Target Specific Virtual Screening: Optimization of an Estrogen Receptor Screening Platform

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In this work, we introduce a four-step scoring and filtering procedure, furnishing target specific virtual screening (TS-VS), which serves to minimize false positives resulting from conformational artifacts of the docking process and is optimized to converge on novel chemotypes of estrogen receptor alpha (ERα). As a proof of concept, VS of a commercial compound database was undertaken (SPECs database release: Aug 2005, 202 054 compounds in total), resulting in the identification of both previously known and novel putative ER scaffolds. Application of distance constraints within TS−VS allowed facile identification of three novel active ligands with ERα binding affinities (IC_{50}) of 1.4 μM, 57 nM, and 53 nM. Importantly, they all exhibited ERα over ERβ selectivity, with the most selective being 17-fold. The ligands also displayed low micomolar antiproliferative activity (7−15 μM) in the human MCF-7 breast cancer cell line.

Introduction

A brief survey of the literature currently available (http://www.ncbi.nlm.nih.gov/) in the area of docking applied to virtual screening (VS[a]) reveals over 250 related entries since late 1997. This field has emerged in the past decade as a key element for both the pharmaceutical industry and the academia in the discovery of new lead compounds that possess specific therapeutic properties.

Structure-based VS is typically performed by docking a molecule into a receptor active site and determining the optimal orientation by conformational, translational, and rotational movement.[1−3] Subsequent scoring of these complexes is undertaken to assess the correct binding modes of the complexes, allowing ranking by affinity.[4] This ranking allows prioritization and selection of compounds for biological testing.

Several studies have examined the ability of docking and scoring combinations to retrieve a set of known actives from databases of decoys.[5−9] Evaluation of their efficacy has been determined through analyses of enrichment (E) rates[10] or their ability to "correctly" reproduce binding modes observed in crystal structures as measured by rmsd.[6] The minimum requirement of a docking tool would be to reproduce known binding modes for a set of complexes as measured by rmsd, however, this metric loses information about intermolecular interactions focusing only on ligand coordinates. Cole et al.[11] importantly point out that rmsd calculations can be flawed because docked solutions can exhibit a low rmsd, but they can also have substituents oriented incorrectly with respect to residues of the active site. Marcou et al.[12] recommend omitting the use of rmsd as a measure of quantifying docking success. It is imperative that good E rates are achieved based on accurate identification of true binding modes to ensure false positives (FPs) resulting from conformational artifacts are minimized. Recent studies have focused on the use of interaction fingerprints[12−14] as a post-docking strategy to reduce FPs. Rognan's group shows scoring by similarity of these fingerprints outperforms conventional scoring functions. The importance of post-docking filters has also been highlighted with the introduction of stand-alone programs such as VISCANA,[15] Silver,[16] and PostDock.[17]

Rather than comparing the performance of post-docking filters with scoring functions, our approach was to implement both methods concurrently and so ensure that only true binding modes are ranked. Our resultant platform, target specific virtual screening (TS-VS), consists of a rigid-body docking algorithm (LIGIN), a "rough" scoring function, normalized complementarity (NC), a post-docking filter, ligand protein contacts (LPC), and final ranking with an empirical scoring function (Figure 1). TS-VS was conceived as a method to target specific biological systems of study rather than deliver a generic tool and its validation was based on the retrieval of active modulators of the human estrogen receptor (ER).

As a stand-alone docking tool, LIGIN has been previously tested in CASP2 experiments involving binding pocket identification,[18] modeling the quinone binding site in the D1 protein of the photosystem-2 reaction center[19] and the inhibitory/stimulatory binding sites for tentoxin within chloroplast F0F1-ATPase.[20] However, in the context of virtual screening, LIGIN has not yet been evaluated. The LIGIN methodology is fully described elsewhere,[21] so for the purposes of this article we briefly describe the main features in the computational section.

Post-docking, the NC function is calculated using LPC software[22] as it differs from the function in LIGIN by inclusion of a wall term that accounts for interatomic clashes. A threshold value is set allowing removal of docked ligands overly exposed to solvent because of "poor" positioning in the active site. This step is highly beneficial when applied to docking against targets such as nuclear receptors, which inherently possess well-buried cavities. The large quantity of cocrystal data for the nuclear receptor superfamily, our previous work on this target, and knowledge about its modulation through key interactions with

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[a] Abbreviations: TS-VS, target specific virtual screening; VS, virtual screening; ER, estrogen receptor; E, enrichment; LPC, ligand protein contacts; NC, normalized complementarity; FP, false positive; vHTS, virtual high-throughput screening.

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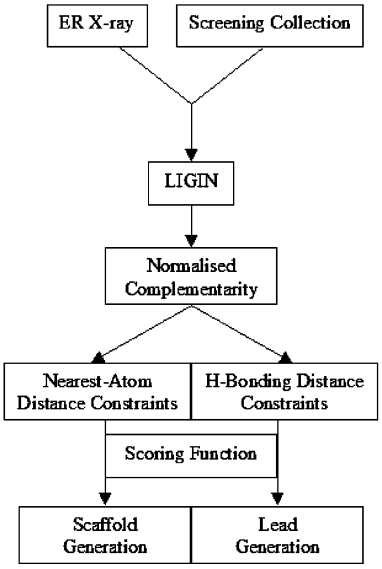


Figure 1. Overview of VS protocol.

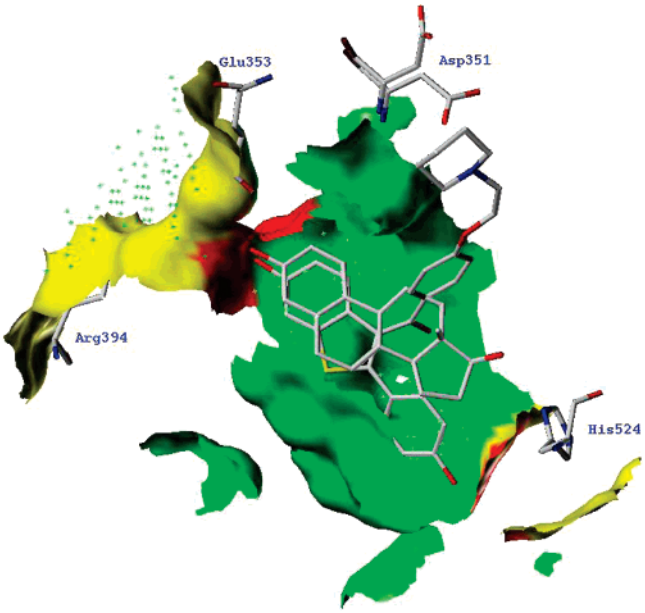


Figure 2. Residue−ligand interactions of the agonist estradiol ERα (pdb ID: 1ERE) and antagonist raloxifene ERα (pdb ID: 1ERR).

residues Glu353, Arg394, His524, and Asp351 (antagonist) prompted us to use it in this study[23−25] (Figure 2). The desire was to deliver a TS-VS utility that would yield high E and low FPs and be clearly validated through experiment.

Post-docking, LPC also generates information about interatomic distances between ligands and residues of the active site of the ER, with specific details about H-bonding interactions. Only those ligands exhibiting true binding modes based on two types of distance constraints, nearest-atom and H-bonding are retained. When nearest-atom distance constraints are used, it is possible to suggest novel chemistry by permitting compounds whose scaffolds may be adequately oriented for ER binding but may not be revealed in a focused virtual screen due to the presence of inappropriate substituents. Alternatively, applying specific H-bonding distance constraints, only those molecules bearing substituents that could interact through H-bonding to specific residues (e.g., Thr347, Glu353, Leu387, Arg394) are retained. Importantly, Asp351 was not included in the filter list of essential H-bonding residues so as to permit the identification of modulating scaffolds, not necessarily antagonists, whose activity could be optimized toward agonism or antagonism by enumeration of a virtual library and follow-up synthesis.

Following the application of either nearest-atom or specific H-bonding constraints, a final scoring component is executed on the remaining complexes to allow prioritization. In the delivery of a target-specific virtual screening utility, selection of an appropriate scoring function component is paramount. Arbitrary or uninformed use of scoring functions can and does lead to negatively impacted hit rates and compound rankings, with increased FP rates and multiple false negative annotations. To select the scoring function most applicable to docking in ERα, 15 popular scoring functions, that is, X-Score, Fresno, six scoring functions implemented in Sybyl6.91 (D-Score, PMF-Score, G-Score, ChemScore, F-Score, and DrugScore), six implemented in FRED2.11 (ChemScore, ChemGauss, ChemGauss2, PLP, ScreenScore, ShapeGauss), and one from Surflex (Hammerhead), were evaluated. This evaluation was performed without the implementation of distance constraints in the process so as to prevent any undue bias. The optimal individual scoring function was then selected and included as the last component in the protocol so that only reasonably docked structures were actually scored. Integration of these "in silico" methods with wet-lab experimentation has allowed us to optimize our suite of algorithms and discover lead compounds of the ERα, delivering a validated TS-VS platform.

Experimental Section

Computational. Active and Decoy Sets. A total of 40 known antiestrogens were selected from literature, with activities ranging from nanomolar to low micromolar potency and converted to SMILES format using ACD/ChemSketch 8.17. The set was passed through FILTER[26] to remove those antiestrogens that were not considered to be "drug-like", leaving only 19 remaining. Our laboratory and others have highlighted the importance of incorporating a set of actives in a decoy set that reflect the properties of the rest of the decoy set when validating a VS protocol.[23,27] We sought to optimize the protocol toward discovery of inhibitors of ERα that would also possess more "drug-like" properties, and our choices of filter parameters reflected this. A subset of the Derwent World Drug Index (WDI)[28] was then extracted and passed through FILTER using the same filtering properties, such as molecular weight <200 or >550, number of hydrogen bond donors $0 < x < 6$ and acceptors $0 < x < 10$, and calculated logP < 7. The set remaining totaled 10 343 compounds. From this, 500 molecules with stereochemical information denoted and 481 without were randomly selected. This was done to best reflect the portion of marketed drugs that contain chiral centers, so representing a "real world" virtual dataset. The two sets were merged with the 19 actives to produce a set of 1000 compounds with similar characteristics.

A larger database comprising 9999 compounds was formed using the WDI and CHEMBANK. A single potent antiestrogen was added to the set to make up the 10 000.[25] This set has been previously described in our study of database preprocessing.[29] This set was employed to validate the vHTS protocol more thoroughly.

Conformer Generation and Storage. Cheminformatic preprocessing of databases of molecules has been assessed by our group in relation to ERα in a previous study.[29] We have demonstrated the impact it has in the context of virtual screening and prioritization of compounds for biological evaluation using the rigid-exhaustive docking algorithm, FRED 2.01.[30] Multiple protonated, tautomeric, stereochemical and conformational states were enumerated and their associated effects on E rates and FP rates were examined using datasets of 1000 and 10 000 compounds, respectively. Unexpectedly, the initial SMILES[31] representation of a compound prior to preprocessing had a significant impact on the E obtained. It is concluded that only the generation of 10 conformers of each

compound using OMEGA 1.81[32] is needed to produce excellent E when docking in the ERα. Noteworthy, this is ER-specific and might not translate to other targets. Interestingly, addition of multiple protonation, tautomeric, and stereochemical states does not provide additional benefit. As a result, we have chosen the same method of conformer sampling using OMEGA 1.81 in the current protocol.

To begin with, OMEGA 1.81 was utilized to convert all databases from sdf format to a multiconformer mol2 database, with subsequent conversion to multi-PDB file using OpenBabel 1.100.2.[33] A set of C subroutines automates these processes and also splitting of the multiconformer file into separate conformers.

Protein Preparation. The crystal structure 3ERT was downloaded from the Protein Data Bank, and crystallographic waters were removed. The subsequent structure was imported to Macromodel 6.5[34] and rewritten in PDB format to ensure bonds were represented correctly in this format. LIGIN does not take hydrogen atoms into account in the docking process and so no addition or minimization of them was needed.

Docking Protocol. A description of the LIGIN docking program employed at this stage is provided. LIGIN is executed when three main files are present, INPUT, PROT, and LIG. The LIG file consists of each conformation of a ligand in the database in standard PDB format. The PROT file is generated from the crystal structure (3ERT)[35] and contains the coordinates of the protein atoms and other atoms in the target but does not include information about the ligand chosen. The input file is then generated from a set of arbitrary rules that classify and assign a number to particular atom types numbered 1−8: (1) Hydrohilic: N and O atoms that can donate and accept H-bonds (e.g., oxygen of hydroxyl group of Ser or Tyr). (2) Acceptor: N or O atoms that can only accept H-bond. (3) Donor: N atom that can only donate H-bond. (4) Hydrophobic: Cl, Br, I, and all C atoms that are not in aromatic rings and do not have a covalent bond to a hydrophilic atom. (5) Aromatic: C atoms in aromatic rings. (6) Neutral: C atoms that have a covalent bond to at least one atom of class 1 or two or more atoms from class 2 or 3; N atom if it has covalent bonds with 3 carbon atoms; S and F atoms in all cases. (7) Neutral-donor: C atom that has a covalent bond with only one atom of class 3. (8) Neutral-acceptor: C atom that has covalent bond with only one atom of class 2.

To reduce the sampling time, the coordinates of the LIG files are translated to those of the cocrystallised ligand (4-hydroxytamoxifen) to ensure docking begins in the binding site. LIGIN begins by generating a number of ligand positions in six dimensions in the binding site of the receptor. The basic presumption is that two atoms will be in contact if they share a common surface area with a distance between them smaller than $R_a + R_b + 2R_w$, where R_a and R_b are van der Waals radii of the atoms and R_w is that of the solvent molecule. A final evaluation of the fit of a molecule in the active site is given by the calculation of a complementarity function (CF)

$$\mathrm{CF} = S_1 - S_i - E \quad (1)$$

where S_1 and S_i are the sum of all "legitimate" (complimentary) and "illegitimate" (uncomplimentary) contact surface areas, respectively, between ligand and residues of receptor. E is a repulsion term similar to that used in energy force fields. A "wall" term is also incorporated, similar to the repulsive term used in the Lennard-Jones potential to account for intermolecular clashes. As the CF value would be ultimately dependent on the size of the ligand, it is normalized by dividing by the solvent accessible surface of the uncomplexed ligand, producing the NC value.

The docked positions obtained have their respective hydrogen bond lengths optimized to allow for refinement of the final structure. After searching for the global maximum of the complementarity function, the program creates ≤20 files (CR1, CR2, CR3, and so on) containing the coordinates of the ligand in PDB format that correspond to the "global" (CR1) and "local" maxima (CR2, CR3, and so on). Merging of the PROT file and each CR file is carried out to produce the final docked complexes. Each step in the process, namely, extraction of ligand information from an SQL database, generation of each INPUT file for the associated LIG files, translating the coordinates of the LIG files to the endogenous ligand, execution of LIGIN, and merging of the output CR files with the PROT file are all carried out by a series of C routines that construct the fully automated suite.

TS-VS Validation: NC, Distance Constraints, and Scoring. In a prescreening phase, the NC according to LPC[22] was calculated for each of the docked complexes for a set of 19 active ERα inhibitors known to potently modulate ERα. The lowest NC value was set as the threshold value for follow-up docking studies.

Next, a post-docking filter was introduced, consisting of distance thresholds set between atoms of a ligand and certain residues known to be important in the ER binding process. From a calculation of the interatomic contacts using LPC on the crystal structure 3ERT, the core putative H-bonds were deemed to be Glu353, Arg394, Leu387, Thr347, and Asp351. The 19 actives used earlier to assess the lowest NC value were redocked, and distance thresholds were set for each of these residues according to the range of distances observed for all 19 actives (e.g., 2.4 <= $glu353 && $glu353 <= 4).

A decoy set of 1000 compounds seeded with the same 19 actives was subsequently docked according to the above procedure with the NC threshold set but without any distance constraints set. The remaining docked complexes were scored using the following scoring functions: F-Score, D-Score, PMF-Score, G-Score, Chemscore, and Drugscore as implemented in Sybyl 6.91, Chemscore, Chemgauss, Chemgauss2, Shapegauss, PLP, and Screenscore as implemented in FRED 2.11, Hammerhead as implemented in Surflex, and two standalone scoring functions, Xscore and Fresno. The optimal scoring function was selected through analysis of E data and now became the final component of the procedure as in Figure 1.

Finally, the two training sets (1000 and 10 000 molecules) described in the previous section were docked, filtered, and scored accordingly.

Virtual Screening—Proof of Concept. A virtual screen of the SPECS database screening collection (Release: Aug 2005, 202 054 compounds in total) was carried out using our fully optimized protocol to initially suggest new scaffolds of inhibitors of ERα. A total of 10 conformers of each molecule were generated using Omega 1.81[32] and docked and scored according to the protocol detailed in the TS-VS validation section. A visual inspection of the compounds that passed was undertaken, and a number of scaffolds were selected.

Taking the same docked complexes, the set was refiltered using LPC, with additional constraints imposed to guarantee H-bonding of ligand atoms occurred with residues Thr347, Glu353, Leu387, and Arg394. Asp351 was not selected as a H-bonding constraint to allow both agonist and antagonist cores to be discovered, as agonists can readily be converted to antagonists through the addition of an antiestrogenic side-chain. The remaining compounds were finally scored with the last scoring component of the procedure.

Biochemical Testing. Receptor Binding Assay. Competitive binding affinity experiments were carried out, as described elsewhere,[36] using purified baculovirus-expressed human ER-alpha (HrERα) and applying Fluoromone (ES2), a fluorescein-labeled estrogen ligand. Both were contained in the ER competitor assay kits, which Green obtained from Invitrogen Corporation. HrERα was stored at −80 °C, and not subjected to any vortexing.

HrERα Titration. HrERα was serially diluted from 400 nM to 0.391 nM in screening buffer (40 mM Tris-HCl, pH 7.5; 50 mM KCl; 5% glycerol; 10% dimethylformamide; 0.02% sodium azide; 50 μg/mL bovine gamma globulin) to a final volume of 100 μL in borosilicate test tubes. ES2 was added to each tube at a concentration of 1 nM, and the tubes were mixed by shaking lightly. After incubation for 1 h at room temperature, the FP assays were carried out using a Beacon 2000 fluorescence polarization instrument

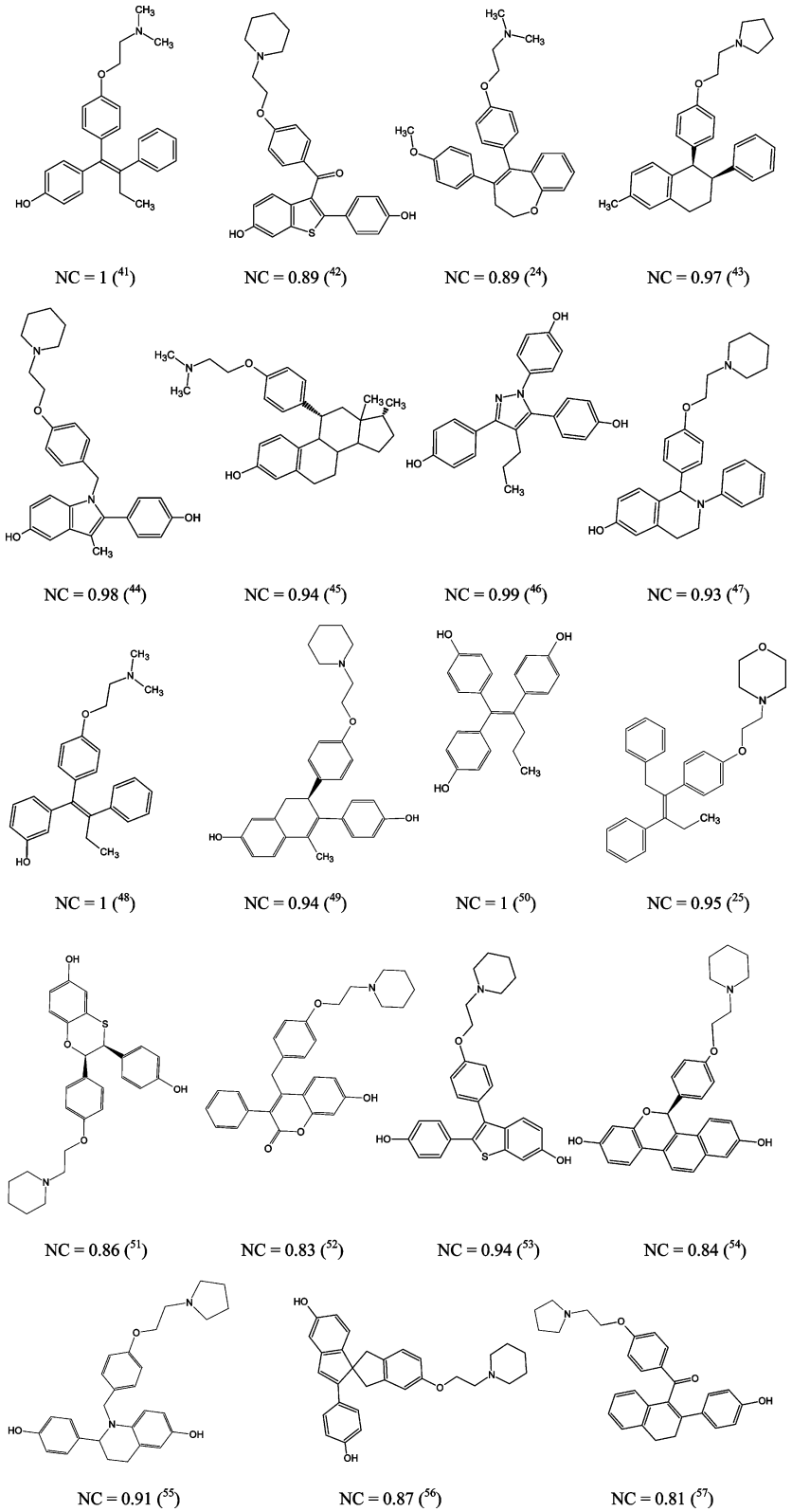


Figure 3. Total of 19 active ligands extracted from literature. NC represents the NC value, and the references for each structure are enclosed in parenthesis.

(PanVera Corporation) with 360 nm excitation filter and 530 nm emission filter. Fluorescence anisotropy was measured for each solution, and the amount of ER that gives 80% of the maximal shift in mP was selected as the concentration to use for competitive binding studies.

Competitive Binding Assay. Competing compounds were prepared at a standard concentration of 10 mM in DMSO. HrERα and ES2 were combined on ice (4 °C) in a glass vial to produce the receptor−fluoromone complex. The vial was gently inverted 2−3 times, again ensuring no vortexing of the mixture. In duplicate, the compounds were serially diluted in ethanol to ensure the final concentration of DMSO and ethanol was below 1% in solution. A total of 1 μL of each solution was diluted in 49 μL of buffer and added to 50 μL of the receptor−fluoromone complex in borosilicate test tubes. Following a 45 min incubation, the samples underwent FP measurement. E_2 was used as a negative control and 50 μL of

Table 1. Enrichment of Inhibitors for ERα Using 15 Different Scoring Functions

database size	0.6%	1.2%	1.8%	2.4%
Chemscore	0	4.526	12.07	11.315
D_Score	0	0	0	0
PMF_Score	0	0	0	0
G_Score	0	0	0	0
Drugscore	0	0	0	0
F_Score	0	0	0	0
Chemgauss	36.21	31.68	27.16	22.63
Chemgauss2	45.26	31.68	27.16	22.63
FRED_Chemscore	0	0	0	0
PLP	18.11	18.11	12.07	11.32
Screenscore	27.16	22.63	15.09	13.58
Shapegauss	36.21	27.16	24.14	22.63
Hammerhead	0	0	0	0
Fresno	0	9.05	6.04	11.32
Xscore	18.11	18.11	18.11	13.58

Table 2. Comparison of E Rates for ChemGauss2 before (wo) and after Addition (w) of Distance Constraints

database size (%)	Chemgauss2 (wo)	Chemgauss2 (w)	theoretical max
0.60	36.21	45.26	45.26
1.2	31.68	45.26	45.26
1.8	27.16	42.24	45.26
2.4	22.63	31.68	45.26

Table 3. Comparison of FP Rates for ChemGauss2 before and after Addition of Distance Constraints

true positive (%)	Chemgauss2 (wo)	Chemgauss2 (w)	theoretical max
80	19.62	0.95	0
90	40.55	2.49	0
100	99.29	5.59	0

the receptor−fluoromone complex in 50 μL of buffer was used as the positive control.

Antiproliferative Studies. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was carried out as previously described.[37] The ER(+) breast cancer MCF-7 cell line was maintained in 75 cm^2 culture flasks (Greiner) containing (Dulbecco) Eagles minimum essential medium in a 5% CO_2 atm with 10% fetal calf serum. The medium was also supplemented with 1% nonessential amino acids.

MTT Assay. Cells were trypsinized and seeded at a density of 1.5×10^4 in a 96-well plate and incubated at 37 °C and 5% CO_2 atm for 24 h. All compounds were prepared at a standard concentration of 10 mM in DMSO and serially diluted to produce a range of concentrations spanning 1 η−100 μm. A total of 2 μL of each compound solution were added to the cells and reincubated for an additional 72 h. Control wells contained 2 μL of vehicle (DMSO) in all cases. At the end of the incubation period, culture medium was removed and all cells were washed with 100 μL of PBS. A total of 50 μL of MTT solution was added to each well, and the plates were incubated in darkness for ∼2 h at 37 °C. The converted dye was solubilized with 200 μL of DMSO and pipetted up and down several times to ensure the dye dissolves completely. Absorbance of the converted dye was measured at 570 nm, with control cells set to 100% cell viability.

Cytotoxicity Studies. Lactate dehydrogenase (LDH) assay was used to measure cellular toxicity effects of the various doses of each compound and was examined using a colorimetric determination kit (Promega).[38]

LDH Assay. The assay was carried out concurrently with the MTT assay following dosing of the compounds and incubation for 72 h as above. Prior to removal of the culture medium in the MTT assay, 50 μL aliquots of medium were removed to a fresh 96-well plate. A total of 50 μL of LDH solution was added to each well and the plate was left in darkness for ∼20−30 min at room temperature. A total of 50 μL of stop solution was then added to each well, and the absorbance read at 490 nm on a micro-plate reader. Control of 100% lysis was measured by the addition of 20 μL of lysing solution 45 min prior to harvesting.

Results and Discussion

A common problem in VS is that some compounds are ranked well by scoring functions post-docking, although their respective pose is barely in the binding site leading to false E.[39] Second, and importantly, Warren et al. have recently observed that from an assessment of 35 scoring functions, none were able to reliably identify the best-docked pose against a set of different targets.[40] Obviously, this leads to difficulty in choosing the optimal docking algorithm to select in a VS campaign against a particular target. To overcome this pitfall, we have introduced the TS-VS platform to ensure that only realistic binders and not conformational artifacts are prioritized.

Normalized Complementarity. Our initial scoring function discerns the "buriedness" of a pose within the binding site termed NC, as calculated by LPC software.[22] A docked molecule producing a score of ∼1 is one that is 100% contained in the binding site and thus the solvent accessible surface is 0. This first filter is sensitive enough to ensure that molecules are actually docked in the cavity of the receptor and not overly exposed to solvent. Figure 3 shows the list of 19 active ligands extracted from literature with demonstrated modulation of ERα. These ligands were used to inform the process to set a threshold value (0.8) that must be overcome to allow a molecule to move to the next stage in the process.

Applying this methodology to the validation set of 1000 compounds seeded with 19 actives, only 860 passed (all seeded actives included) and docked sufficiently well within the hydrophobic cavity of the ER.

Scoring. It was necessary to select the optimal scoring function at this stage before application of the distance constraint filters to prevent introduction of bias into the final scoring process. Our intention, however, was to use the optimal scoring function as the final component in the full VS procedure after application of the distance constraint filters to score only true binders.

The ability of 15 scoring functions to discriminate between actives and inactives from the remaining 860 docked poses was examined. Table 1 depicts the E calculated for 0.6% (top 5), 1.2% (top 10), 1.8% (top 15), and 2.4% (top 20) of the ranked hit list for each scoring function. It is immediately clear that ∼50% of the scoring functions provide no E at all. The increasing order of merit of each is D_Score = PMF_Score = F_Score = G_Score = Drugscore = FRED_Chemscore = HammerHead < Fresno < Chemscore < Plp < Xscore < Screenscore < Shapegauss < Chemgauss < Chemgauss2.

Chemgauss2 is a smooth Gaussian function composed of shape-based interactions between all heavy atoms, hydrogen bonding interactions, and aromatic interactions. From Table 1, Chemgauss2 is undoubtedly the best performing scoring function.

Distance Thresholds Combined with Scoring. Having identified ChemGauss2 as the optimal scoring function for the ER for use in the final step in the protocol, it was necessary to take the 860 molecules that passed the NC threshold of 0.8 and refilter, applying distance constraints between the nearest interacting atom of a ligand and atom of the residues Thr347, Glu353, Leu387, and Arg394. The residues, Met343, Leu349, Leu384, and His524, were added to the list for constraining, as

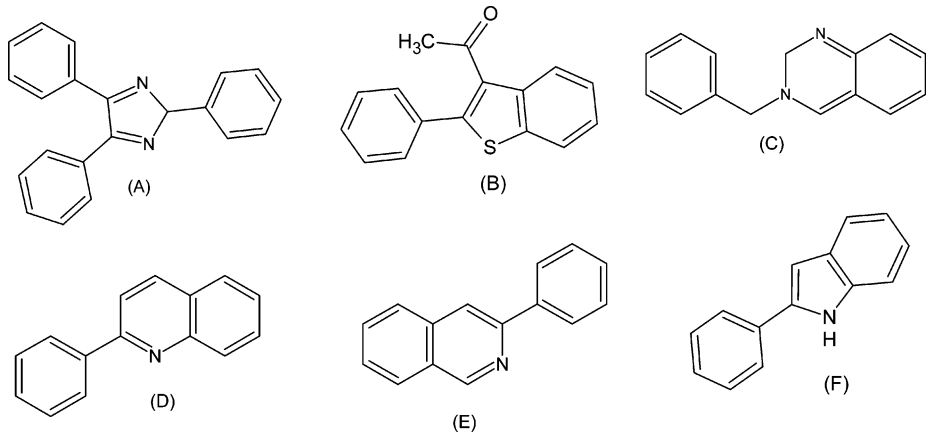


Figure 4. Several known selected scaffolds identified by vHTS protocol outlined.

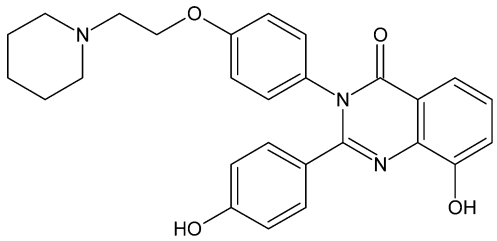


Figure 5. Quinoline structure developed by American Home Products.

they appear to provide additional important interactions in the binding process for antiestrogens.[58] A large reduction in the number of docked complexes needing to be rescored resulted from this process, with all actives and only 52 inactives passing this stage. It is important to note that this reduction in the docked molecule listing makes prioritization significantly easier for any scoring function and assists in ranking, particularly if suboptimal scoring functions are the only tools available to the cheminformatician. Corroborating this, Chuaqui et al. described a 1D profile-based approach, structural interaction fingerprint (p-SIFT), to filter out poorly docked poses and found that once incorrect poses that contribute to FP scores were removed, differences in the performance of individual scoring functions were factored out.[59]

As illustrated in Table 2, the optimized protocol significantly improves E over the data set. Prior to incorporation of distance constraints, the E rates observed using Chemgauss2 were 36.21 in the first 0.6% and 31.68 for the first 1.2%, respectively. On addition of the target-specific constraints to "focus" the docking, a maximum E of 45.26 was observed for both 0.6% and 1.2% levels of the dataset. What is important to note at this stage is that no specific H-bonding constraints have been applied, but only interatomic distance constraints. Halgren et al.[60] also point out that the common definition of E does not account for the actual rank of each active in a scored hit list, and for this reason, we also calculate FP rates for our program in the validation process as another indicator of success. Again, it can be observed that the FP rates are significantly lower after introduction of distance constraints, as evidenced in Table 3.

Finally, to more rigorously test the procedure and its application of the ER, we screened a set of 10 000 compounds (known inactive but with similar molecular properties to known antiestrogens) seeded with a single known antiestrogen used previously by us to show the importance of preprocessing a database prior to docking.[29]

From the ranked database of 10 000 compounds, our procedure managed to select the single antiestrogen in 14th place. Typically, on completion of a virtual screen, a certain percentage of the top ranking compounds is biologically evaluated. In our test case, and in most screens in our laboratory, the top 0.5% is ordered for further biological testing, translating to the top 50 compounds selected in this case. Therefore, the single antiestrogen would have been retrieved from the set successfully, with significant E and savings over random or traditional HTS.

Virtual Screening—Deployment. The procedure detailed above at this stage is sufficient for discrimination between actives and inactives in a training set. It is clear that if a molecule from a compound collection docks in the correct manner with appropriate interatomic distances from the required residues to pass the LPC filter it may not be immediately active because the necessary H-bonding substituents would not be present. Generally, a VS platform with pharmacophoric preferences incorporated (e.g., Flexx-Pharm,[61] Gemdock[62]) will retrieve molecules that fulfill a number or features and as a result novel chemotypes are retrieved, but many potential ones are also missed because of the specificity of such protocols. Advantageously, at this stage of the TS-VS procedure, all scaffolds that adopt the correct shape within the active site are retained. While these ligands may not currently possess the appropriate H-bonding substituents to be immediate binders, they could be tailored to do so using classical chemical modification. Thus, TS-VS is serving to suggest novel scaffolds for follow-up studies. Final scoring of the selected scaffolds with ChemGauss2 allows prioritization according to those compounds with the best interactions. To highlight this process, we again carried out a virtual screen of the SPECS database (Release: Aug 2005, 202 054 compounds in total) employing this method. A selection of some the known scaffolds obtained are illustrated in Figure 4.

Scaffolds B and F represent known scaffolds present in the raloxifene moiety and ZK-119010 moiety, respectively. Scaffold A represents a triarylimidazole-type scaffold. Stauffer et al.[46] have detailed the differences in binding affinity when the cores (diazoles, imidazoles, pyrazoles) are replaced by one another and with the rings containing the same substituents in the same positions. Although the imidazole core still permitted binding to the ER, it was less efficacious than the pyrazole core. Fink et al.[63] have also previously demonstrated the high affinity binding of 1,3,5-triaryl-alkyl-pyrazoles to ERα. Scaffolds C, D, and E all contain a quinoline core that has been previously shown to be effective when incorporated in an antiestrogenic moiety, as illustrated in Figure 5.

Next, specific H-bonding interactions were incorporated in this section to allow immediate identification of potential ER binders from the same screen of the SPECS database carried

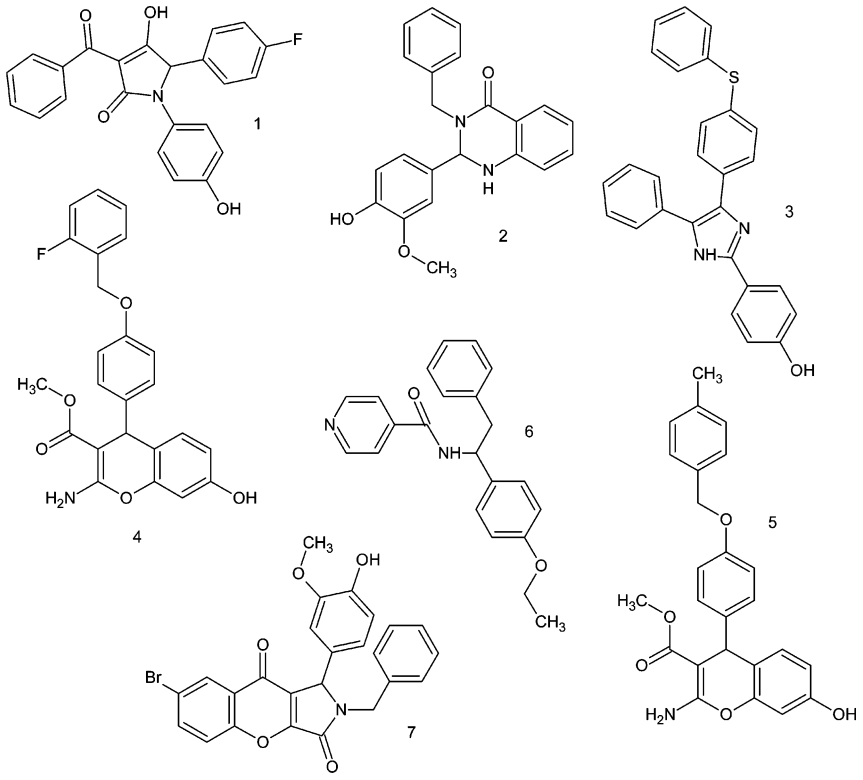


Figure 6. Hits identified by vHTS and chosen for biochemical testing.

out to suggest novel scaffolds. The set was filtered using LPC with H-bond constraints set (Arg394, Glu353, Thr347, Leu387). Following visual inspection (compounds selected based on number of interactions with key residues) of the 13 ranked compounds remaining, and as a proof of concept, a set of seven compounds from the original database of >200 000 (Figure 6) were selected, purchased, and evaluated for their ability to bind to the ER by fluorescence binding assay using 10 μM as an activity cutoff. Of these seven tested ligands, three demonstrated ERα binding affinities above the 10 μM cutoff, with measured binding affinities of 1.1 μM (compound **2**), 53 nM (compound **4**), and 56 nM (compound **5**) for human ERα.

Figure 7 illustrates predicted binding modes of the seven compounds in ERα.

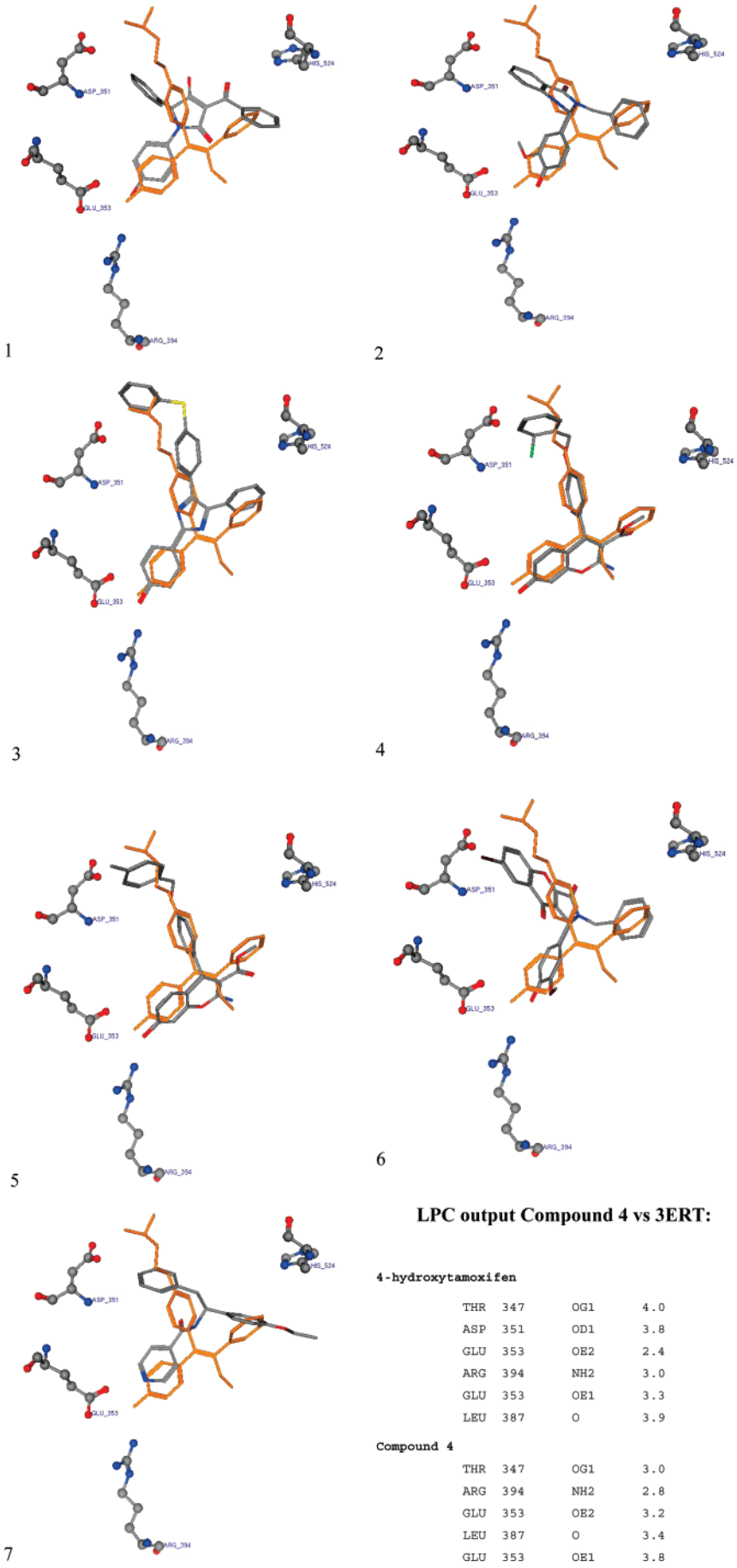
From Figure 7 it is clear that compounds **4** and **5** adopt an orientation very close to that of 4-hydroxytamoxifen (orange). The presence of free hydroxy substituents allows an interaction with Glu353 and Arg394 and also His524 as observed from the LPC output of the docked complex of compound **4**. These compounds were shown to possess the best binding affinity, validating and corroborating the computational analysis. Compound **4** possesses fluorine on the *ortho*-position, which appears to reduce the binding affinity because of steric interactions with Asp351, compared with compound **5**, which possesses a methyl group on the *para*-position of the side-chain ring. The affinity of compound **2** for ERα is probably due to the presence of a *meta*-methoxy group and a *para*-hydroxy group near Glu353 and Arg394, which permits a strong H-bond interaction to occur.

To determine whether our TS-VS methodology was indeed specific enough to select hits that would preferentially bind ERα over the ERβ isoform, we examined binding of these compounds to ERβ by the same experimental method. Compounds **2**, **4**, and **5** exhibited binding IC_{50} values of 6.2 μM, 780 nM, and 915 nM, respectively, demonstrating 4.4-, 13.7-, and 17-fold selectivity for ERα over ERβ.

Finally, to evaluate the ability of these compounds to inhibit proliferation of human MCF-7 breast cancer cells, an MTT functional assay was also carried out. The compounds exhibited (2) 15 μM, (4) 11.4 μM, and (5) 7 μM inhibitory activity, comparing well with the clinical standard, tamoxifen (4.6 μM). All compounds assayed for their antiproliferative effects were concurrently tested to assess the extent of their cytotoxity using the LDH assay outlined in the Experimental Section. All three compounds possessed cytotoxic effects comparable to tamoxifen, indicating their actions to be also cytostatic rather than cytotoxic.

As discussed elsewhere,[23] the key to turning an estrogenic substance into an antiestrogen is by inclusion of a basic side-chain such as that of a dimethylaminoethyl chain of tamoxifen. It is interesting to note that none of the compounds possessed the predicted ability to interact directly (i.e., via H-bonding) with what is usually considered to be the key antiestrogenic residue, Asp351, but yet they all exhibited inhibitory activity close to that of tamoxifen.

Compounds **4** and **5** have been previously synthesized by Elagamey et al.,[64] and compound **2** was synthesized by Chernobrovin et al.,[65] however, no indication of their pharmacological activity has been reported with respect to the ER to date. Güngör et al. have independently described the synthesis and biological activity of a different series of arylquinazolinone and 3-arylquinazolinethione derivatives that possess low nM ER binding, and preferential binding affinity to ER beta.[66] The 4*H*-chromene-3-carboxylate scaffold of compounds **4** and **5** has been incorporated in ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate and found to induce apoptosis in tumor cells by binding to bcl-2 protein.[67] This is the first report of associated ER activity for these compounds, and additional virtual and chemical library enumerations incorporating focused structural modifications to the core scaffolds are underway in our laboratory to optimize activity.



LPC output Compound 4 vs 3ERT:

4-hydroxytamoxifen

THR	347	OG1	4.0
ASP	351	OD1	3.8
GLU	353	OE2	2.4
ARG	394	NH2	3.0
GLU	353	OE1	3.3
LEU	387	O	3.9

Compound 4

THR	347	OG1	3.0
ARG	394	NH2	2.8
GLU	353	OE2	3.2
LEU	387	O	3.4
GLU	353	OE1	3.8

Figure 7. X-ray of 4-hydroxytamoxifen (orange) in active site of ERα (3ERT), with docked structure of hits 1−7 overlaid, and comparison of LPC output of compound **4** docked vs 4-hydoxytamoxifen (3ERT).

Conclusion

As indicated previously, a common occurrence and pitfall in many VS campaigns is the retrieval of FPs among true positives. We have shown that incorporation of two components from our TS-VS protocol, namely, NC scoring and distance constraints, can significantly reduce FPs, and subsequently, ranking by a universal scoring function is far more effective after their inclusion. The choice of the docking algorithm to use in VS is highly dependent on the target of interest, and we suggest that assessing the binding modes of a set of known actives using distance constraints may be more effective than calculation of rmsd, as rmsd only accounts for ligand coordinates and negates any information about potential interactions. Constraining the distances in different ways, that is, nearest-atom or H-bonding, we have been able to suggest both new saffolds and also retrieve actual validated hits of both ERα and ERβ. Our full TS-VS procedure positively identified one micromolar (compound **2** = 1.4 μM) and two novel nanomolar (compound **4** = 56 nM, compound **5** = 53 nM) ligands of ERα by virtual screening of 202 054 compounds, of which only seven were selected for biological testing. The compounds also exhibit low micromolar inhibition of MCF-7 proliferation and were also shown to be selective in targeting ERα over ERβ (e.g., compound **5** = 17-fold selective). The procedure is fully automated, and access to a mid-sized 130 Intel Xeon 3.06 GHz processor cluster[68] allows us to carry out VS via these methods in a short time. This procedure is currently being extended to carry out virtual screening to identify compounds selective for ERβ.

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