false positives that scored in the lower half of all compounds in at least one of the virtual screens.

genuinely inhibit the enzymatic activity of ANG. Moreover, the conditions under which the HPLC assays were conducted should have eliminated any "phony" inhibitors of the type described recently by McGovern et al.,[67] which form large aggregates that inhibit by sequestering or adsorbing the enzyme/receptor target rather than through specific binding. The nonspecific inhibition reported by these authors was observed only at enzyme concentrations [E] that were orders of magnitude lower than that which we used for ANG. For example, with β-lactamase the effect largely disappeared when [E] was increased from 1 to 10 nM, a concentration that is still 500-fold lower than that used for all K_D determinations with ANG. Indeed, the relatively low stoichiometry of inhibitor to ANG (4:1 to 20:1) by itself would seem to exclude the possibility that this phenomenon underlies the measured inhibition. Furthermore, the artifactual inhibition was shown to be reduced substantially when a carrier protein (BSA) was included in the assay at 0.1 mg/mL[67]; our ANG concentration itself (70 μg/mL) is nearly equivalent to this. Finally, we note that the inhibition plots of k_{cat}/K_m versus [I] for all of the present

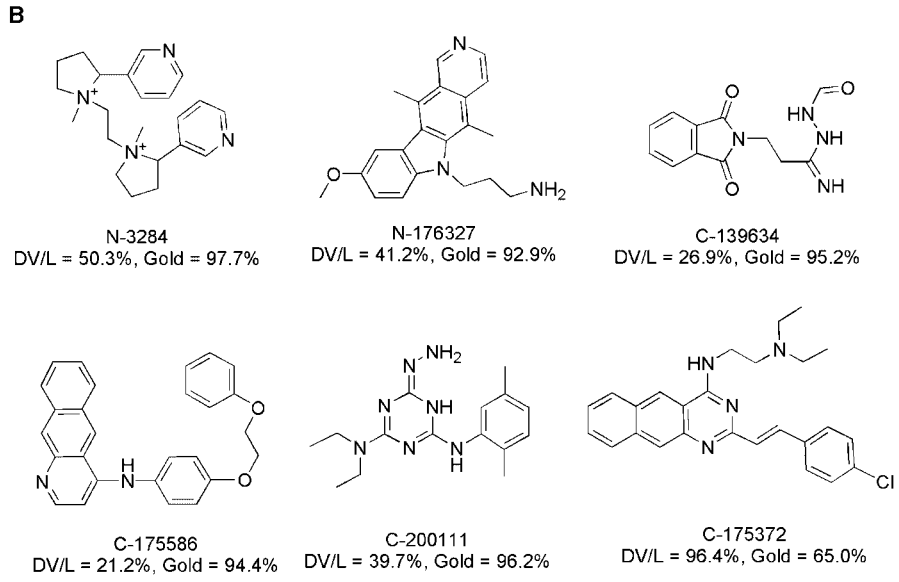

Figure 4. (Continued.)

true hits conform well to the theoretical shape for simple, reversible inhibition (Eq. 1).[58]

Other types of nonspecific factors (e.g., a general attraction of the cationic active site for anionic compounds) also seem unlikely to account for inhibition by the true hits. The active site of ANG is extremely "unsticky," even for molecules such as nucleotides that resemble the natural RNA ligands of ANG and have strong negative charges.[55,56] For example, standard mononucleotides have K_D values > 5 mM, and the most avid nucleotide inhibitor tested to date, 5′-diphosphoadenosine 2′-phosphate, has a K_D of ~500 μM under our HTS assay conditions (see Refs. 55 and 56). Moreover, with four of the true hit compounds (N-65828, C-181431, C-180553, and C-180582) studies with analogs and ANG variants reveal that relatively minor changes in enzyme and/or ligand structure can markedly enhance or reduce inhibitory potency[58] (J.L.J. and R.S., unpublished results). The effects of structural changes are entirely consistent with the model complexes obtained by docking.

Control Assay to Examine the Basis for HTS False Positives

Because a large percentage of the original HTS hit list turned out to be false positives, we undertook a control study to examine the basis for the apparent activity of these compounds during HTS. In general, false positives are expected to arise during fluorimetric or spectrophotometric HTS assays from two primary sources. One of these (delivery errors) is "trivial" in the sense that it can be minimized or avoided by improving the equipment used. Repeating the assay would also expose this type of false positive, although with large numbers of compounds this can be time-consuming and expensive, particularly when using common robotics that are not capable of dispensing selected compounds. The other source of error (compound optical absorbance or insolubility that artifactually reduces fluorescence emission) is "intrinsic" to the assay method and can be eliminated experimentally only through appropriate control assays.[16,17,68,69] The incidence of such intrinsic errors in our HTS was evaluated by plating the NCI Diversity Set compounds in wells containing precleaved fluorescent substrate and then measuring fluorescence as in the original screen. We found that a substantial portion (73%) of the HTS hits determined to be false positives interfered appreciably with fluorescence measurement. None of the true hits had any effect on fluorescence. Thus, control assays of this type can, in principle, provide a powerful means to eradicate false positives. At the same time, there is a definite risk that some true hits (or even entire families of structurally similar true hits) will be eliminated by relying solely on these measurements. Moreover, for larger libraries, control assays (as with replicate assays to identify trivial false positives) can be labor-intensive and can deplete valuable materials. In view of these drawbacks, a purely in silico approach for enriching HTS hit lists by eliminating intrinsic false positives would be extremely advantageous. Consequently, we explored a method for computationally based virtual library screening.

Virtual Library Screening

Database docking was performed with two different programs, DockVision and GOLD, to test the validity of the approach beyond one software's specific performance and to examine the value of consensus scoring with multiple VS methods. Figure 2 shows the frequency of docked ligand scores from each program. The DockVision/Ludi (DV/L) distribution is relatively narrow [Fig. 2(A)]. The DV/L mean score for all compounds was 247, corresponding to a predicted K_D value of 3.4 mM, and the top 10% of compounds scored above 333 (predicted K_D = 468

μM). The GOLD fitness distribution was skewed by a large number of poorly performing ligands compared to DV/L [Fig. 2(B)]. The average fitness of all library members was 25.80, and the top 10% of ligands scored better than 35.80. The GOLD fitness score is not designed for conversion to free energy of binding. In this regard, it should be noted that the purpose of rapid library docking is to prioritize hits rather than to predict binding affinity constants. If desired, more accurate binding affinity estimates may be obtained subsequently by other prediction methods (for a review, see Ref. 64).

The percentile rank of each true hit within the total docked library was examined to judge the accuracy of both VS methods. As shown in Figure 3, both DV/L and GOLD rank the full group of HTS hits only marginally higher than a random sample. This is to be expected because 93% of the HTS hits show no experimental activity. In contrast, the scores for true hit compounds docked onto ANG were consistently higher than for a random sampling, showing the predictive capacity of VS. With DV/L [Fig. 3(A)], 33% of the true hits were ranked in the top 10% of the total docked library, and 58% were ranked in the top quarter. In GOLD, the active compounds were ranked even higher [Fig. 3(B)]. For example, 42% of the true hits were in the top 2% and all but one ranked in the top 38%.

Figure 4(A) shows the chemical structures of all of the true hit compounds, together with the measured K_D values and the percentile ranks in the two VS programs. Figure 4(B) displays some false-positive HTS hits that, like many of the false positives, were ranked in the bottom 50% from one or both virtual screens. Hydrogen bond acceptors and negative charges are prevalent among the true hits. In the modeled complexes for those actives that scored highly in VS, many of the same types of interactions used for nucleotide binding have been found. This is exemplified by the docking pose of ChemBridge compound 181431 from GOLD (Fig. 5). To further explore which ligand parameters influenced docking/scoring, we determined the correlation between all Ludi ligand parameters and the overall Ludi score for every ligand in the DV/L virtual screen (Fig. 6). The parameter with the highest correlation was the penalty for rotatable bonds, followed by the hydrogen bond score. It is important that we found that there is essentially no correlation between the number of atoms in ligands and Ludi scores, even though bias in favor of higher molecular weight ligands has been observed in other VS studies.[23,24,26] (A comparison of GOLD fitness scores vs molecular weights showed no correlation as well; correlation coefficient = 0.014).

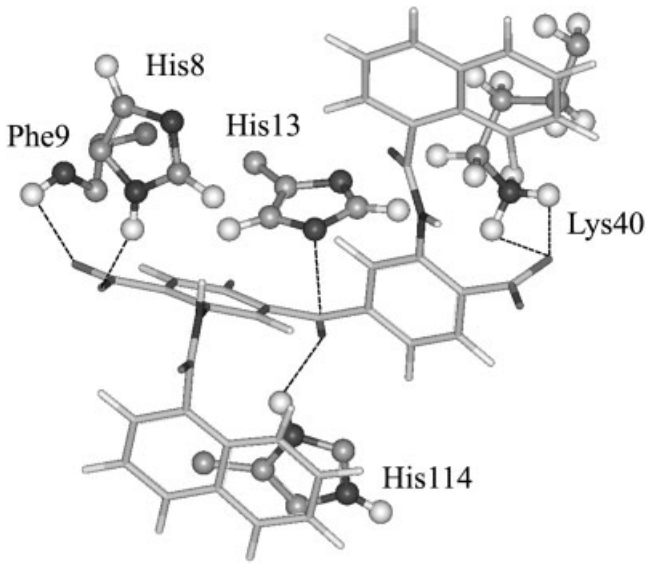

Fig. 5. Docking pose of ChemBridge 181431 predicted by Gold. The ANG catalytic residues His13, Lys40, and His114 are shown.

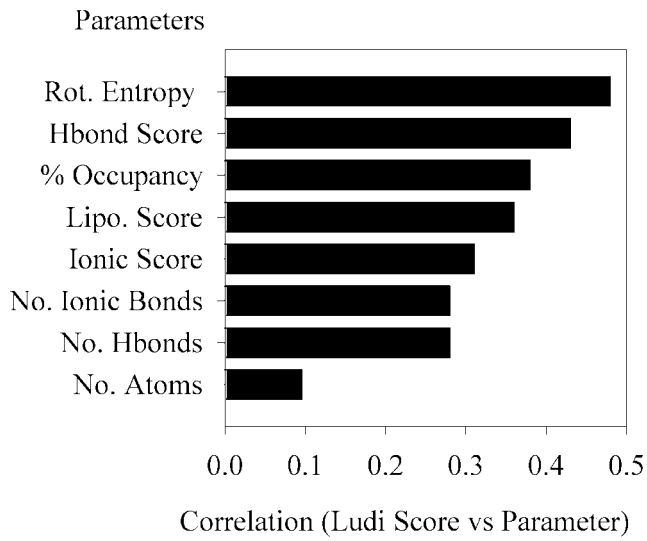

Fig. 6. Correlation of ligand variable parameters with predicted free energy of binding from the DockVision/Ludi virtual screen.

HTS Hit Enrichment

The extent to which VS can enrich the primary HTS hit list is dependent on the hit threshold selected for VS. Threshold setting is often limited by the number of compounds practical for follow-up assays; however, within these constraints it may be possible to adjust the cutoff depending on whether the objective of the screen is to obtain a high number of actives or to obtain few false positives.[33] The hit enrichments at several thresholds for both VS programs were compared (Fig. 7). For GOLD, the optimal cutoff occurs as the VS percentile rank threshold approaches 2%, where GOLD enriches the HTS hit list by 5.7-fold. This scoring scheme decreases the number of false positive hits by 21-fold, from 166 to 8, while identifying 42% of the true hits. Overall, GOLD shows greater enrichment of true hits than DV/L at every cutoff. For DV/L, the 2% threshold was also optimal, but in fact, only one of the true hits was identified at this threshold. These data suggest that a more advanced criterion than enrichment is desirable for evaluating hit lists.

Guner and Henry[66] have described a "goodness-of-hit" (GH) metric that may be used to judge the value of a hit list (see Materials and Methods). The GH metric was used for several HTS/VS schemes to better determine ideal VS

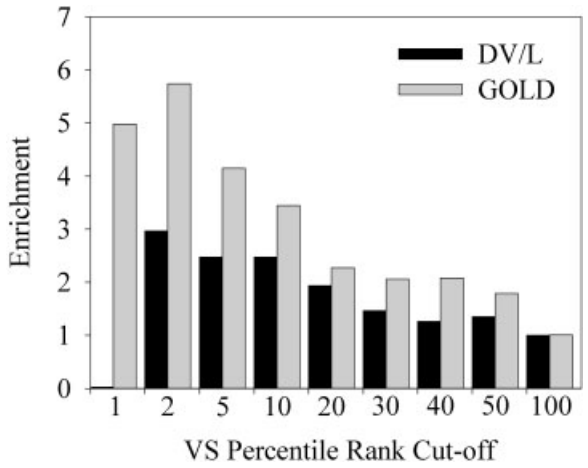

Fig. 7. Hit enrichment at various VS percentile rank cutoffs. Enrichment is defined as the percent yield of active compounds in a hit list (in this case from VS) divided by the percent yield of actives in the entire dataset (in this case from the HTS hit list). Note that the maximum theoretical enrichment for this study is 15-fold, representing a perfect hit rate (100% yield), divided by the HTS percent yield (6.7%). The VS percentile rank cutoff of 100% denotes selection of the entire HTS hit list, where no enrichment occurs by definition.

scoring thresholds (Table I). The scoring schemes are rated relative to the HTS hit list (GH = 0). As expected, GH does not always correlate with the enrichment factor. For example, a DV/L percentile rank cutoff of 10% results in a marginally better hit list than a DV/L cutoff of 2%, although the enrichment values suggest the opposite. All of the HTS/VS scoring schemes involving GOLD result in higher GH values than DV/L. It is striking that the hit list representing the intersection of the HTS hit list with the top ranking 2% of ligands scored in GOLD yields an impressive GH value of 0.37.

We also tested the possibility that consensus scoring between both VS functions and HTS might yield superior hit rates. Table I shows the results of the three consensus scoring schemes (see Materials and Methods). We found that the intersection of both VS functions at a 25% threshold resulted in the highest enrichment (6.6-fold) and gave the highest GH value (0.40) for any consensus scheme. Overall, 33% (4 of 12) of the total actives could be identified by examining only 5% (9 of 178) of the HTS hits, resulting in 33-fold fewer false positives. If this were applied to an HTS study where 100,000 starting compounds yielded 1000 hits (1% hit rate) of which 66 were actives (6.6% yield), only 50 of the 1000 primary HTS hits would need to be subjected to a final rigorous confirmation assay to find 22 of the active substances. Most of the true hits (8 of 12) could be identified by using the mean-rank score at the 25% cutoff, with an intermediate yield (28% correct). An even higher number of actives (10) were obtained by using the intersection score at a 50% threshold or the mean-rank score at a 33% threshold, in each case screening about a quarter of the hits. Finally, the inclusive strategy resulted in less desirable hit lists than the intersection strategy; although a high percentage of the total actives were typically identified, the inclusion of false positives from either VS method resulted in larger hit lists with lower overall enrichments.

HTS/VS: Practical Applications

Enrichment of HTS by the VS approach presented has greatest application in screening large compound collections against a target with a well-characterized active site or region. No ligand chemical descriptors beyond three-dimensional structure are necessary. As noted, there is a certain degree of flexibility in improving % actives versus % yield, depending on the aim of the screen. In addition, if a docking/scoring function has already been established for a specific target system, only the actual HTS hits would need to be subjected to VS, as opposed to docking the entire library in parallel; for example, if the HTS hit list had been docked by itself in the current study, GOLD would have placed 58% of the true hits in the top 10%, corresponding to nearly sixfold enrichment. Docking of HTS primary hits exclusively could be performed with an increased number of energy calculations per ligand for improved accuracy because fewer compounds would need to be screened. Alternatively, VS scoring of HTS hits may be used simply as a prioritization method to determine the sequence of HTS hits for testing in follow-up assays.

The potential for hit enrichment by VS is not limited to post-HTS data processing: if applied before HTS, high-throughput docking can be used to identify subsets of HTS libraries likely to contain increased percentages of actives. With advanced robotics, it would then be possible to limit HTS to these smaller numbers of test compounds. Although this general approach has been described previously,[36] we are not aware of any published studies in which its value has been assessed experimentally. Therefore, we evaluated the utility of several of the GOLD and/or DV/L scoring schemes for enriching our original HTS libraries (Table II). The greatest enrichment, 21-fold, was achieved with GOLD by itself at the 2% cutoff (5 actives out of 362 compounds). Although enrichments were lower at less stringent thresholds, some of the consensus schemes yielded a much greater number of actives while still reducing library size to a useful, if less substantial, degree. For example, the hit list for the mean-rank method at a 33% cutoff contained 10 of the 12 true hits and was one fourth the size of the HTS library.

Potential Impact of Target Flexibility on the HTS/VS Method

Although considerable progress toward inclusion of protein flexibility in VS docking programs has been made recently,[20,70–74] the methods currently in use still have limited capability in this area. One of the programs used in our study, GOLD, allows only for movement of the terminal (nonhydrogen) atom of side-chains, whereas the other, DockVision, keeps the protein structure completely fixed. Obviously, ligands that require a significant change in target conformation for binding may not be docked accurately by these methods and, therefore, may be poorly ranked. With ANG, there was some reason for concern in this regard. The crystal structure of free ANG reveals that

TABLE I. Consensus Scoring of HTS and VS at Different Scoring Thresholds

Consensus scheme[a]	% Actives[b]	% Correct[c]	Hit list enrichment[d]	Goodness-of-hit[e]
HTS only	100 (12/12)	6.7 (12/178)	1	0.0
HTS + DV/L 2%	8 (1/12)	20 (1/5)	3.0	0.17
HTS + DV/L 5%	17 (2/12)	17 (2/12)	2.5	0.16
HTS + DV/L 10%	33 (4/12)	17 (4/24)	2.5	0.18
HTS + DV/L 25%	58 (7/12)	12 (7/58)	1.8	0.16
HTS + GOLD 2%	42 (5/12)	38 (5/13)	5.7	0.37
HTS + GOLD 5%	42 (5/12)	28 (5/18)	4.1	0.29
HTS + GOLD 10%	50 (6/12)	23 (6/26)	3.4	0.26
HTS + GOLD 25%	58 (7/12)	13 (7/54)	1.9	0.17
HTS + DV/L-GOLD Intersection 25%	33 (4/12)	44 (4/9)	6.6	0.40
HTS + DV/L-GOLD Intersection 50%	83 (10/12)	22 (10/46)	3.2	0.29
HTS + DV/L-GOLD Inclusive 5%	50 (6/12)	21 (6/28)	3.2	0.25
HTS + DV/L-GOLD Inclusive 10%	67 (8/12)	19 (8/43)	2.8	0.24
HTS + DV/L-GOLD Inclusive 25%	83 (10/12)	10 (10/103)	1.4	0.10
HTS + DV/L-GOLD Mean-Rank 25%	67 (8/12)	28 (8/29)	4.2	0.33
HTS + DV/L-GOLD Mean-Rank 33%	83 (10/12)	24 (10/42)	3.6	0.31

[a]See Materials and Methods for consensus scheme definitions.
[b]The number of true hits identified by a consensus scoring scheme divided by the total number of true hits from HTS. Number of true hits identified out of 12 is shown in parenthesis.
[c]The number of true hits divided by the total number of compounds in a hit list. Number of true hits and hit list size are shown in parenthesis.
[d]Hit list enrichment is the % correct divided by the % correct for HTS only scoring (6.7%).
[e]See Materials and Methods.

TABLE II. Pre-HTS Library Enrichment by VS

VS method	% Actives[a]	% Correct[b]	Library enrichment[c]
None	100 (12/12)	0.066 (12/18111)	1
DV/L 10%	33 (4/12)	0.22 (4/1811)	3.3
GOLD 2%	42 (5/12)	1.4 (5/362)	21
DV/L-GOLD Intersection 10%	17 (2/12)	0.76 (2/262)	11.6
DV/L-GOLD Intersection 25%	33 (4/12)	0.28 (4/1407)	4.2
DV/L-GOLD Intersection 50%	83 (10/12)	0.20 (10/4894)	3.1
DV/L-GOLD Inclusive 5%	50 (6/12)	0.35 (6/1734)	5.2
DV/L-GOLD Inclusive 10%	67 (8/12)	0.24 (8/3368)	3.6
DV/L-GOLD Inclusive 25%	83 (10/12)	0.13 (10/7671)	2.0
DV/L-GOLD Mean-Rank 10%	25 (3/12)	0.59 (3/505)	9.0
DV/L-GOLD Mean-Rank 25%	67 (8/12)	0.31 (8/2596)	4.7
DV/L-GOLD Mean-Rank 33%	83 (10/12)	0.24 (10/4175)	3.6

[a]The number of true hits identified by a VS method divided by the total number of true hits in the HTS library.
[b]The number of true hits divided by the number of compounds remaining after VS.
[c]The % correct divided by the % of true hits in the whole library.

the subsite for binding the central pyrimidine moiety of RNA substrates is completely obstructed by the residue Gln117,[50] and evidence from modeling and mutagenesis indicates that a major reorientation of the C-terminal segment 117–123 to open this site is required for ANG to bind and cleave RNA.[51,75] It is conceivable that some of the active inhibitors obtained by HTS also induce or require this conformational change. These would then be expected to perform poorly during VS, thereby constituting VS false negatives. However, several considerations, taken together, suggest that the vast majority of the present inhibitors bind to the closed (native) rather than the open conformation of ANG. First, 11 of the 12 true hits were ranked by GOLD in the top 50% of compounds (9 were in the top 30%), and 10 were ranked by DV/L in the top 50%. Given the extensive nature of the predicted rearrangement in ANG to open the pyrimidine site, it seems unlikely that compounds binding to the open conformation would have docked this favorably to the native structure. Second, no structural reorganization occurs in the complexes of ANG with phosphate and pyrophosphate,[60] indicating that binding at the catalytic site (which most of the true hits also occupy in the docked complexes) does not induce the conformational change. Third, binding to the open conformation would involve an entropic energy penalty; in RNA substrates (which bind extremely weakly[75]), this is driven by the catalytic reaction, but for nonsubstrates it would appear to be entirely disadvantageous. Fourth, there is precedent for small-molecule inhibitors binding to native enzyme structures in systems where substrate binding involves a change in conformation. For example, binding of the HIV protease inhibitor UCSF8 (a modified

form of a compound identified by VS[76]), does not require the movement of the enzyme flap that normally occurs on binding of peptide-based substrate analogs.[77] Finally, we note that if, despite the preceding observations, some of the true hits were eliminated by VS because of an inappropriate (i.e., native) target structure, this would suggest that the use of a consensus HTS/VS strategy might be even more effective with a less mobile target.

CONCLUSIONS

We have examined a method for improving HTS hit rates by scoring hits in consensus with VS of the corresponding three-dimensional chemical databases. The rationale for the method was that false-positive hits are not likely to dock well in the target active site compared to true actives, and thus could be eliminated computationally with minimal time and expense. This hypothesis was tested in a case study using the enzyme angiogenin, which has been screened for small-molecule inhibitors by HTS. Using DockVision/Ludi and GOLD as two independent docking and scoring methods, we found that active compounds with midmicromolar K_D values ranked highly overall within the total docked data set, as well as within the full group of HTS primary hits. VS could predict active compounds with varying efficacy depending on the program and scoring cutoff applied. When VS with GOLD was used in consensus with the HTS hit list, the hit rate could be enriched by nearly sixfold in the optimal scheme; lower, but still potentially useful, enrichments were achieved with DockVision/Ludi. We also examined the consensus hit lists with a more stringent statistical method, the "goodness-of-hit." By this criterion, we again found that GOLD yielded more productive hit lists than DockVision/Ludi. Combined use of both VS methods in consensus with HTS resulted in 6.6-fold enrichment of the HTS hit rate, with dramatic reduction in the number of false positives. It is possible that for a different target system, DockVision, Ludi, GOLD, or a combination of them may perform more accurately, and consensus HTS/VS scoring will be even more productive.

Although midmicromolar affinity hits are often obtained in primary screens, larger-scale HTS with more avid-binding enzymes frequently produce leads with higher binding affinity. It is reasonable to assume that in these cases the consensus HTS/VS strategy would be even more effective, because VS accuracy improves for tighter binders.[22] However, it should be noted that important information may be gained from examining lower-affinity compounds. For example, novel binding motifs or pharmacologically useful frameworks can be determined, even from small chemical fragments.[78,79]

We also examined the use of VS as a tool to enrich whole libraries before performing HTS. One VS scheme (GOLD 2%) retained 42% of the true hits while reducing the number of compounds needed for screening by 50-fold. However, the optimal schemes required to keep substantial majorities of actives, involving consensus between both VS methods, resulted in only approximately fourfold decreases in library size. Consequently, in our case study, the VS consensus methods may be less valuable as pre-HTS filters than as tools for minimizing false positives after HTS.

At the present time, the hit rates obtained here by HTS/VS of a "real-life" library (i.e., one that has not been supplemented with known high-affinity binders) would be difficult to achieve by VS alone or even with consensus scoring between multiple VS functions. Only a few other methods for enriching HTS hit rates have been reported. One of these improved primary hit rates by nonparametric recursive partitioning of the test set, based on distributions of chemical and biological descriptors in a training set.[12] This procedure generally produced hit lists with higher % actives, but lower % yields than the method we presented, and the test set under consideration was ultimately enriched by approximately fourfold. In contrast to docking, partitioning methods can require a large number of chemical descriptors to be generated for each ligand, and, importantly, only incorporate indirect information about the target itself during hit selection. Furthermore, in the case of a new protein target, established inhibitors will not be available to train the partitioning algorithm.

Overall, our findings show that VS can be a highly effective and facile tool for enriching the hit rate from high-throughput screens, whether conducted as a precursor to HTS or as a means to weed out false positives afterward. HTS/VS methods will be increasingly beneficial as structural proteomics yields new three-dimensional target structures and libraries for HTS continue to expand.

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