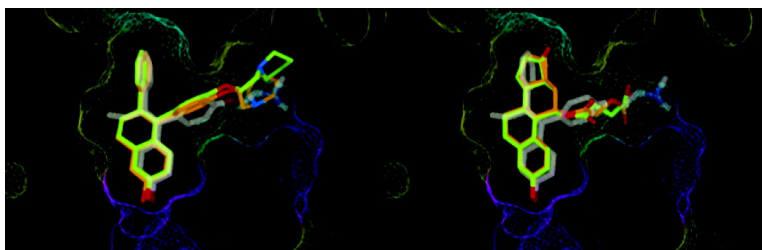


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Fast Structure-Based Virtual Ligand Screening Combining FRED, DOCK, and Surflex

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A protocol was devised in which FRED, DOCK, and Surflex were combined in a multistep virtual ligand screening (VLS) procedure to screen the pocket of four different proteins. One goal was to evaluate the impact of chaining “freely available packages to academic users” on docking/scoring accuracy and CPU time consumption. A bank of 65 660 compounds including 49 known actives was generated. Our procedure is successful because docking/scoring parameters are tuned according to the nature of the binding pocket and because a shape-based filtering tool is applied prior to flexible docking. The obtained enrichment factors are in line with those reported in recent studies. We suggest that consensus docking/scoring could be valuable to some drug discovery projects. The present protocol could process the entire bank for one receptor in less than a week on one processor, suggesting that VLS experiments could be performed even without large computer resources.

Introduction

Many research scientists are under ever increasing pressure to identify new therapeutic compounds. Drug discovery has traditionally made progress by a combination of random screening and rational design with the help of experimental high-throughput strategies, protein crystallography, NMR, combinatorial chemistry, molecular modeling, etc. Yet the process is not trivial. Escalating costs, shorter timelines, and increasing number of targets, among many others, highlight the need of using *in silico* tools. Structure-based virtual screening methods have been developed to assist the drug discovery process and have been shown to provide valuable information in numerous cases.^{1–8} While it is known that experimental high-throughput screening⁹ is cost intensive (yet sometimes the only way to initiate chemistry programs) and eventually out of reach to many academic laboratories, it is interesting to note that the cost of computer approaches such as docking-scoring approaches (licenses of modeling programs, Linux/Unix farms, computer recycling) is also significant. If it is important to reduce experimental cost, then it should be also important to reduce biocomputing cost by developing faster methods.^{10,11} Along this line of reasoning, a key question arises: is it possible to get reasonable success in the screening of one receptor pocket with a relatively large bank using one workstation and “freely available packages to academic users” in less than a week?

Several studies have been designed to address some of the questions above (i.e., evaluation of docking algorithms, scoring functions). For example, several reports focusing on comparing docking/scoring methods,^{12–21} on reproducing X-ray poses, on the screening of small

libraries, or on tailoring docking/scoring parameters with possible benefit of consensus scoring, have been published but very few have tested the advantages of combining methods on speed and accuracy.²² It was underlined that independent benchmarks are definitively important for assessing the performance of the various docking methods,²³ but clearly it is very challenging to compare and contrast effectiveness of virtual ligand screening (VLS) methods as the way of analyzing the results by different research groups differ. For instance, it has been shown that VLS results can be significantly influenced by the composition of the compound library (e.g., small compound collections could give highly misleading results).²⁴ VLS validation typically involves pulling known actives out of a random library, but if the random compounds are, on the average, much smaller than the actives, then the method will appear very efficient while indeed such test is of limited value.²⁴ Nevertheless and despite ongoing debates, some VLS packages have been reported to perform better (Glide, GOLD, Surflex, ICM, LigandFit) than others (Slide, DOCK, FlexX, FRED).^{12–14} However, a more reasonable statement would be that all applications have weaknesses and strengths and that their accuracy tends to be depending on the way programs are run. Furthermore, it is also of importance to evaluate speed during validation of VLS packages as some tools are too slow and not suitable for large database screening.

There are many issues regarding VLS methods, but in line with the questions above and considering the fact that several reports have assessed one VLS docking package in conjunction with one or several scoring functions, we decided to study in this article if a combination of VLS programs could perform well with regard to speed and accuracy. To this aim, we combined three VLS methods, FRED,²⁵ DOCK,²⁶ and Surflex.²⁷ These tools have been recently reported or are well established in the field of drug design and are presently

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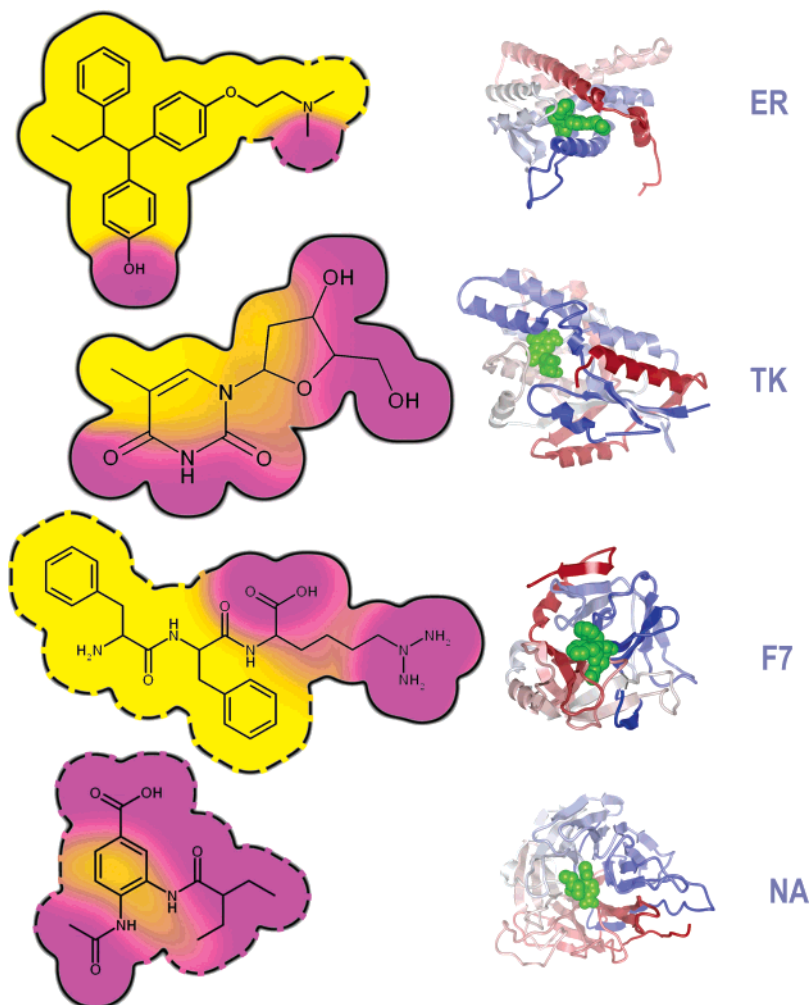


Figure 1. Nature of the binding site of the selected targets. Left: the pockets of ER, TK, F7, and NA are shown. Dashed lines indicate regions of the pocket that are accessible to the solvent while solid lines indicate buried regions of the binding site. The pockets can be essentially buried and nonpolar (yellow) like ER or exposed and polar (magenta) like NA. Right: The overall fold of each protein is shown as ribbons, colored from the N-terminal (blue) to the C-terminal (red). The cocrystallized compounds are shown as green CPK spheres.

freely available to academic users. VLS was performed on four different proteins (different folds and binding site characteristics) with a relatively large compound library (65 660 unique drug-like compounds). Our data indicates that it is possible to complete our multistep VLS protocol on one target pocket in less than a week on one workstation with reasonable accuracy.

Results and Discussion

In the present article, we aim at defining a multistep protocol that allows fast and efficient structure-based VLS studies, even with limited computer resources. To be closer to a real-life virtual screening experiment, we decided to use a relatively large compound collection to run the tests, four different protein targets and a total of 49 actives (Figure 1, Table 1). We selected three VLS packages: a fast rigid-body docking approach implemented in the program FRED and two (pseudo)flexible ligand docking tools, as implemented in DOCK and Surflex (Figure 2). FRED is known to be extremely fast when used in its rigid-docking mode,^{12,15} DOCK has already been successfully applied on many protein targets,^{26,28} and Surflex has also been shown to be very efficient on numerous protein receptors.^{12,27}

Table 1. The 49 Active Compounds

protein	no. of heavy atoms	molecular weight	no. of rotatable bonds	K_i
ER (10 actives)	29 to 45	390 to 458	7 to 15	~ 1 nM
TK (10 actives)	13 to 21	186 to 369	4 to 7	~ 200 to 1 μ M
F7 (19 actives)	25 to 33	339 to 494	9 to 12	30 to 0.02 μ M
NA (10 actives)	17 to 25	237 to 350	4 to 10	~25 μ M to 0.7 nM

Binding Mode Accuracy. We first investigated how each program (FRED, DOCK, Surflex) included in our VLS protocol performed with regard to positioning known actives into the binding site of the target proteins, ER (PDB code 3ert), TK (PDB code 1kim), F7 (PDB code 1dva), and NA (PDB code 1b9s). It is important to note here that this exercise is not equivalent to the so-called bound docking problem that attempts to reproduce the structure of a complex starting from the crystallographic coordinates of a receptor and its ligand. Rather, the 3D structures of the ligands were generated in silico and were thus not extracted from the PDB files.

All these four targets possess binding sites with different degrees of burial and polarity. The docked poses of each known inhibitor were visually inspected

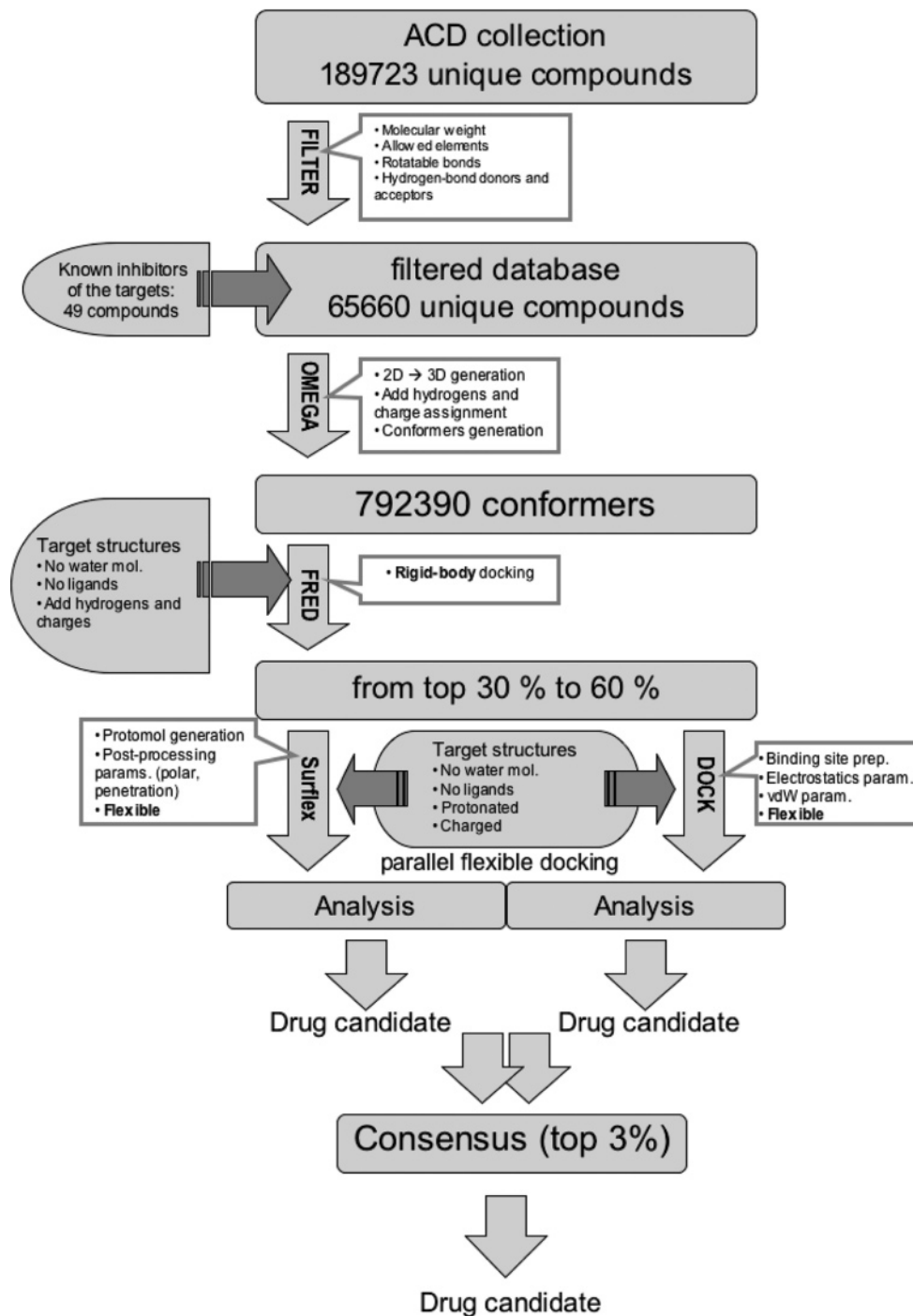


Figure 2. Flowchart of our funnel-like multistep VLS protocol. Grey boxes show quantity of compounds/conformers in each of the steps. Grey arrows indicate programs or procedures applied to the entry compound collection (upstream) and the resulting database (downstream). Grey ovals represent additional input information needed for different steps in conjunction with the compounds to be processed (dark grey arrows). Some characteristics about the different programs are also listed.

and subjectively classified, in a manner similar to a recent report.¹³ The results for docking accuracy of each program used in our VLS protocol are presented in Table 2 and examples of ‘close’ (i.e., highly similar to the X-ray structure, RMSD $\sim < 2$ Å) or ‘binding site’ (good orientation in the binding pocket, ~ 2 Å $<$ RMSD < 2.6 Å) docking solutions are shown in Figure 3 together with the crystallographic solution (inaccurate poses RMSD $\sim > 2.6$ Å). The numbers of acceptable poses (“close” and “binding site” solutions) with corresponding inhibitors in correct orientations in their

respective active site are as follows: Surfex: 34, Dock: 31, FRED: 22, among the 49 known actives.

On average and in our hands (tuned protocol, see Methods), Surfex was the most successful with regard to docking accuracy. The advantage of Surfex’s algorithm for the docking step seems to be due in part to an efficient positioning of the fragment/ligand into the binding site (see comments below, DOCK and Surfex comparison). It was previously shown that the accuracy of docking experiments decreases with the number of rotatable bonds of the ligand.^{22,29–31} We therefore

Table 2. Docking Accuracy

receptor	close				binding site				inaccurate				n/a		
	FRED	SF	D25	D50	FRED	SF	D25	D50	FRED	SF	D25	D50	FRED	SF	DOCK
ER	6	7	3	3	-	3	-	-	4	-	3	3	-	-	4
TK	2	4	4	5	4	3	1	3	4	1	4	1	-	2	1
F7	1	7	12	11	5	4	1	4	13	6	6	4	-	2	-
NA*	1	6	1	1	3	1	2	4	6	3	4	2	-	-	3
total	10	24	20	20	12	11	4	11	19	10	15	10	-	4	8

^a n/a, not available: compounds could not be fitted into the binding pocket/discarded by the scoring function; close: the compounds were positioned in the binding pocket like the X-ray structure; binding site: the compounds were positioned acceptably in the binding pocket; inaccurate: the compounds were positioned upside down compared to the X-ray structure. *For NA the values D25/D50 correspond to respectively 50/75 configurations per cycle (see text). SF: Surflex. D25: DOCK, 25 configurations per cycle. D50: DOCK, 50 configurations per cycle.

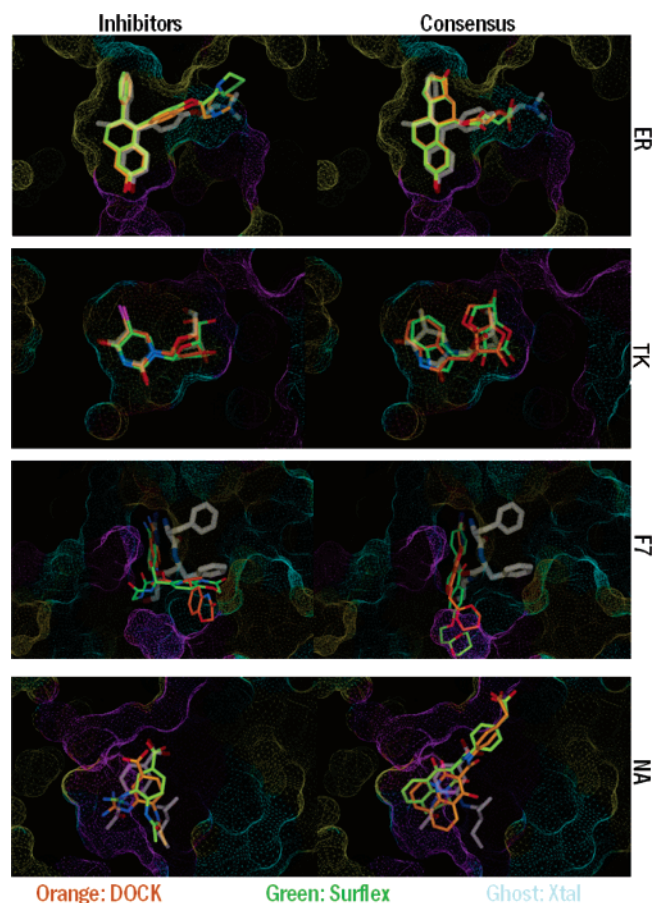


Figure 3. Docking poses. The pockets are shown as vdW surface wireframes, colored according to the residues properties: nonpolar (yellow), polar (cyan), and charged (magenta). The cocrystallized compounds are shown as semitransparent (ghost) sticks, with white carbon atoms. The docking solutions derived from the programs DOCK and Surflex are shown as solid sticks, with carbon atoms shown in orange (DOCK) or green (Surflex). Left: known inhibitors docked into the respective binding site of different proteins. Nafoxidene (ER); idu1 (TK); 2-[3-(4-carbamimidoyl-phenyl)-ureido]-N-[1-(3-oxo-3,4-dihydro-2H-benzo[1,4]oxazin-6-yl)-ethyl]acetamide (F7) and bana113 (NA). Right: promising consensus solutions (among the top 10) docked into the binding sites. 1,3,5(10)-estratrien-3,11- α -diol-17-one 11- α -hemisuccinate (ER), ascorbigen (TK), 7-O-[2-(1,3-dioxanyl) ethyl]-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (F7), and [4-[(1-hydroxy-3-oxo-6,7-dihydro-3H,5H-pyrido[3,2,1-*ij*]quinoline-2-carbonyl)amino]phenyl]acetic acid (NA).

decided to carry out DOCK flexible docking with 25 and 50 conformations per cycle in an attempt to improve its performance. In the most difficult case, NA (with a highly exposed binding site and micro/nanomolar

inhibitors³²⁻³⁷), the numbers of conformations explored were 50 and 75, and this indeed gave better results (Table 2) while increasing the number of conformations further did not really change the results significantly (data not shown).

We did not observe a clear correlation between the docking accuracy and hydrophobicity/solvent-accessibility of the binding site. For example, F7 presents a mixed environment (inhibitors' activity ranging from 0.02 to 30 μ M, as described³⁸) and TK's site is totally closed, yet both programs, Surflex and DOCK, showed good performances.

DOCK was not very successful for ER. The binding site of this target has an extensive and nearly closed hydrophobic pocket. The only opening is due to the presence of an antagonist on this structure (3ert) that impedes the complete closure of the pocket by a nearby α -helix. In our docking experiments we used the same 10 ER antagonists (low-nanomolar affinity) as Bissantz et al.²⁰ and Halgren et al.³⁹ When we employed the standard AMBER force field implemented in DOCK, we could verify that the volume of the buried portion of the binding pocket (PDB entry 3ert) is smaller than five out of ten docked inhibitors. Thus, although we used soft vdW potentials to partially overcome clashes, several ER inhibitors could not fit into the binding site. Therefore, as was proposed in ref 39, the nonbonded radii could be scaled down to allow the known binders to dock correctly. For such pockets, additional structural modeling could be carried out in order to examine different conformations of the pocket, via, for example, the use of different X-ray structures of the same protein or MD simulations. This problem is partially circumvented in Surflex, as some clashes between the ligand and the receptor atoms are allowed to a certain degree.

FRED, due to its rigid-body approach, presented the worst accuracy among the tested programs (results shown for FRED were obtained using default parameters). Only the shape complementarity (contact energy term) was taken into account, which explains the number of incorrect poses for F7 where the influence of a salt-bridge between the protease D189 residue and the ligands is not taken into consideration. More accurate docking solutions may be achieved if additional optimization steps (e.g., OH group rotation, torsion, and solid body minimization) available in FRED are included in the docking calculations as shown in ref 15. It should be emphasized that in our study, FRED was used for a rapid weeding of the compounds that did not fit well into the pockets. FRED was chosen since it is

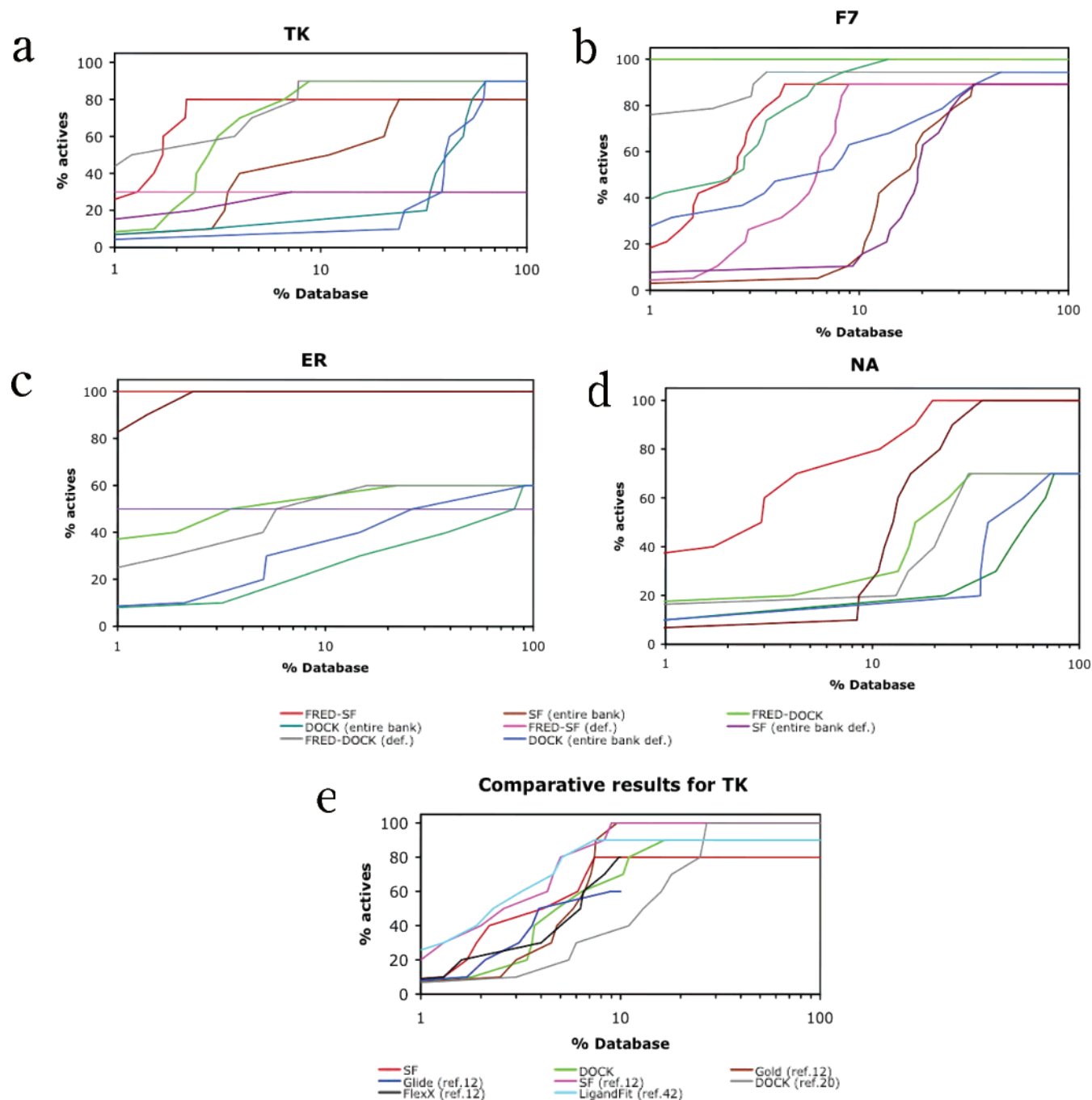


Figure 4. Enrichment graphs for the four protein targets and comparison of enrichment achieved in different studies for TK. (a–d) Results are shown for our protocol with FRED-Surflex (red), FRED-DOCK (green), default parameters FRED-Surflex (magenta), default parameters FRED-DOCK (grey), starting with 65 660 compounds: Surflex (our parameters, brown), Surflex (default parameters, purple), DOCK (our parameters, dark green), DOCK (default parameters, blue). 100% refers to 65 660 screened compounds. (a) TK; (b) F7; (c) ER; (d) NA. In part b (F7), FRED-DOCK (our parameters) retrieved all actives within the top 1%. In this figure, the purple/magenta line corresponds to the superimposition of results obtained from FRED-Surflex (default parameters) and Surflex (65 660, default parameters) computations. In part d, FRED-Surflex (default parameters) and Surflex (65 660, default parameters) did not retrieve any active compound. (e) Our results are shown for Surflex (red), DOCK (green). Data taken from the literature DOCK (grey),²⁰ LigandFit/Ligscore (light blue),⁴² Surflex (magenta),¹² FlexX (black),¹² Glide (dark blue),¹² Gold (brown).¹² A library of 1000 unique compounds was screened.²⁰

extremely fast when compared to other programs performing similar rigid-body fittings.

VLS Protocol Performance. The goal of this work was to propose an efficient procedure for fast VLS by compromising satisfactory ligand screening, speed, and computational cost. Four target proteins were used to validate our proposed VLS multistep protocol. The

efficacy for the targets of the overall VLS protocol is presented in Figure 4. Our data are also compared to screening results evaluated with other VLS methods/protocols (Figure 4e).

The overall reasoning behind our multistep approach is based on previous reports that underlined the value of using sequentially several filtering steps.^{39–41} How-

ever, in addition to these previous reports, we decided to also investigate if consensus docking could be beneficial to a VLS project.

The first step was the application of physicochemical property filters (ADME/Tox, Lipinski's rule-of-five, see Methods) in order to reduce the number of molecules to be docked. This step was followed by a very fast rigid-body docking procedure. The first step was planned in order to rapidly trim-off molecules that have a high probability of failure at a later stage of the drug discovery process while the second step aimed at removing unlikely binders. The reduced compound databases were then submitted to more time-consuming (pseudo)-flexible docking steps, followed by scoring. Two flexible docking algorithms were used. The overall scheme of our protocol is presented in Figure 2.

Fast Rigid Docking. In the first selection step, an ADME/Tox screening tool was used (FILTER), reducing the initial ACD library (189 723 compounds) to a list of 65 660 molecules (including the 49 known actives). From these unique compounds, 792 390 3D conformers were generated by OMEGA, using parameters ensuring satisfactory sampling of the ligands' conformational space. These conformers were then docked as rigid bodies to the four different targets using FRED.

The program DOCK offers an alternative to FRED for the task of rigid-body docking step (data not shown). By constraining the compounds flexibility, DOCK could achieve results similar to those obtained by FRED in the case of F7, albeit five times slower (DOCK clocked 2.5 s per compound as compared to 0.4 s per compound with FRED on the same computer system).

DOCK and Surflex Comparison. Figure 4 (4a–d) gives an overview of database screening efficacy for the second step of our VLS protocol involving flexible ligand docking as implemented in DOCK and Surflex. It should be noted that our VLS experiments were performed on a large compound collection of 65 660 molecules while most comparative studies have been performed with much smaller collections, ranging, in average, from 1000^{20,39,42} to 10000 compounds.^{14,16} It is also important to underline that the size and the composition of the database to be screened may lead to biases on the final docking performance of the tested protocols. This is especially true with smaller subsets of compounds, where the presence of several known inhibitors with similar features tends to favor its docking over the limited representation of the database variability. Thus, we consider that our results could represent a more realistic situation of the database screening process during a real-life drug discovery project.

For evaluation purposes, we report virtual screening results using enrichment graphs for all screened databases/targets (Figure 4). We compare the performance of our protocol with DOCK and Surflex default parameters.

Each of the flexible docking methods – Surflex and DOCK – used in our VLS protocol ranked 44 and 32 respectively out of the 49 known inhibitors in the top 5% of the screened library. We thus retrieved in the top 5%, 90% of the known actives with Surflex and 65% with DOCK. Using Surflex and DOCK with default parameters after FRED, we found 29% and 59%, respectively, active compounds in the top 5%.

With regard to TK, 80% and 70% of the known inhibitors were retrieved in the top 5% of the database by our VLS protocol using Surflex and DOCK, respectively (Figure 4a). Overall, our protocol performed better than default parameters for Surflex. We use less stringent postprocessing parameters that are more permissive to atomic clashes, thereby helping to account implicitly for the lack of flexibility from the receptor side. The importance of considering water-mediated interactions explicitly for accurate docking between ligands and proteins has been pointed out in some cases,⁴⁰ in particular for TK with purine/pyrimidine inhibitors.⁴³ Nevertheless, although no waters were involved in our protocol, the docking was very successful, with a high number of correctly positioned ligands (Table 2).

The comparative analysis of our VLS results for F7 shows that with our approach, Surflex and DOCK could retrieve 90% and 100%, respectively, of the known inhibitors in the top 5% (Figure 4b). Our parameters give significantly better results than default ones because they take into account the polar nature of the binding pocket.

In the case of ER our protocol using Surflex ranked all known inhibitors in the top 5% (Figure 4c). This suggests that the less harsh postprocessing parameters resulted in a better acceptance of the compounds otherwise rejected by default Surflex parameters (Figure 4c). For ER and with our parameters, DOCK retrieved 50% of the active compounds in the top 5% (40% with default parameters). This seems to be due to the fact that several compounds had severe clashes with the receptor and were rejected despite the use of soft vdW potentials.

The results obtained for the last target NA are presented in Figure 4d. With our parameters, we obtained better results than with default values with both programs. Indeed, with Surflex default parameters, no active compounds were ranked. With our parameters, Surflex performed better than DOCK and could rank 70% of the known inhibitors in the top 5% of the database. Apparently the relatively weak performance with DOCK (ranking only 20% of the known inhibitors in the top 5%) (Figure 4d) can be partially attributed to a well-known problem concerning the high scores achieved by larger compounds.

Additional evaluations were carried out on the four protein targets, starting from the 65 660-filtered compound collection (before the FRED step). In all cases, our multistep protocol (FRED-Surflex, FRED-DOCK) performed better than the protocol starting with the 65 660-collection and using flexible docking with DOCK or Surflex, either with default or our pocket-optimized parameters. Filtering the molecules with the OMEGA/FRED rigid-docking step achieved better enrichment after DOCK and Surflex as compared to flexible docking of the entire collection. Analysis of our results indicated that in average, 50% of the compounds selected by FRED were also well scored by Surflex and DOCK after flexible docking (entire databank) due to favorable vdW contacts. For the remaining compounds (well ranked after flexible docking by DOCK or Surflex of the entire databank but not selected after FRED), some showed very favorable electrostatic energy (and low vdW con-

tacts). However, it has been shown that the shape of the pocket and its hydrophobicity are crucial for the binding.⁴⁴

The others molecules are striking because they have bad scores as evaluated by FRED (Gaussian scoring, low shape complementarity) while they were found to have good vdW contacts after flexible docking by DOCK or Surflex. It seems that the pseudoflexible approaches (fragmentation methods) used by DOCK or Surflex succeeded to fit some molecules into the binding pockets with favorable contact scores while they have relatively low shape complementarity as evaluated by FRED. Possibly, errors in handling flexibility of some ligands led to ranking these compounds better than the real actives. It has indeed been noted that difficulties in the treatment of receptor flexibility tend to make the results worse,⁷ and we suggest that such observation also apply to the ligands.

Overall, compounds selected by FRED display favorable vdW contacts. The second step (flexible docking/scoring) selects molecules that have in addition favorable electrostatic energy.

We performed additional investigations in order to allow direct comparison of our results with those achieved by different protocols or methods^{12,20,42} (Figure 4e). This evaluation was performed on TK using the same previously screened databank of 990 molecules plus 10 known actives.²⁰ In our hands and with our parameters, both Surflex and DOCK performed well as they retrieved in the top 5%, 50% of the known actives, similar to Glide or Gold. The best performing methods for TK and this databank seem to be Surflex (results in the present article and the ones reported by Kellenberger et al.,¹² also using a nondefault penetration violation cutoff of -6) and LigandFit.⁴² DOCK with our parameters performed better than previously reported.²⁰

Scoring Functions. Scoring functions are based on molecular mechanics force fields (sometimes including solvation/entropy) or are empirical or knowledge-based.⁴⁵ In most cases, the final score results from the summation of different terms eventually weighted by some factors (e.g., empirical functions). In general, various set of protein–ligand complexes are used to calibrate these scoring functions. As a result of such procedure, it is understandable that there is no universal scoring function that outperforms the others. Such observations suggest that consideration about the overall nature of the binding pocket could help in improving the performance of scoring functions. Although extremely diverse in their properties and features, a simple classification of the pocket can certainly be made based on some properties, such as accessibility, hydrophobicity or polarity. On the basis of a similar binding site classification, Wang et al.⁴⁶ demonstrated that the performance of different scoring functions is influenced by the nature of the protein–ligand interactions. Clearly, scoring functions tuned to a wide variety of binding sites would fail quite frequently, unless some weighting factors or some force field parameters are corrected on a case-by-case basis. We therefore advocate for either the development of docking/scoring functions directed to specific binding sites or tuning of the docking/scoring parameters. This latter method was our approach in the present study. We coarsely changed the postprocessing

Table 3. A Comparison of Enrichment Factors for Surflex (SF), DOCK, and SF-DOCK Consensus in the Top n Compounds^a

	SF	DOCK	SF-DOCK consensus
ER ($n = 291$)	201.0	67.0	89.3
TK ($n = 600$)	21.7	0	54.2
F7 ($n = 449$)	15.2	144.8	121.9
NA ($n = 268$)	72.8	24.3	24.3

^a The number n refers to the number of common compounds found in the consensus docked/scored list (see Methods for details).

default parameters of Surflex as well as some parameters in DOCK (see Methods). As shown above, these modifications led to very reasonable results. For instance, the two targets with closed pockets (TK, ER) were allowed to have slightly more clashes than those targets with open pockets. Also, in the presence of extended polar surface within the pocket, we tuned the weight of the factor(s) describing such interaction. This was the case of F7 and NA: both demanded stronger polar interaction to be considered in the scoring function.

Consensus Docking and Scoring. The consensus-scoring scheme has been applied with relative success recently, as described in several reports.^{21,47} However, opinions diverge²⁴ and it should be pointed out that most studies using consensus scoring are performed with different scoring functions but only one docking method. If a compound is wrongly positioned in the binding pocket, several scoring functions will be applied on the same badly docked ligand, while, it is known that no one docking/scoring method currently performs consistently well across different protein targets. One possible alternative could be to use different docking methods, followed by scoring each docked set independently and generating a list of common compounds. This approach could give interesting results in the future as mentioned in.⁴⁸ In practice, consensus docking/scoring could have both strengths and weaknesses. The disadvantage could be possible loss of active compounds if poorly docked by one software even when correctly docked by the other program or if one program performs poorly with regard to docking. The benefits of such approach could be a more consistent and less active-site dependent performance across different targets relative to one single docking program and possible reduction of false positives which are less likely than active compounds to be favorably docked by two different packages. Overall, we found reasonable enrichment factors for Surflex-DOCK consensus for all targets but NA (Table 3). Clearly, NA is a difficult case as its binding site is fairly open and both docking and scoring tend to be troublesome. In some ongoing projects in our laboratory for which no active ligands are known, consensus docking/scoring is helping us to reduce the list of compounds to be tested experimentally (unpublished data).

Timing. CPU time is still considered to be a critical parameter in many VLS projects. The timing with our VLS protocol for F7 and TK is reported in Table 4 and compared with values obtained starting from the 65 660 compounds for Surflex and DOCK, with default as well as our parameters.

Our whole protocol starting from an initial database of ~ 189000 unique compounds, on a Linux machine (1.5 Gb RAM, single 2.8 GHz Xeon processor) including:

Table 4. Timing^a

target	total heavy atoms	OMEGA 2D-3D	FRED rigid	SF our param.	DOCK our param.	total VLS protocol	SF def. param.	DOCK def. param.	SF_65K our param.	SF_65K def. param.	DOCK_65K our param.	DOCK_65K def. param.
TK	2304	3	2	84	54	143	96	31	191	212	142	98
F7	1974	3	3	92	60	158	99	30	171	169	143	85
average time per ligand, s						8.3 s			9.9 s	10.4 s	7.8 s	5.0 s

^a SF: Surflex flexible docking/scoring starting from the filtered database by FRED and FILTER. DOCK: DOCK flexible docking/scoring starting from the filtered database by FRED and FILTER. SF_65K: Surflex flexible docking/scoring starting from the 65 660-compound collection. DOCK_65K: DOCK flexible docking/scoring starting from the 65 660-compound collection.

filtering, generation of the 3D conformers database (OMEGA), FRED, Surflex and DOCK timed approximately 1 week for F7 or TK.

The docking time per compound with our VLS procedure was 8.3 s on average, thus apparently faster than the HierVLS hierarchical docking protocol reported in⁴⁰ taking in average 4.3 min per compound (calculations performed on a single 866 MHz Pentium III Linux PC). Our protocol seems also faster than the Glide approach,³⁹ where the authors reported good docking performance achieved with a subminute timing per compound (Athlon MP1800+ Linux).

In our case, two main steps allowed a fast reduction in the number of molecules to be screened by the flexible docking methods: the initial FILTER step and the subsequent rigid body docking step. In the first step, properties filtering decreased dramatically the number of compounds to be passed to the next step, trimming off approximately 65% of the initial database within an extremely short period of time (~6 min). The following rigid-body step (FRED) took about 3 h.

The next flexible docking step was executed with two different methods. The timing is similar for each algorithm using the settings described in the methodology section: about 90 h for Surflex and about 55 h for DOCK. This latter timing for DOCK is two times higher than with default parameters due to the use of 1000 orientations and 0.5 distance tolerance for the orientational search.

Our complete VLS protocol, involving rigid body docking and two flexible methods, can be run in less than a week, which is comparable to performing flexible computations with Surflex or DOCK with our parameters starting from the filtered 65 660 compound collection. Moreover, our complete VLS protocol gives better enrichments compared to running Surflex or DOCK alone (Figure 4a-d).

Final Comments and Conclusions

In the present paper we proposed a multistep VLS protocol based on sequential docking/scoring steps, with the goal of decreasing the time and resources needed for VLS projects without reducing the overall performance. The results are comparable to those previously published, with the desirable aspect of being performed on the screening of a large compound collection. The processing of the putative hits is fast, allowing computations on few workstations and the use of several docking packages in parallel. Several steps can be automated, but skilled human interventions are still necessary in order to obtain high-quality performance. Tuning docking/scoring parameters according to the pocket properties significantly improved the perfor-

mance of both docking methods: Surflex excelled over its already confirmed high-performance while DOCK results were improved severalfold when compared to a previous comprehensive independent benchmarking report. These observations reinforce the idea that tuning the parameters according to the nature of the binding pocket could be more efficient, in practice, than searching for the ideal scoring function (although the functions and the parameters can obviously be improved).

We obtained better results starting from the FRED-filtered bank (thus using multiconformers initially and after a rigid body docking/filtering step) with both DOCK and Surflex than starting with the same programs but with flexible docking and the filtered 65 660-compound collections. Also worth mentioning, we noted that the program DOCK offers an alternative to FRED for the task of rigid body docking. We further suggest that consensus docking/scoring could be valuable to some drug discovery projects.

To sum up, we believe that current docking tools are mature enough for VLS projects and that combination of such tools together with tuning of some parameters can allow for very fast screening of drug-binding pockets even with limited computer resources.

Methods

Target Structures. Four different protein targets were chosen according to availability of known ligands and diversity of active/binding-site properties (Figure 1). The cocrystallized structures of the estrogen receptor (ER) with an antagonist (file 3ert), thymidine kinase (TK) (file 1kim), coagulation factor VIIa (F7) (1dva), and neuraminidase (NA) (1b9s) were retrieved from the PDB⁴⁹ and processed in order to remove the ligands and water molecules. Hydrogen atoms were added to the protein structures with the program ICM.⁵⁰

Structural analysis of the targets was used to define the pocket properties. The pockets (surface and residues) were defined with ICM. The degree of burial was calculated as the fraction of the solvent-accessible surface area of the protein pocket that became buried upon ligand binding. The degree of burial for the four pockets is as follows: TK (very buried pocket, 91.0%) > ER (75.4% buried) > F7 (47.3%) > NA (very open pocket, 30.5%) (Figure 1). The polarity of the binding pockets was evaluated as the percentage of nonpolar residues present in each cavity (defined following amino acid tables as present at <http://www.russell.embl.de/aas/>): ER, 75% nonpolar; TK, 56%; F7, 47% and NA, 35% (thus very polar).

Compound Library. All compounds with molecular weight between 100 and 1000 were retrieved from the February 2004 release of the Available Chemical Directory (ACD) database (MDL Information Systems, San Leandro, CA), resulting in 189 723 unique molecules (Figure 2). The bank was then filtered with FILTER v.1.0.2 (OpenEye Scientific Software, <http://www.eyesopen.com>), a molecular screening tool that uses a combination of physical-property calculations and functional-group knowledge to assess libraries and ultimately remove nondrug/lead-like compounds. We used the default "filter-light"

parameters with minor modifications in order to not overreduce the screening library. The main parameters that we decided to use involved: molecular weight (minimum value = 100 Da, maximum value = 1000 Da but with additional constraints in term of min/max number of carbons (min = 5, max = no limit), rings (min = 0, max = 7), rotatable bonds (min = 0, max = 20), allowed elements (H, C, N, O, F, S, P, Cl, Br, I), hydrogen bond donors/acceptors (max = 6/10), sum formal charges (min = -2, max = 2), XlogP (min = -2, max = 6), 2D polar surface area (min = 0, max = 150 Å²), and rejection of about 100 toxic functional groups. The resulting library contained 65 660 compounds in SMILES format (including the 49 known actives, see below).

Concomitantly, known inhibitors for each of the four targets were harvested in the literature and/or databases. The PDB codes listed below were used to extract compounds and assess docking accuracy. Overall we collected 49 of such compounds, 10 for ER (3ert, 1err),^{20,39} 10 for TK²⁰ (1kim, 1ki7, 1ki6, 1e2p, 1e2m, 1e2n, 1e2k, 2ki5, 1ki2, 1ki3), 19 for F7³⁸ (1dva) and 10 for NA^{32–35} (1a4g, 1b9s, 1b9v, 1b9t, 1inv, 1inf, 1vcj, 1liv). When a ligand could not be extracted from the PDB, it was rebuilt from the literature. The main characteristics of these organic ligands are reported in Table 1.

The program OMEGA v.1.1 (<http://www.eyesopen.com>) was used to convert all compounds to 3D multiconformer structures and to add hydrogen atoms/Gasteiger partial charges. For the first docking step, where the method is depending on rigid body shape-fitting (see section 2.3.), multiconformer structures for each compound were indeed required. The algorithm implemented in OMEGA dissects the molecules into fragments, reassembles and regenerates many possible combinations, and then submits each conformer to a simplified energy evaluation (modified Dreiding force field, see <http://www.eyesopen.com>). Then, all conformers below an energy threshold are compared and those within a certain RMS distance are clustered into one single representation. We found that an optimal compromise between speed, number of conformers generated, and structural diversity for our library required changing the parameters associated with the (1) RMSD value (a value below which two conformations are considered to be the same), RMSD was set to 1.0 Å (GP_RMS_CUTOFF; default 0.8 Å), and the (2) energy window (a value used to discard high energy conformations), this parameter was set to 10.0 kcal/mol (GP_ENERGY_WINDOW; default 3.0 kcal/mol), with up to 50 conformers generated per compound. This yielded a total of 792 390 structures with an average of 12 conformations per unique compound. It is important to note that the objective of using these parameters is to allow an acceptable fast rigid-body filtering instead of precisely reproducing the docking conformation and pose. Moreover, the relatively larger energy window parameter that we used was set in order to increase variability of the conformers, since in several cases the conformation adopted by small compounds cocrystallized into an active site can be relatively far from global minimum energy conformations.

Docking Step 1: Rigid Body Shape-Fitting. The docking funneling step was approached with a rigid-body, shape-fitting algorithm implemented in the program FRED v.1.2.9²⁵ (Figure 2). This program generates an ensemble of different rigid body orientations (poses) for each compound conformer within the binding pocket and then passes each molecule against a negative image of the binding site. Poses clashing with this 'bump map' are eliminated. Poses surviving the bump test are then scored and ranked with a Gaussian shape function.

We defined the binding pocket using the ligand-free protein structure and a box enclosing the binding site. This box was defined by extending the size of a cocrystallized ligand by 4 Å (addbox parameter of FRED). This dimension was considered here appropriate to allow, for instance, compounds larger than the cocrystallized ones to fit into the binding site. One unique pose for each of the best-scored compounds was saved for the subsequent steps. To define how many compounds should be kept after FRED, we decided to run test cases on F7, TK, and NA over the full ChemBridge compound collection ([\[chembridge.com/\]\(http://chembridge.com/\)\). About 450 000 compounds were downloaded and filtered with FILTER \(as described above, about 350 000 compounds remained after filtering\) and converted in 3D with OMEGA \(same protocol as above\). To this set, the active compounds \(39 molecules and generation of multiconformer with Omega\) corresponding to the three test proteins were added. It was possible to retrieve all known active for F7 and TK when selecting the top 30–40% FRED scores, while for NA, it was necessary to keep the top 60–70% scores, as the pocket is not well defined as compared to the two other targets. We thus kept, after FRED runs over our entire ACD library \(filtered ACD + 49 actives\), the top 20 000 unique compounds for ER, the top 25 000 unique compounds for TK, the top 15 000 unique compounds for F7, and the top 30 000 unique compounds for NA. These calculations led to the generation of a focused library for each target that could be used for the subsequent flexible docking steps. All active compounds were present in these libraries.](http://</p></div><div data-bbox=)

Docking Step 2: Flexible Ligand Docking. After selecting the compounds that could potentially fit into the binding pocket based on shape complementarity alone (Figure 2), we performed the flexible ligand-docking step with two different programs, Surflex and DOCK, both relatively fast in their implementations and following different protein–ligand docking/scoring approaches (see below). The results from this parallel docking and scoring served as starting point for consensus selection strategy (see below, Consensus Docking and Scoring Selection).

Surflex. This program (v. 1.22)²⁷ is a newer implementation of the Hammerhead methodology described by Welch, Ruppert, and Jain (1996).³⁶ Similar to its predecessor, it performs through (1) generation of a pseudo-binding site (called protomol), followed by (2) fragmentation of each individual ligand that are then aligned to the protomol in order to yield poses that maximize molecular complementarity with the binding site. (3) A full molecule is then positioned from the aligned fragments and scored using an empirically derived function including charged and hydrogen bond polar terms, solvation, entropic, and hydrophobic complementarity terms. (4) Best poses (default = 10) are then subjected to gradient-based optimization and returned along with their scores and can be postprocessed at a later stage using user-defined parameters.

The definition of the protomol is a sensitive step, and the docking performance will depend on the area considered to form the binding site and how far from a potential ligand the site should extend (proto_bloat), as well as how deep into the protein the atomic probes used to define the protomol can penetrate (proto_thresh). We considered that these two parameters needed to be tailored and adapted to a binding pocket type (buried or fully solvent exposed). In Surflex, the binding pockets can be defined either from a cocrystallized ligand or from a list of residues known to be part of the interaction site (or predicted de novo). We carefully analyzed each one of the four proteins and applied the following values for the 'proto_thresh (or penetration)' (default 0.5) and 'proto_bloat (or extended binding site)' (default 0) parameters, respectively: ER = 0.1 and 2.0; TK = 0.2 and 4; F7 = 0.1 and 2 and NA = 0.2 and 2.0.

DOCK. Hydrogens and partial charges based on AMBER⁵¹ were assigned on the protein receptor using the InsightII program (Accelrys Inc., <http://www.accelrys.com>) as required in DOCK (v.5.1).⁵² DOCK applies a sphere-matching algorithm to fit ligand atoms to spheres in the binding pocket. We used the SPHGEN program⁵³ to create overlapping spheres within a radius of 5 Å complementary to the protein surface. A Connolly surface of each binding pocket was generated using InsightII with a probe radius of 1.4 Å. The binding pocket included all receptor residues at a distance of 6 Å from any atom of the reference ligand. In the case of NA (very open binding site) a distance of 4 Å was used.

The implementation of flexible docking in DOCK is based on an incremental built algorithm that starts by selecting a rigid anchor fragment within a ligand, which is then placed into the binding site. Then the small molecule is divided into

nonoverlapping segments, which are then arranged concentrically about the anchor segment. The complete ligand is constructed by adding the remaining components followed by minimization. We used a maximum of 1000 orientations for the anchor fragment. Manual matching mode was used with 0.5 distance tolerance. To speed up the calculations, we set 25 configurations per cycle for growth of the ligand, except for the NA binding site where 50 configurations per cycle were used. Simplex minimization was applied to each growth step of the ligand.

Scoring–Ranking–Postprocessing. Each of the docking methods has a built-in scoring function. Surflex incorporates an empirically derived function tuned to predict binding affinities through a combination of hydrophobic complementarity, polar complementarity, entropic terms, and solvation terms. Additionally to Surflex's output that describes a calculated affinity constant, this program also offers a post-ranking trimming tool, essentially through two cutoff parameters: penetration violations (vdw clashes) and polar contribution to the final score. The postprocessing outputs two different ranks—the 'combo' rank where the score of each compound is penalized by the penetration value/polar value above the penetration cutoff and a simple-trim rank, where compounds exceeding the penetration cutoff/polar value are simply excluded from the ranking. We used the second simple-trim rank for this study for we could see in our preliminary tests that it yielded a better screening enrichment. Due to the different features of the four binding sites, we applied diverse values to these two parameters (polar and penetration respectively) to partially take into account the nature/shape of the binding pocket described above. Namely, we were more permissive for penetration violations when the pocket was clearly closed, as is the case of ER and TK (a value of -10.0 was assigned for the penetration parameter) and more rigorous for open pockets such as F7 and NA (the value was -3.0). We also adopted different numbers to account for polar contributions depending on the binding site: lower values for nonpolar pockets, ER and TK (the value was 1.0) and higher values for clearly polar pockets (a value of 2.0 was assigned to both F7 and NA). In short we used the following parameters for polar contribution and penetration violation cutoffs, respectively: ER = 1.0 and -10.0 ; TK = 1.0 and -10.0 ; FVIIa = 2.0 and -3.0 and NA = 2.0 and -3.0 .

The docking/scoring function (S) of DOCK is an energy grid-based potential ($S = E_{vdW} + E_{el}$). We used the Lennard–Jones "softer" 6–9 potentials for vdW interactions and distant-dependent dielectric function ($\epsilon = 4R$) for electrostatic interactions. In the case of polar active sites (F7, NA), we used for the docking/scoring function $S = E_{vdW} + 2E_{el}$ while for more hydrophobic binding sites (ER, TK) we applied $S = E_{vdW} + E_{el}$.

Surflex and DOCK Default Parameters. Calculations were also performed using default parameters as found in the User Guide manuals. We screened the database selected after the FILTER/OMEGA/FRED step for the four protein targets. We also screened the filtered database containing 65 660 compounds (thus before FRED and with OMEGA single conformer mode).

For Surflex, we used the default protomol: 'proto_thresh (or penetration)' = 0.5 and 'proto_bloat (or extended binding site)' = 0. In the postprocessing step, we used polarity = 1.0 and penetration = -3.0 .

The DOCK default parameters were Lennard–Jones 6–12 potentials and distant-dependent dielectric function ($\epsilon = 4R$) without changes in the ratio between the vdW and electrostatic terms in the docking/scoring function. We used a maximum of 500 orientations for the anchor fragment and 25 configurations per cycle for growth of the ligand. Manual matching mode was used with 0.25 distance tolerance. Simplex minimization was applied to each growth step of the ligand.

Consensus Docking and Scoring Selection. We decided to perform consensus docking and scoring. We established a cutoff value for the top 3% (1950) compounds ranked by FRED-DOCK and FRED-Surflex and harvested the compounds that

were common to these two lists, resulting in the generation of a consensus "docked/scored list". To evaluate the efficiency of consensus docking-scoring we computed the enrichment factors (EF) of Surflex, DOCK, and Surflex-DOCK consensus.

$$EF = (a/n)/(A/N)$$

where N is the number of compound in the library (65 660), A , is the number of all active compounds (10 or 19 depending on the proteins), a , is the number of active compounds found in the top n compounds; n , is the number of common compounds found in the consensus docked/scored list.

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