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A practical guide to large-scale docking

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Abstract

Structure-based docking screens of large compound libraries have become common in early drug and probe discovery. As computer efficiency has improved and compound libraries have grown, the ability to screen hundreds of millions, and even billions, of compounds has become feasible for modest-sized computer clusters. This allows the rapid and cost-effective exploration and categorization of vast chemical space into a subset enriched with potential hits for a given target. To accomplish this goal at speed, approximations are used that result in undersampling of possible configurations and inaccurate predictions of absolute binding energies. Accordingly, it is important to establish controls, as are common in other fields, to enhance the likelihood of success in spite of these challenges. Here we outline best practices and control docking calculations that help evaluate docking parameters for a given target prior to undertaking a large-scale prospective screen, with exemplification in one particular target, the melatonin receptor, where following this procedure led to direct docking hits with activities in the subnanomolar range. Additional controls are suggested to ensure specific activity for experimentally validated hit compounds. These guidelines should be useful regardless of the docking software used. Docking software described in the outlined protocol (DOCK3.7) is made freely available for academic research to explore new hits for a range of targets.

Introduction

Screening chemical libraries using biophysical assays has long been the dominant approach to discover new chemotypes for chemical biology and drug discovery. High-throughput screening (HTS) of libraries of 500,000 to 3 million molecules has been used since the 1990s[1], and multiple drugs have had their origins in this technique[2]. While the libraries physically screened in HTS were an enormous expansion on those used by classical, pre-molecular pharmacology[3], they nevertheless represent only a tiny fraction of possible 'drug-like' molecules[4]. DNA-encoded libraries[5], where molecules are synthesized on DNA that encodes their chemistry, begin to address this problem by offering investigators libraries of 10^8 molecules, sometimes more, in a single, highly compact format; and multiple such libraries can be used in a single campaign. However, as DNA-encoded libraries are restricted to reactions on DNA, reaction chemistries are limited to aqueous solutions, thereby limiting the type of chemical reactions and subsequent chemical libraries available with this technology[6].

Computational approaches using virtual libraries are an attractive way to explore a much larger chemical space[7]. Large numbers of molecules—certainly into the tens of billions, and likely many more—may be enumerated in a virtual library. Naturally, very few of these compounds can ever be actually synthesized because of time, cost and storage limitations, but one can imagine a computational method to prioritize those that should be pursued. In practice, this idea has had two limitations that have prevented wide-scale adoption: the virtual libraries have rarely been carefully curated for true synthetic accessibility[8], and there were well-founded concerns that computational methods, such as molecular docking, were not accurate enough to prioritize true hits within this large space[9].

In the last several years, however, two advances have at least partly addressed these problems:

First, several vendors and academic laboratories have introduced 'make-on-demand' libraries based on relatively simple two- or three-component reactions where the final product is readily purified in high yields[10]. At Enamine, a pioneer in this area, >140 reactions may be used to synthesize products from among >120,000 distinct and often highly stereogenic building blocks, leading to a remarkably diverse and, critically, pragmatically accessible library of currently over 29 billion molecules[11].

Second, structure-based molecular docking, for all of its problems, has proven able to prioritize among these ultra-large libraries, if not at the tens of billion molecule level, then at the 0.1–1.4 billion molecule level[12-14], finding unusually potent and selective molecules against several unrelated targets (Table 1). Indeed, simulations and proof-of-concept experiments suggest that, at least for now, as the libraries get bigger, docking results and experimental molecular efficacies improve[12].

If docking ultra-large libraries brings new opportunities, it also brings new challenges. Docking tests the fit of each library molecule in a protein binding site in a process that often involves sampling hundreds-of-thousands to millions of possible configurations. Each molecule is scored for fit using one of several different scoring functions[15-18]. To be feasible

for a billion-molecule library on moderately sized computer clusters (e.g., 500–1,000 cores), this calculation must consume not much more than 1 s/molecule/core (1 ms/configuration).

This need for speed means that the calculation cannot afford the level of detail and number of interaction terms that would be necessary to achieve chemical accuracy. For instance, docking typically undersamples conformational states, ignores important terms (e.g., ligand strain) and approximates terms that it does include (e.g., fixed potentials)[19,20].

Owing to these approximations and neglected terms, docking energies have known errors, and the method cannot even reliably rank order molecules from a large library screen[21,22]. What it *can* hope to do, however, is separate a tiny fraction of plausible ligands from the much larger number of library molecules that are unlikely to bind a target. This level of prioritization is enhanced with the careful implementation of best-practice guidelines and controls. It is the goal of this essay to provide investigators with such best practices and control calculations for ultra-large library docking, though they equally apply to modest-sized library docking (Table 1). These will not ensure the success of a prospective docking campaign—the only true test of the method—but they may eliminate some of the more common reasons for failure.

We begin by describing protocols and controls that can be used across docking programs and that are general to the field (Fig. 1). There are by now multiple widely used and effective docking programs[23-31], employing different strategies for sampling ligand orientations and ligand conformations in the protein site, for handling protein flexibility, and for scoring ligand fit once they have been docked. Notwithstanding these differences, there are strategies and controls that may be used across docking programs, including how to prepare protein sites for docking calculations, benchmarking controls to investigate whether one can identify known ligands from among a large library, and controls to investigate whether one's calculations contain biases towards particular types of interactions. Since the true success of a virtual screen is the experimental confirmation of docking hits, we also propose a set of control assays to validate initial in vitro results. We then turn to protocols that are specific to the docking program we use in our own laboratory, DOCK3.7—these necessarily get into fine details, and will be of most interest to investigators wanting to use this particular program.

General guidelines for virtual structure-based drug discovery

Structure preparation and suitability for docking

Any structure-based campaign begins with a suitable target site.: The most promising starting point for a virtual screening campaign is typically a high-resolution ligand-bound structure. Ligand-bound (*holo*) structures usually outperform ligand-free (*apo*) structures as the geometries of the binding pocket are better defined in the bound state than in the unbound state[32,33]. If there is no available *holo* structure, tools such as SphGen[34], SiteMap[35] and FTMap[36] can be used to identify potential ligand binding sites.

Generally, small, enclosed binding pockets that well complement a ligand perform better than large, flat and solvent-exposed binding sites typical of protein–peptide or protein–protein interactions. For instance, neurotransmitter G-protein-coupled receptor (GPCR)

orthosteric sites, such as the β_2 adrenergic, D_4 dopamine, histamine H_1 and A_{2a} adenosine receptors[37-40] typically have higher hit rates and more potent docking hits than do peptide receptors like the CXCR4 receptor[41], and these often perform better than the more open sites typical of soluble enzymes like β-lactamase[12,42]. In most cases, targeting protein–protein interaction surfaces, outside of a few that are well defined[43], often leads to disappointing results.

Modifying the high-resolution protein structure.: It is not always a good idea to use the structure exactly as it was found in the database.

- Dealing with mutations. For stability, crystallization and other biochemical reasons, high-resolution protein structures are sometimes determined in a mutant form; such mutations should be reverted to the wild type especially if they are within the ligand site to be targeted. Missing side chains and loops in the experimental structures should be added as well if they are close to the binding site, while those that are weakly defined by the experimental observables (e.g., low occupancy, high displacement parameters (B values), poor electron density) should be examined critically

- What about water molecules? When the resolution permits, water molecules can also be included in the target preparation, often treating them as nondisplaceable parts of the protein structure. Typically, water molecules enclosed in the targeted binding pocket or involved in interactions between the co-crystallized ligand should be considered as they may determine side-chain conformations or offer additional hydrogen-bonding sites. Some docking programs will allow water to be switched on or off during the docking[44] or include other implicit solvent terms[45]

- Buffer components. Buffer components such as PEG and salts are likely specific to the crystallization conditions, and should be removed

- Cofactors. Cofactors like heme or metal ions should be considered if they are involved in ligand recognition

- Hydrogen atoms. Due to the resolution limits of most experimentally determined structures, hydrogen atoms are often unresolved. The position of many hydrogen atoms can be readily modeled according to holonomic constraints (e.g., backbone amide hydrogen atoms). For those that are not, such as hydroxyl protons on serine and tyrosine residues, imidazole hydrogens on histidine, and the adjustment of frequently erroneous terminal amide groups for glutamine and asparagine residues[46], programs like Reduce[47] (default in DOCK3.7), Maestro (Schrödinger)[48], PropKa[49] or Chimera[50] can be used to protonate the target of interest. While these automated protocols usually produce reasonable protonation states, special care should be taken for the residues within the ligand binding pocket or residues that form a catalytic/enzymatic site. Lastly, although less frequently encountered, glutamic and aspartic acids can adopt protonated forms under specific circumstances[51]

- In summary, the protonation of the protein structure is critical for the success of docking to more accurately depict the Van der Waals (VDW) surface and dipole moments of the binding pocket

Homology modeling—When no experimental structure has been determined for the target protein, structural models can be generated if a template structure with high sequence identity is known. Common programs used for homology modeling include Modeller[52], Rosetta[53], ICM[27] and I-Tasser[54]. These two principles can improve the chances of success:

- Typically, the higher the sequence identity between the target and the template, the better the accuracy of the model[55]. Particular focus should be given to identity within the target binding pocket; if there is a choice, choose the template that has the highest identity in the binding pocket

- Incorporation of a ligand during the modeling process or ligand-steered homology modeling approaches[56,57] will help prevent the pocket from collapsing inward, and will better orient the side chains of binding residues[32,58,59]

When it is unknown how a ligand binds within the pocket, orthogonal experimental data can guide the modeling such as iterating between docking and modeling[60,61]. In the case of MRGPRX2[62] and GPR68[63], for instance, the authors predicted multiple binding poses of a known active ligand and used mutagenesis and binding assays to test these predictions. A binding mode and receptor structure were identified that was consistent with the mutagenesis data and used for subsequent preparation in the prospective screen. Despite many difficulties, homology models have been successful in identifying novel ligands from prospective docking campaigns[41,62-76]; though it is also true that, given the choice, most investigators will prefer to use a well-determined experimental structure.

Control calculations—Docking undersamples ligand–protein configurations and conformations, and its scoring of these configurations for fit remains highly approximate. Unlike methods like quantum mechanics, or certain lattice calculations in statistical mechanics, docking has surrendered 'ground truth' to be able to pragmatically search among a large and growing chemical space. Accordingly, control calculations are critical to the success of a docking campaign. As with experimental controls, they do not *ensure* prospective success, but they do guard against obvious sources of failure, and can help one understand where things have gone wrong if they do. Through a key control, we assess whether the prepared binding pocket and docking parameters can prioritize known ligands over presumed inactive molecules. In an optimized binding pocket, these known actives should rank higher against a background of decoy molecules in a retrospective screen, and reasonable poses should be predicted.

As it is more likely to know true actives than true inactives, it is common practice to use property-matched decoys[77], which are compounds that have similar physical properties as the actives, but unrelated topologies, and so are presumed inactive. The DUDE-Z server (tldr.docking.org) was built specifically to generate decoys for a given list of ligands by matching the following physical properties: molecular weight, hydrophobicity (LogP),

charge, the number of rotatable bonds, and the number of hydrogen bond donors and acceptors[78].

The performance of the parameterized binding pocket to enrich known ligands over decoys can be evaluated by receiver-operator characteristic (ROC) curves, quantifying the true positive rate as a function of false positive rate (Fig. 2)[17,77,79-81]. The area under the curve (AUC) is a well-regarded metric to monitor the performance of a virtual screen by a single number[17] (Fig. 2a). The log transformation of the false positive rate enhances the effect of early enrichment for true positives[17] (Fig. 2b). This is important because, in a docking campaign with hundreds of millions of molecules, only compounds ranked within the top 0.1% are often closely evaluated (see ranks in Table 1). For example, if a retrospective docking challenge shows that known actives are only identified starting around the tenth percentile, novel actives may be missed in a prospective screen. In this setting, higher LogAUC values correspond to better discrimination between actives and inactives and provide a sanity check on the ability of the docking parameters to identify actives.

A second criterion is the pose fidelity of the docked ligands to their experimental structures. The validity of predicted binding poses can be assessed qualitatively by visual inspection of reported key interactions between protein and ligand or, in the best case, quantitatively by calculating root mean square deviations between predicted and experimentally determined poses[82]. During pocket modeling and docking parameter optimization, one will often insist that the retrospective controls lead to both high LogAUC values and good pose fidelity; often there will be some trade-off between the two. While calibrating the scoring functions, it is also important to monitor the contributions of each energy term to the total score and ensure they match the properties of the binding pocket. If one term dominates, the scoring may have been overoptimized to that term, while other protein–ligand interactions are underweighted, leading to dominance by a certain type of molecule. For instance, if the docking score of a polar solvent-exposed pocket is inappropriately dominated by VDW energies, large molecules may score high due to nonspecific surface contacts with the target protein.

In addition to property-matched decoys, other chemical matter can be used to evaluate different aspects of the docking model (Fig. 3). A test set including molecules with extreme physical properties (Extrema set), such as a wide variety of different net charges, can be screened to measure whether the docking model succeeds in prioritizing molecules with net charges corresponding to known actives[78]. If there is a difference in the charge of top-ranked compounds from the extrema set and the charges of the known ligands, the scoring may be biased. In another useful control experiment, a small fraction of the purchasable chemical space (e.g., readily available 'in-stock' compounds from multiple vendors) representing the characteristics of the ultra-large make-on-demand screening library can be docked against the protein model. This control serves two purposes: to test whether the positive control molecules remain among the highest scored compounds and to examine if intriguing novel compounds rise to the top of the rank-ordered docking list. It may even be fruitful to purchase and experimentally test a few promising and structurally diverse in-stock compounds as it could inform the docker if the binding pocket model is likely to find hits

in the ultra-large library screen. If top ranked compounds do not form expected interactions, further optimization may be beneficial.

Another control, if available, are true inactives from a previous discovery campaign. These can be used as a background against known actives to provide a 'real-world' benchmark of the performance of the system. Lastly, for a protein that has little or no known chemical matter (i.e., reported ligands), enrichment calculations with known actives against a background of property-matched decoys may be impossible. Here, the docking parameters can be calibrated by docking 'Extrema', which challenges the docking with extremes of physical properties, and 'in-stock' compound sets, which probe how the docking will perform on a representative subset of the library[78,83]. It remains true that, without known ligands as positive controls, one is at a substantial disadvantage at setting up docking campaigns, increasing the risk of failure.

Prospective screen—Once the docking model is calibrated, large libraries of molecules can be virtually screened against the target protein. For this virtual screen, it makes sense to focus on compounds that are readily available for testing. The ZINC20 database (http://zinc20.docking.org/) enumerates over 14 billion commercially available chemical products, of which ~700 million are available with calculated 3D conformer libraries ready for docking. Most of the enumerated compounds belong to the make-on-demand libraries of Enamine and WuXi. Further, ZINC20 allows one to preselect subsets of molecules for docking, reducing computation time. For instance, ZINC20 allows users to download ready-to-dock subsets of molecules within user-defined ranges of molecular weight (MWT), LogP and net charge, as well as predefined sets such as fragments (MWT   250 amu) or lead-like molecules (250   MWT < 350 amu; LogP   3.5). The result of a prospective screen is a list of molecules rank-ordered by docking score.

Hit-picking—A well-controlled docking calculation can concentrate likely ligands among the top-ranked molecules. But even if it was able to do so among the top 0.1% of the ranked library, in a screen of 1 billion molecules this would still leave 1 million molecules to consider, and with the errors inherent in docking, many of these will be false positives. Accordingly, we rarely pick the top *N*-ranked compounds by docking to test experimentally, but rather will use additional filters to identify promising hits within the top scoring 300,000–1,000,000 molecules. These filters can catch problematic features missed by the primary docking function, ensure dissimilarity to known ligands and promote diversity among the prioritized compounds.

Compounds may be filtered for both positive and negative features[84] (Table 2). For instance, one may insist that a docked orientation has favored interactions with key residues. Conversely, molecules with strained conformations should be discarded. Molecules with unsatisfied, buried hydrogen-bond donors and acceptors may also be deprioritized. Compounds with metabolic liabilities[85,86] or that closely resemble colloidal aggregators[87] can also be filtered out, despite otherwise favorable scores. Further, as closely related compounds will likely dock in similar poses with similar score, we typically cluster compounds by 2D structure similarity after all other filters have been used and only select the best scoring cluster representative for testing. In such a large dataset, clustering can be

computationally expensive, so reduced scaffold clustering such as Bemis–Murcko[88] analysis is useful to efficiently parse the compounds. In principle, many of these filters could be included directly in the scoring functions, but balancing them against other scoring terms can demand extensive optimization and will likely increase the compute time, which will become a hindrance for ultra-large library docking. Lastly, visual examination of the docked poses has been useful for selection of compounds to purchase. Following the criteria in Table 2, we typically inspect up to 5,000 compounds visually after applying automated filtering and clustering steps.

Experiments to test docking hits—The success of a docking campaign is ultimately measured by its ability to reveal novel chemotypes that can be shown to experimentally bind to the target, typically in binding or functional assays (Fig. 4). However, common artifacts should be controlled for: chemotypes likely to interfere with specific assays[89] (e.g., the controversial[90] pan assay interference chemotypes), covalent adducts, redox cyclers[91] and aggregators[87,92].

Among the most common of these mechanisms is colloidal aggregation, which can account for >90% of all primary hits[89,93]. This aggregates sequester proteins with little selectivity, partially denaturing and inhibiting them[32,92,94-96], occasionally even activating them[97,98], a common problem both in HTS and also in docking screens[92]. Aggregators tend to have high LogP values and limited aqueous solubility, so we prefer to dock and test molecules with LogP 3.5. Chemical stability or reactivity can also contribute to experimental artifacts, and reactive scaffolds should be avoided. The ZINC20 database allows users to select screening libraries in the lead-like chemical space (i.e., low LogP) and exclude reactive compounds[9,99]. Unless controlled for in the experimental setting (Box 1), these artifacts can lead to false positives.

Once convinced that one's hits are not artifactual, more detailed testing on-target is warranted. For all targets, this involves determining concentration response curves, typically with 7–12 points at half-log intervals, with the transition being sampled over at least two log orders of concentration (Fig. 4). For enzymes, determining a true Ki will illuminate mechanism, though this can be laborious and it might only make sense to do this for characteristic lead molecules. For receptors such as GPCRs and ion channels, functional assays are typically performed to determine if the new ligand is acting as an agonist, an antagonist or an allosteric modulator. For GPCRs, initial screening assays may differ from secondary confirmatory assays, and showing that a molecule is active in more than one assay is often quite useful.

Before initial hits are advanced for optimization, it is important for make-on-demand libraries to confirm the identity of the hit compound. Limitations of virtual library enumeration, chemical synthesis and downstream purification can result in mismatches between the in silico predicted compound and the in vitro tested compound. For example, in the screen against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) macrodomain protein, a number of purine derivatives with an N9 substitution were requested but N3-substituted compounds were synthesized instead[83]. Accordingly, it is worth fully characterizing promising compounds before costly lead optimization is undertaken; we

ourselves have made the mistake of designing analogs based on an in silico starting scaffold that was different than the true hit delivered from the chemists. At a minimum, the identity and purity of the compounds should be determined. High-resolution mass spectrometry or quantitative 1D proton nuclear magnetic resonance spectroscopy can be used to detect gross variation between the tested sample and the expected molecule; these data can be obtained from most vendors, or the experiments could be performed independently.

The most direct experiment to test a docking *pose* prediction is the determination of an experimental protein structure in complex with the docking hit (Fig. 4). Such structures illuminate crucial details of ligand recognition, including adjustments of binding site residues or the docking hit in the binding pocket[12,100,101]. The key question to answer with high-resolution structures is whether docking worked for the right reasons, i.e., does the predicted binding mode agree with an experimental structure? Previous studies suggest that hits from virtual screening generally compare fairly well with experimental high-resolution structures; i.e., key anchor points are predicted correctly[12,83,102,103]. However, as docking is typically performed against rigid protein structures, conformational changes, especially in flexible loops, will complicate pose prediction[104]. Water-mediated protein–ligand interactions are generally difficult to explore de novo[45,105], though experimentally determined and structurally conserved water molecules can be included in virtual screens[100]. Lastly, there are few cases where docking hits showing on-target activity revealed binding positions at unexpected and nontargeted subsites. For example, the β_2 adrenergic receptor allosteric modulator AS408 was predicted to bind to an extracellular subpocket but crystallographically determined to bind to the membrane-facing surface of the receptor[106]. In contrast, the pose of an inverse agonist also identified at the β_2 adrenergic receptor from in silico docking[39] was confirmed by X-ray crystallography[107], demonstrating the variability in outcomes even at the same target.

If the protein target can be readily expressed and purified in milligram quantities, crystallography and cryo electron microscopy can guide hit discovery early in the campaign. In campaigns against targets where high-resolution structures are more challenging to obtain (e.g., for transmembrane receptors), an experimental protein–ligand complex might only be achievable for the most potent hit compound. Nonetheless, it is usually worth the effort to confirm the predicted binding site and ligand pose, or to identify unexpected interactions between the discovered hit and the target[106].

Next steps: selecting analogs for hit to lead optimization—In the fortunate event that the virtual screen was successful and docking hits were confirmed by different experiments, the newly obtained scaffolds are blueprints for exploring structure–activity relationships and lead optimization.

A concern about molecules from make-on-demand libraries is that they will initially be delivered as racemic or stereomeric mixtures since the production of pure stereoisomers requires more sophisticated synthesis routes. If confirmed hits were observed from stereomeric mixtures, the measured potency of the mixture may be artifactually lower if only one stereoisomer is active at the target (e.g., the concentration of the active stereoisomer is

less than the total mixture concentration). Purified stereoisomers can typically be purchased from the make-on-demand compound supplier or separated in-house.

Hit optimization can be performed in several ways. Synthetic chemistry groups may obtain synthesis routes of the parent compound from the supplier allowing the generation of medicinal chemistry-inspired analog series[108]. Alternatively, chemoinformatic tools can be employed to virtually search the purchasable chemical space for structurally related scaffolds or identifying molecules with a common substructure to the parent hit compound (analog-by-catalog). Searching for similar molecules within the Enamine REAL space can be conducted using the *SmallWorld* (sw.docking.org) search engine[99]. Molecules with an identical substructure as the parent compound can be found using *Arthor* (arthor.docking.org)[99]. Additionally, the supplier of the parent compound might be able to provide a collection of molecules resembling the hit scaffold. While we cannot provide clear guidance or a best-practice protocol for hit-to-lead optimization, analog-by-catalog of docking hits obtained for the melatonin 1 (MT_1) receptor and AmpC led to compounds with improved potencies compared with the initial parent compounds[12,13]. Currently, we recommend subtle changes to the starting compound or modifications to test particular interactions as suggested from the docked pose.

It is our hope that the above guidelines will be useful for outlining a docking campaign from start to finish. As shown, the prospective docking step is actually only one small component of the overall pipeline (Fig. 1). Control calculations using retrospective datasets and docking setup optimization make up the bulk of the process. Hit picking from the prospective screen requires careful perusal of the data, and experimental design of the confirmatory assays is critical for defining success of the campaign. We ourselves find that these guidelines insulate against the more common sources of failure in large library docking campaigns. Lastly, we want to mention that docking campaigns against protein targets without experimental structure, i.e., requiring homology modelling, or without known active chemical matter for retrospective control calculations are particularly risky and should not be initiated naively.

This concludes our general guidelines for large library docking. In the last section, we turn to a detailed protocol to set up, optimize and prospectively screen a target of interest using DOCK3.7, though any docking program can use the controls presented.

While the protocol should be general for most proteins, we provide example data from a recent campaign against the MT_1 receptor[13], a target particularly well suited for docking: a crystal structure had been determined; the orthosteric pocket is compact and almost completely encloses the ligands, simplifying the biophysics; many such ligands exist for retrospective calculations and for optimization; and in vitro assays to test docking hits were well established. We note that the MT_1 receptor is ideal for large library docking, and so the achieved hit rate, and the hit potencies, were unusually high. Still, the docking optimization strategies below have been useful against a wide spectrum of targets, including the Nsp3 macrodomain of SARS-CoV-2[83] and highly solvated pockets as in β-lactamase[12], and were developed against the spectrum of targets in the DUD-E benchmark[77], ranging from ion channels to kinases to soluble enzymes. It should be clear that the optimization strategies

sketched remain rooted in retrospective controls, and so will work best against targets with precedented ligands, and against targets with well-formed and readily liganded binding sites.

Docking campaigns with DOCK3.7 and ZINC20

In DOCK3.7, ligands are placed in the target pocket by mapping ligand atoms onto predefined hotspots, so-called matching spheres. Matching spheres are generated from the coordinates of heavy atoms from an input bound ligand structure, if available, and supplemented with coordinates based on the negative image of the binding site generated from the program SphGen[34]. Ligand rigid fragment (e.g., rings) are mapped onto matching spheres using a bipartite graph algorithm[79,109]. Different ligand conformations and orientations are sampled in the binding pocket using precalculated 3D conformer libraries (flexibases, db2 files)[9,99]. During conformer library building, each molecule is divided into its rigid fragments, and different conformations of rigid fragment substituents are generated.

Ligand poses are evaluated using a physics-based scoring function (E_{score}) combining VDW, electrostatic (ES) and ligand desolvation (lig_desol) energy terms (Equation 1):

$$E_{score} = E_{VDW} + E_{ES} + E_{lig\_desol} \tag{1}$$

In order to allow rapid scoring of new poses (typically 1,000 poses per molecule per second), contributions of the target protein pocket are mapped onto pregenerated scoring grids. The VDW surface of the binding pocket is converted into a scoring grid by ChemGrid[15]. Electrostatic potentials within the binding pocket are estimated by numerically solving the Poisson–Boltzmann equation using QNIFFT[16,110]. Context-dependent desolvation energy scoring grids, both polar and apolar, are generated by the Solvmap program[17]. Thereby, ligand desolvation energies are computed as the sum of the atomic desolvation of each ligand atom scaled by the extent to which it is buried within the binding site. Atomic desolvation energies for ligand atoms are calculated during ligand building using AMSOL[111] and are included in the ligand conformer library. Both, the electrostatic and ligand desolvation scoring grids depend on the dielectric boundary between the low dielectric protein ($\varepsilon_r = 2$) and high dielectric solvent ($\varepsilon_r = 80$) environments. Consequently, these scoring grids can be fine-tuned by modulating the protein–solvent dielectric interface (see below Steps 41–44).

The following protocol (Fig. 5) prepares a starting structure (Steps 1–4), generates the scoring grids and matching spheres (Steps 5–10), and collects a set of control ligands for model evaluation (Steps 11–33). Control ligands are docked retrospectively (Steps 34–58) to determine the model's ability to identify actives from a pool of inactives. Optimization of sampling (Steps 59–64) and scoring (Steps 65–77) is evaluated with the same controls. Model biases are evaluated using an Extrema control set (Steps 78–88) to examine for charge preferences in the scoring grids and with a small-scale in-stock screen (Steps 89–97) to ensure a computationally expensive large-scale prospective screen (Steps 98–103) is likely to yield interesting chemical matter. Hit picking (Steps 104–108), though critical to success of these prospective experiments, is beyond the scope of the protocol as it is highly user- and target-specific, and we suggest referring to Table 2 for insight. Additionally, we

caution against regarding hits from a screen as true positives until common artifacts are ruled out (Box 1). All controls in silico and in vitro are applicable to any docking program or computer-aided drug discovery campaign. The details of grid preparation and modification are DOCK3.7-specific, but the principles are transferrable to other software.

Materials

Software

- DOCK3.7.5: apply for a license from http://dock.docking.org/Online_Licensing/dock_license_application.html. Licenses are free for nonprofit academic research. Once your application is approved, you will be directed to a download for the source code. The code should run without issue on most Linux environments, but can be optimized by recompiling with gfortran if needed. Questions related to installation can be addressed to dock_fans@googlegroups.com

- Python: the current code uses python2.7, which will also need to be installed. Additional python dependencies that are required for running scripts can be found in the file `$DOCKBASE/install/environ/python/requirements.txt`

- AMSOL: free academic licenses can be obtained from https://comp.chem.umn.edu/amsol/

- (Optional) 3D ligand building software: if interested in 3D ligand building in-house (not necessary for this protocol), licenses will also need to be obtained for ChemAxon (https://chemaxon.com/), OpenEye Omega (https://www.eyesopen.com/omega) and Corina (https://www.mn-am.com/products/corina). We note that, for many campaigns, 3D molecular structures with all necessary physical properties may be downloaded directly from ZINC20. This tutorial makes use of a webserver for 3D ligand building that is suitable for small control sets. Please apply for an account at https://tldr.docking.org/, which is free of charge

- Chimera: this application is recommended for grid visualization with the VolumeViewer feature (see Troubleshooting). The ViewDock feature, also within Chimera, is useful to examine the docking results

Equipment

- Hardware: initial tests of this tutorial (grid preparation and small retrospective screens) can be performed on a single workstation, but more intensive docking will need access to a high-performance computing cluster (we regularly use 1,000 cores)

- Queuing system: DOCK3.7 comes with submission scripts for SGE, Slurm and PBS job schedulers. If using a different scheduler, scripts may need to be adapted for your specific computing cluster

- Protein structure: structures can be downloaded from www.rcsb.org or generated by the user in the case of homology models

- Example data: an example set of files used in this protocol including ligand and decoy sets, default docking grids and optimized docking grids can be downloaded from http://files.docking.org/dock/mt1_protocol.tar.gz. The example dataset uses the MT_1 structure (PDB: 6ME3) co-crystallized with 2-phenylmelatonin

Equipment setup

Define the pathway to critical software—The environment will need to be defined in order to run the following commands correctly. The two most important paths that need to be defined are to DOCKBASE and python. We provide an environment script in the example dataset that will need to be modified for a user's settings. Always run the following command with updated paths specific to your file directory before working with DOCK3.7:

```
source env.csh
```

Procedure

Section 1: set up binding pocket for docking calculations ● Timing 30 min to 1 h

1. Download the structure from the PDB or use in-house structures. Preference should be given to structures of high resolution and with a ligand bound in the site that will be docked into. Apo structures are useable, but tend to yield poorer results due to unconstrained binding site geometries[32].

2. Visualize the binding pocket in PyMOL or Chimera to decide on the relevance of protein cofactors.

    - Delete components from the structure that do not contribute to ligand binding such as lipids, water molecules and buffer components

    - Fusion proteins engineered for protein stability that are near the binding pocket should be removed, and the resulting chain break should be either capped or the loop remodeled

    - Protein cofactors such as heme or ions should also be kept or discarded depending on their relevance to ligand binding in the pocket

    - Additionally, examine the residues in the binding pocket for incomplete sidechain, multiple rotamers or engineered mutations, and revert or rebuild as necessary

    - It is helpful to examine the pocket with the electron density if available for making these decisions. For the example MT_1, we deleted the fusion protein, capped non-native termini and reverted two mutations in the binding pocket (6me3_cleaned.pdb)

3. Save the `rec.pdb` file. This file will contain any component that will remain static during the docking such as structural waters and cofactors.

4. Save the `xtal-lig.pdb` file. This file will contain the atoms of the bound ligand, which will be used to generate the matching spheres that guide ligand sampling in the pocket.

5. Generate the scoring grids and matching spheres with blastermaster from these two inputs (`rec.pdb` and `xtal-lig.pdb`)

```
$DOCKBASE/proteins/blastermaster/blastermaster.py --
addhOptions=" -HIS -FLIPs " -v
```

? TROUBLESHOOTING

6. Inspect the output, in particular the `rec.crg.pdb` file located in the new `working` directory to ensure protonation of polar residues and side chain flips of glutamine and asparagine side chains look accurate. Examine histidine tautomers (HID, HIE and HIP) as well. If everything looks as it should, proceed to Step 10; otherwise, proceed to Step 7.

Generating a `rec.crg.pdb` file manually

7 If the automatically protonated `rec.crg.pdb` file does not fit to the expected protonation state of key residues, the `rec.crg.pdb` file can be generated manually using various protein modeling software packages including Rosetta[112], Chimera[50] or Maestro[113]. This may include manually flipping side chain rotamers and setting the pH value for pK_a calculation of charged residues. After the modeling step is completed, new `rec.pdb`, `rec.crg.pdb` and `xtal-lig.pdb` files need to be provided to blastermaster. To be compatible with the united atom AMBER force field, `rec.crg.pdb` should only contain polar hydrogen atoms, and explicit histidine tautomer names. `rec.pdb` and `xtal-lig.pdb` only need to contain heavy atom coordinates. In Step 8, we provide a script that produces the required files.

8 Assuming the protein modeling software resulted in a fully protonated protein–ligand complex, protein atoms should be listed as ATOM records, while ligand atoms should be listed as HETATM records. Further, ensure that cofactors are given the correct heading in the PDB file (i.e., static metal ions should be given ATOM records if they are to be included in the scoring grids).

```
bash $DOCKBASE/proteins/protein_prep/prep.sh
protonated_input.pdb $PWD
```

The outputs are new `rec.pdb` and `xtal-lig.pdb` files, as well as a `rec.crg.pdb` file inside a `working` directory. HID, HIE and HIP naming is generated automatically from the protonation state of the HIS residues and again should be checked for accuracy.

Note: this script requires a path to the Chimera binary file and may need to be modified by the user.

9   Generate scoring grids and matching spheres from the new files (`rec.pdb`, `xtal-lig.pdb`, and `working/rec.crg.pdb`), and examine the outputs as in Step 6:

```
$DOCKBASE/proteins/blastermaster/blastermaster.py --
addNOhydrogensflag -v
```

Checking the files

10   Check that all files are generated. At the end of a successful blastermaster run, the directory should contain an `INDOCK` file and two directories: `working` and `dockfiles`. The `working` directory contains all the intermediate files used for grid generation. The `dockfiles` directory contains the scoring grid files and matching spheres file. The INDOCK file contains all parameters to control the docking program, such as sampling options and location of input files, i.e., docking grids and 3D ligand conformer libraries (see the INDOCK Guide in Supplementary Information). In our experience, the automated grid generation with `blastermaster.py` successfully produces reliable docking parameters; however, nonstandard amino acids or particular atom types require additional information and adjustments of force field parameters. Instructions on how to check and adopt the grid generation are provided in the Troubleshooting section below and in the Blastermaster Guide (Supplementary Information). For the provided example files generated for MT_1, the protein–ligand complex was modeled using the automated preparation pipeline in Maestro, Schrodinger[48].

Section 2: collect and build the control ligand set ● Timing Minutes to hours for ligand building, depending on the number of molecules

11   Collect the known actives (positive controls) for retrospective analysis. For a given target, these can be found in the scientific literature, patent literature or public databases such as IUPHAR/BPS[114], ChEMBL[115] or ZINC[9,99,116], or available in-house.

Curate the actives list—▴ CRITICAL While it may be possible to find dozens of actives, it is likely that many come from the same chemical series. For a rigorous control analysis, redundant (i.e., highly similar) compounds should be clustered and the most potent compound selected.

12   Sort all knowns by potency.

13   Cluster these based on 2D similarity using any preferred method. We suggest calculating clusters based on ECFP4 Tanimoto similarities with a cutoff of 0.35.

14   Use the most potent compound from each cluster as the representative of that scaffold. It is best to refine these actives to a set that is representative of the chemical space intended for prospective screening (i.e., with limits on molecular weight) so that the retrospective analysis will match the prospective aims.

15 Find out whether the known ligands of the target are neutral or charged as one criteria of the docking parameter calibration is the ability to score ligands with corresponding charges well.

16 Save a final list of actives' SMILES as `ligands.smi`. Typically, 10–30 diverse actives represent a good control set. For targets with less than this value, the controls are still useful but may not be as informative. For the example of MT_1 we extracted a set of 28 agonist and antagonists from the IUPHAR/BPS database[13].

Build the 3D conformer library of the actives

17 Go to the 'build3d' application on tldr.docking.org[79].

18 Add the `ligands.smi` file to the 'Input' section.

19 Select 'db2' for the file type.

20 Click 'Go' to submit.

21 Download the `build3d_results.tar.gz` file, and move it to your work directory.

22 Decompress the tar file:

```
tar xvfz build3d_results.tar.gz
```

23 The results are in a `build3d_results/` directory with two subdirectories `failed/` and `finished/` indicating the build status of the compounds. In example data provided, all ligands were built to completion.

24 To build a split database index file, or path file to the ligands, use the following command:

```
ls -d $PWD/build3d_results/finished/*.db2.gz > actives.sdi
```

25 To generate a list of actives' IDs:

```
ls build3d_results/finished/*0.db2.gz | awk -F"/" '{print
$NF}' | awk -F"_" '{print $1}' > actives.id
```

Curate and build the property-matched decoy set

26 Go to the 'dudez' application on tldr.docking.org[78].

27 Submit the `ligands.smi` file to the input section.

28 Click 'Go' to start the calculation. The ZINC database will be scanned for compounds that match the following six properties of the input ligands: molecular weight, LogP, charge, number of rotatable bonds, number of hydrogen bond donors, and number of hydrogen bond acceptors. Each compound will then be compared with the actives for 2D similarity, with similar compounds being discarded. A final set of 50 decoys per input active will be calculated.

29 Download the `decoys_dudez.tar.gz` file, and move it to your work directory.

30   Extract the files:

```
tar xvfz decoys_dudez.tar.gz
```

The files will live in a new directory `new_decoys`.

31   To obtain the split database index file of the decoys:

```
ls -d $PWD/newdecoys/decoys/*.db2.gz > decoys.sdi
```

32   To obtain a list of all the decoys' IDs:

```
awk '{print $2}' newdecoys/decoys.smi > decoys.id
```

33   **Collect output files**. At this point, four files should be generated: actives.sdi, actives.id, decoys.sdi and decoys.id.

Section 3:   run retrospective docking calculations to test the binding pocket parameters
● **Timing Minutes to hours depending on number of molecules and compute cores**

34   In the directory where blastermaster was run (i.e., contains the `INDOCK` file and `dockfiles` directory), copy over the ligand and decoys `.sdi` files and `.id` files.

35   Combine the two `.sdi` files:

```
cat actives.sdi ligands.sdi » controls.sdi
```

36   Check values in the `INDOCK` file

- Set the `atom_maximum` to 100. This value is a hard cutoff such that if a ligand has more than this number of heavy atoms it will not be docked. For retrospective calculations, we want all ligands to be docked and scored regardless of size, so we use a large value here

- Set the `mol2_maximum_cutoff` to 100. This value is a cutoff for saving 3D coordinates of docked poses. If a ligand scores worse (i.e., more positive) than this cutoff, the pose will not be saved. Setting a low value is useful in large-scale prospective screens to save on disk space and computation time, but for retrospective screens we want all information saved for analysis

37   Set up the docking directory

```
$DOCKBASE/docking/setup/setup_db2_zinc15_file_number.py ./
controls controls.sdi 1 count
```

This script will separate the `controls.sdi` file into one directory called `controls0000`. For this first calculation, the size of the sdi file is manageable on a single core, though splitting it will be faster (i.e., 10). For control calculations with 10,000–100,000 ligands and for large-scale prospective screens, the sdi file should be split into multiple directories and the jobs distributed over multiple cores. A `dirlist` file is generated that lists all of the split directories prepared for docking.

38 Dock the control set. At this point, it is possible to submit the data to a computer cluster or do the calculation on a single computer.

    A. Docking without submitting to a cluster

        i. Move into the `controls0000` directory. You will find `INDOCK` (pointing to the `dockfiles/` directory) and a `split_database_index` file. These are all the inputs that the DOCK program needs to run the calculation.

        ii. Run the docking calculation:

```
$DOCKBASE/docking/DOCK/bin/dock.csh
```

? TROUBLESHOOTING

        iii. The output files from the docking program are `OUTDOCK`, listing docking scores of successfully docked molecules, computational performance or potential error messages, and a zipped mol2 file (`test.mol2.gz`) containing the 3D poses of docked molecules.

        iv. Move back into the directory that contains `dirlist` when done.

    B. Docking with submitting to a cluster

        i. In the directory that contains the `dirlist` (created in the previous step), run one of the following commands depending on cluster architecture:

            • SGE: `$DOCKBASE/docking/submit/submit.csh`

            • Slurm: `$DOCKBASE/docking/submit/submit_slurm.csh`

            • PBS: `$DOCKBASE/docking/submit/submit_pbs.csh`

            ▴ **CRITICAL STEP** If you use another cluster scheduler, adopt these scripts to your needs.

39 When the job has completed, the last line of the `OUTDOCK` file should contain an elapsed time. If this is not the last line of the `OUTDOCK` file, an error has occurred (see Troubleshooting) and the following script will not run.

? TROUBLESHOOTING

40 Extract the scores of all docked compounds

```
python $DOCKBASE/analysis/extract_all_blazing_fast.py ./
dirlist extract_all.txt 100
```

The arguments are the `dirlist`, the name of the file to be written (`extract_all.txt`) and the max energy to be kept (this value should match the `mol2_maximum_cutoff` value in the `INDOCK` file). The important output file for future analysis is the `extract_all.sort.uniq.txt` file, containing the rank-ordered list of docked compounds with only the highest score for each compound.

41   Get the poses of the docked compounds

```
python $DOCKBASE/analysis/getposes_blazing_faster.py ./
extract_all.sort.uniq.txt 10000 poses.mol2 test.mol2.gz
```

The arguments are the path where the docking is located (`./`), the name of the `extract_all.sort.uniq.txt` file, the number of poses to get (`10000`, i.e., set to larger than the number of compounds docked for retrospective calculations since we want to get all), the name of the output mol2 file (`poses.mol2`), and the name of the input mol2 files (`test.mol2.gz`), containing 3D coordinates of predicted poses from the docking calculation (located in the `controls0000` directory).

42   Get the poses of just the actives

```
python $DOCKBASE/analysis/collect_mol2.py actives.id
poses.mol2 actives.mol2
```

The arguments are the file containing active IDs, the pose file containing all of the compounds both actives and decoys, and the name of the output file to be written.

43   Calculate the LogAUC early enrichment

```
python $DOCKBASE/analysis/enrich.py -i . -l actives.id -d
decoys.id
```

Inputs are the working directory ('.' if in the directory where the `extract_all` file is located) and the two files containing the IDs of actives and decoys. The output is a `roc.txt` and `roc_own.txt` file. Both files report an AUC and LogAUC and contain the grid points for plotting receiver operator curve (ROC) plots. The `roc.txt` file is calculated over all inputs in the ID files, and the `roc_own.txt` file is calculated only over the compounds that successfully docked.

44   To generate a plot (`roc_own.png`) of the `roc_own.txt` file:

```
python $DOCKBASE/analysis/plots.py-i . -l actives.id -d
decoys.id
```

45   Calculate the charge distribution by DOCK score

```
python $DOCKBASE/analysis/get_charges_from_poses.py
poses.mol2 charges
```

Inputs are the poses files and the name of the output file to be written. The output `charges` file is a list of DOCK scores and the charge of the ligand with that score.

46 To generate a plot of total DOCK score by ligand charge (`charge_distributions_vs_energy.png`) run:

```
python $DOCKBASE/analysis/plot_charge_distribution.py
charges
```

47 Plot the contribution of each score term in the energy function outlined in Equation 1 (`energy_distributions.png`):

```
python $DOCKBASE/analysis/plot_energy_distributions.py
extract_all.sort.uniq.txt
```

48 Collate the output files. The important outputs of these steps are: `actives.mol2`, `roc_own.png`, `energy_distributions.png` and `charge_distributions_vs_energy.png`.

Evaluate the docked poses of the actives

49 Open the `rec.pdb` and `xtal-lig.pdb` in Chimera, and prepare the visualization of the binding pocket to the user's preference.

50 In the Tools tab, select Structure/Binding Analysis and click ViewDock.

51 In the menu, navigate to your directory and open `actives.mol2`.

52 For file type, select any of the DOCK options. This will open a window (ViewDock) to navigate through all of the molecules in this file such as in Fig. 6a.

53 Under the 'Column' tab, several details about the docked ligands can be listed, e.g., Total Energy, Electrostatic or Van der Waals terms.

54 In the Tools tab, under Structure/Binding Analysis, 'Find HBonds' can be used to identify hydrogen bonds between the protein and ligand molecules.

55 Evaluate the docked poses, asking the following questions:

- Do the ligands occupy the part of the pocket as expected?

- Do they make the types of interactions anticipated from what is known about the ligands and the pocket?

- Are there aberrant or unsatisfied interactions? For example, in MT_1, it is known from the crystal structure that ligands can form hydrogen bonds with Asn162 and Gln181. Issues with binding poses are usually a sampling problem, but scoring terms such as electrostatics can influence specific interactions

- If many actives do not dock, is there a reasonable explanation (e.g., selected controls are larger than the pocket volume as has been

observed when attempting to dock antagonists into agonist pockets)?
Optimization of sampling is performed with a matching sphere scan as
detailed in Steps 59–64

Evaluate the enrichment and scoring metrics

56   Evaluate the docking parameters' ability to discriminate actives from decoys.
The `roc_own.png` file shows the plot of the rate of finding actives as a function
of decoys. The AUC is indicative of this discriminatory power (Fig. 6b). A
positive LogAUC value is a sign that actives are enriched over decoys, a value
near 0 represents random selection, and a negative value demonstrates that the
model prefers decoys over actives. This may be a result of poor poses, improper
scoring or both. It is best to optimize poses before pushing forward on scoring
discrimination. Even for good LogAUC values, it is important to evaluate the
poses as in Step 55 as it is possible to get good scoring actives that do not dock
in the correct pose.

57   Examine the energy contributions of the docked poses. In the
`energy_distribution.png` file (Fig. 6c), the total DOCK scores for the
docked poses are broken down into the main components of the scoring
function: electrostatics, VDW and polar ligand desolvation.

Do the contributions of the various score terms match the properties of the
binding pocket? In the MT_1 pocket, the VDW interactions dominate because
of its largely hydrophobic nature. For targets forming salt bridges to ligands,
electrostatic terms should at least be balanced with VDW scores. If the balance
does not match with what is expected for the pocket, the strength of the scoring
terms can be modified in Steps 65–68.

58   Evaluate the charge preference of the docking parameters. If the
electrostatics term dominates in Step 57, the scoring function will
likely prefer highly charged ligands. This can be measured in the
`charge_distributions_vs_energy.png` plot (Fig. 6d), which shows the
charge state of the top scoring molecules. DUDE-Z decoys should have charges
matching the active ligands. However, in property-unmatched decoys such as the
Extrema set[78] (Steps 78–88) and in-stock set (Steps 89–97), it is important to
ensure the docking is not biased toward charge extremes, which can suggest an
overweighting of electrostatic terms; this can be addressed in Steps 65–68.

Section 4:   **optimize poses by modifying matching spheres ● Timing Minutes to build;   1
h to dock**

59   Create a new directory `matching_sphere_scan/` that contains the `INDOCK`
file and `working/` and `dockfiles/` directory from the previous directory.

60   Run the following script to create sets of matching spheres in which the
matching spheres that do not map to atoms in the original `xtal-lig.pdb` file
are perturbed as in Fig. 7a: (~1 min)

```
python $DOCKBASE/proteins/optimization/scramble-matching-
spheres.py -i $PWD/dockfiles -o $PWD -s 0.5 -n 50
```

The arguments are: `-i`, the path to the `dockfiles/` directory; `-o`, the path to where new docking directories should be created; -s, the maximum distance to move a matching sphere (0.5 recommended); `-n`, the number of perturbed sphere combinations to create. For a single receptor target, we recommend 50–100 sets of sphere sets.

61 Each new directory should contain an `INDOCK` file and `dockfiles/` directory. Check that the `matching_spheres.sph` file in the `dockfiles/` directory is unique to each new directory created.

62 For each directory, copy over the `controls.sdi` file.

63 Run the docking calculation and extract the poses as in Steps 37–48.

64 Examine the new `actives.mol2` files and LogAUC values from the different matching sphere sets. Ideally, a set of spheres will increase the LogAUC and improve the binding poses of the actives. For MT_1, the LogAUC increased from ~2 to 5. While not a large change, there was improved placement of the flexible components of the active ligands, which suggests better sampling and pose identification in a prospective screen.

If the poses did not improve during this matching sphere scan, it may indicate that there is a problem with the binding pocket model, the ligand set, the sampling of ligand conformations, or the placement of the crystallographic matching spheres. Examining each of these can help improve the binding poses in this first control calculation. Alternatively, the scoring function needs to be optimized to score the expected pose better (see Steps 65–68).

? TROUBLESHOOTING

Section 5: optimize ligand scoring by modulating the protein–water dielectric interface (adding a layer of dielectric boundary spheres) ● Timing 15 min on cluster; 20–30 min per radius on local machine

65 Create a new directory with the `INDOCK` file and `working/` and `dockfiles/` directories from the best matching sphere set (or the default settings if no matching sphere scan was run).

66 Generate the boundary modifying spheres (Fig. 7b)

```
$DOCKBASE/proteins/optimization/boundary-sphere-scan.sh -b
$PWD -v
```

This version of the script will run on an SGE cluster. Modify the submission script and scheduler as needed for different cluster architecture. To run on a local machine, use the script:

```
$DOCKBASE/proteins/optimization/boundary-sphere-
scan_no_cluster.sh -b $PWD -v
```

67   Combine the different radii boundary spheres (5 min):

```
python $DOCKBASE/proteins/optimization/combine-grids.py -b
$PWD
```

This script generates a set of combinations of boundary modifying spheres across both ligand desolvation and low dielectric spheres. These directories have everything needed to run individual retrospective calculations except for the `controls.sdi` file that will need to be copied into each one. Repeat the retrospective calculations from Steps 37–48.

68   Following retrospective calculations across the combinations of dielectric boundary sphere radii, select a combination that ideally improves LogAUC (Fig. 6b) and helps to balance the score distributions in the `energy_distribution.png` plot (Fig. 6c). If there are a number of equally performing combinations, subsequent control calculations can help identify the best set for your system (see Steps 78–97). In the case of MT_1, we chose electrostatic spheres of radius 1.9 and desolvation spheres of radius 0.1 as they increased LogAUC and enhanced the charge distribution of compounds to favor neutrals to match known MT_1 ligands (Fig. 6d).

Section 6:   (optional): polarize (or depolarize) residues to effect electrostatics ● Timing 15 min

▲ **CRITICAL** If after these scans a particular interaction is still missed or erroneously captured, it may be necessary to modify the partial charges of a particular residue. For example, in the case of MT_1, the docking scores are largely dominated by VDW interactions (Fig. 6c). However, two residues Gln181 and Asn162 form hydrogen bonds with the known ligands. For these specific interactions, a global modification to the dielectric boundary (Steps 65–68) may not be sufficient and, instead, a local modification to partial charges as outlined in Steps 69–77 may enhance favorable scores for these interactions. For targets where this is not necessary, proceed to Step 78.

69   Make a new directory for polarizing residues. Copy over `rec.pdb`, `xtal-lig.pdb`, `working/rec.crg.pdb`, `working/amb.crg.oxt` and `working/prot.table.ambcrg.ambH` from the previous docking setup.

70   In the `rec.crg.pdb` file, rename the residues to be polarized with a unique three-letter amino acid code. For example, in the MT_1 receptor, we chose to polarize `GLN181` and `ASN162` to enhance the polar interactions between actives and these hydrogen bonding residues. Accordingly, we renamed `GLN181` as `GLD` and `ASN162` as `ASM` (see Fig. 8).

```
sed -i 's/GLN A 181/GLD A 181/g' rec.crg.pdb

sed -i 's/ASN A 162/ASM A 162/g' rec.crg.pdb
```

Generate partial charges for the new residues—▲ **CRITICAL** The two files that read in partial charges are the `amb.crg.oxt` and `prot.table.ambcrg.ambH` files

located withing the `working/` directory. These files contain default partial charges for all amino acids and some special residues such as ions. Make the following changes in both `amb.crg.oxt` and `prot.table.ambcrg.ambH` files.

71   Create new sections for the polarized residues in the two files by copying the standard residue partial charges.

72   Rename the polarized residues to match the three-letter codes used in the `rec.crg.pdb` file. The names are all capitalized in the `prot.table.ambcrg.ambH` file as shown in Fig. 8 and all lowercase in the `amb.crg.oxt` file.

73   Redistribute the charge around the atom of interest, making sure that the net charge remains the same. We suggest testing modifications of 0.2 or 0.4 charge units for a given atom. For example, for an `ASN`-to-`ASM` change, where we want to enhance the electronegativity of the sidechain carbonyl (`OD1`), increase the charge by 0.4 units, resulting in a change from –0.470 to –0.870. As –0.4 charge was added to the residue, +0.4 must be distributed to other atoms in the residue to maintain the net charge. One option is to add +0.2 to each of the sidechain amide hydrogens (`HD21` and `HD22`) as in Fig. 8, though the charge could have been distributed to the backbone amide or another atom not involved in binding.

Running the grid generation

74   Before running the grid generation, check that the following files are present: `rec.pdb`, `xtal-lig.pdb`, the modified `amb.crg.oxt` and `prot.table.ambcrg.ambH` files, and a `working/` directory with the modified `rec.crg.pdb` file.

75   Run blastermaster with the following command:

```
$DOCKBASE/proteins/blastermaster/blastermaster.py --
addNOhydrogensflag --chargeFile=amb.crg.oxt --
vdwprottable=prot.table.ambcrg.ambH -v
```

? TROUBLESHOOTING

76   Rerun control calculations as in Step 37–48. When examining the new poses, consider the following questions:

•   Does the polarized residue promote or discourage the desired interaction?

•   Are the new interactions scored more favorably?

77   Rerun the dielectric boundary modifying sphere scan in Steps 65–68. We recommend doing this because the new polarized residue(s) will have altered the electrostatics grid. It may be necessary to test different combinations and strengths (0.2, 0.4) of the polarized residues to identify the best-performing set of parameters that enhances correct interactions, improves poses and ideally improves LogAUC. However, due to the modification to the electrostatic

potential introduced by these changes, it is important to ensure these improvements do not come at the cost of biasing the screen towards overly charged molecules. This bias can be checked in the next section Steps 78–88.

Section 7:   Extrema charge control calculations ● Timing 2–3 h for compound building

Generate the Extrema set—▴ CRITICAL Compounds in the extrema set come from the area of chemical space occupied by the known actives (molecular weight and LogP) but distinct charges (−2, −1, 0, +1, +2). These decoys will be used to measure biases introduced into the scoring function owing to modifications to the electrostatic potential.

78   On tldr.docking.org, navigate to the 'extrema' application.

79   Upload the SMILES of your active ligands used in section II to the webpage.

80   Select 500 or 1,000 molecules per charge, and click the 'Go' button.

81   After a few minutes, a results file will be available to download and extract. Within the file should be five SMILES files grouped by charge. Extract the SMILES file from the download:

```
unzip extrema_results.zip
```

82   Build the extrema set.

83   For each SMILES file, submit the file to the 'build3d' application on tldr.docking.org. Depending on the number of ligands in each file, and the activity on the website, each file should take 2–3 h to build.

84   When all sets are built and downloaded, combine the path to all compounds in a split database index file along with the known actives.

Run the extrema set

85   Navigate to a directory containing the INDOCK file and dockfiles/ directory that you want to test. The dockfiles/ directory should contain the set of matching spheres and the dielectric boundary sphere combination selected from the previous steps.

86   Prepare the docking run. In the previous steps, the control ligand set was small enough to run in a single run. This control set often has 10,000+ compounds, and it is best to split this into multiple jobs. To do this run:

```
$DOCKBASE/docking/setup/setup_zinc15_file_number.py ./
extrema extrema.sdi 100 count
```

87   Submit the docking calculations over a cluster as in Step 38B.

88   Repeat the analysis in Steps 39–48 with a focus on the charge_distributions_vs_energy.png plot (Fig. 6d). An optimal set of docking parameters will have good LogAUC with early enrichment, and the top-ranking compounds will contain charges that match the charges of the known actives. Sometimes multiple boundary-modifying sphere combinations

will be tested in this Extrema calculation to find a set that enhances the scoring preference for compounds similar to knowns.

Section 8: run a 'small'-scale in-stock screen ● Timing 1–24 h depending on download speed

89 To collect an in-stock screening library, go to http://zinc20.docking.org/tranches/home/.

90 At the top of the tranche viewer (Fig. 9), select '3D' representation, set Purchasability to 'In-stock', and select the charge states of interest to your target (i.e., only 0). Next, select the molecular weight range you want to screen. Finally, select a LogP range tailored to your target. If no specific LogP range is desired, we recommend LogP 3.5 to avoid insoluble compounds that might aggregate and yield false positives in the experimental assay.

91 When only the tranches of interest are selected, click the download button in the upper right corner of the screen. Select 'DOCK37' as the download format and 'cURL' or 'WGET' as the download method. When done, click 'Download' to obtain a text file with all the cURL or wget commands needed to download the set of selected tranches.

92 Prepare a split database index file with the path to all of the newly downloaded compounds.

93 Split the docking run over 1,000 jobs to efficiently run the screen as the number of compounds is usually ~1–4 million.

```
$DOCKBASE/docking/setup/setup_zinc15_file_number.py ./
instock instock.sdi 1000 count
```

94 Run the screen on a cluster as in Step 38B.

95 Run the analysis of the screen to generate a LogAUC. It should be on par or better than LogAUC values seen previously, as the set of decoy molecules grows in size and departs further from the set of known ligands.

96 Test out hit picking strategies. Use any metric for filtering hits (Table 2), and visually examine the hits that pass the selected filters.

- Are the compounds forming the expected interactions with the binding pocket?

- Are they sampling in the correct region of the pocket?

If there are any issues, determine if it is a sampling or scoring problem and alter the previous steps (matching spheres or dielectric boundary modifying spheres, respectively) to optimize. If the top poses capture expected interactions, then it is worth moving into a large-scale prospective screen.

97 Choose settings for the large-scale docking experiment. If multiple docking parameters were tested in this section (i.e., section 8), one method for selecting the best settings for large-scale docking is to choose the parameters that yield

the greatest number of viable compounds after all of the post-docking filters have been applied. This will ensure a large number of hits in the large-scale campaign. Alternatively, one can purchase hits from the different screens and see which setup leads to more true actives in an experimental setting. Even if just a few low-potency hits are obtained at this point, it may suggest which interactions are critical for activity in the pocket.

Section 9:   large-scale docking ● Timing 1–5 d, depending on number of compounds and compute nodes

98 Create a new directory for the large-scale campaign with the final set of parameters, i.e., the `INDOCK` file and the `dockfiles/` directory.

99 Change the `mol2_maximum_cutoff` in the `INDOCK` file to a value likely to eliminate ~90% of the docked molecules to save on disk storage as compounds with total scores worse than a certain value (more positive) are unlikely to be considered hits from the large screen and should not be saved (use the results of the in-stock screen to help inform on the best `mol2_maximum_cutoff` values to use).

100 Obtain the ZINC20 library. The ZINC20 library contains a prebuilt 3D conformer library of nearly 700 million fully protonated and tautomerized compounds (i.e., protomers). The library is largely built on Enamine's readily accessible library, which consists of molecules that have a >80% likelihood of synthesis in one or two reaction steps[11]. This library can be downloaded from https://files.docking.org/3D/ or using the tranche browser as in Fig. 9 and consists of ~60 TB of data.

101 Select properties of the ZINC20 library for the screen such as molecular weight, charge and LogP. We recommend screening compounds separately by MWT range (i.e., fragments   250 amu) as larger compounds often score better due to their ability to form additional interactions within the binding pocket[117]. To obtain a split database index (sdi) file for a predownloaded ZINC20 database, use the 'DOCK Database Index' download format in the tranche viewer (Fig. 9) and provide a path to the downloaded database in the 'ZINC DB Root' field.

102 Split the docking campaign over 1,000 or more jobs. Typically, ~100 million compounds can be screened per day on an academic 1,000 core cluster of recent vintage (as of 2020).

```
$DOCKBASE/docking/setup/setup_zinc15_file_number.py ./
largescale docking zinc20library.sdi 1000 count
```

103 Run the screen on a cluster as in Step 38B.

Section 10:   hit picking ● Timing Days to weeks

104 Extract the scores of the top compounds as in Step 40 with the maximum score cutoff set to the mol2_maximum_cutoff set in Step 99

```
python $DOCKBASE/analysis/extract_all_blazing_fast.py ./
dirlist extract_all.txt -50
```

105 Collect between 300,000 and 1,000,000 poses from the best-scoring compounds.

```
python $DOCKBASE/analysis/getposes_blazing_faster.py ./
extract_all.sort.uniq.txt 1000000 poses.mol2 test.mol2.gz
```

106 Use any number of post-docking filters including but not limited to those described in Table 2.

107 From the compounds that remain after filtering, visually examine up to 5,000 molecules depending on how viable the docked compounds look. We recommend that visual examination is done by more than one person, because of the different knowledge base that each individual brings to the selection process (i.e., a medicinal chemist will prioritize different features over a molecular biologist and vice versa)[19]. This step should be done before prioritizing compounds for purchase, as we have found human-picked compounds have better efficacy than compounds obtained from fully automated hit-picking[12]. By using post-docking filters as in Step 106 prior to visual examination, one can search more deeply through the list of top ranked molecules to identify molecules for testing.

108 Choose 50–200 compounds for wet-lab testing depending on price, capability of testing and confidence of success as informed from retrospective calculations.

Troubleshooting

Structure preparation

Alternative conformations or missing side chains should be completely modeled before starting the grid generation (`blastermaster.py`). Otherwise, any superfluous or missing atoms will result in erroneous VDW surface or partial charge calculations (see below).

Blastermaster

Section 1, Step 5—All input and output files as well as options and flags of the blastermaster program are listed in the Blastermaster Guide (see Supplementary Information). Of important note is that blastermaster requires protein and ligand structures to be provided as PDB files called `rec.pdb` (and `working/rec.crg.pdb` in case a protonated structure is used) and `xtal-lig.pdb`. Alternative file names will not be recognized in the default settings.

Section 1, Step 10—If blastermaster does not successfully finish the grid generation, log files in the `working` directory will yield the required information to backtrace the error (see Blastermaster Guide in Supplementary Information). We suggest running blastermaster in the verbose mode (-v) as it allows to easily detect at which step the program failed.

Most common errors are related to unknown atom or residue types in `rec.pdb` (and/or `rec.crg.pdb`). Missing parameters for specific atom types can be added to the following parameter files:

```
$DOCKBASE/proteins/defaults/radii (required for surface calculation
by the dms program)
```

```
$DOCKBASE/proteins/defaults/amb.crg.oxt (partial charges for qnifft)
```

```
$DOCKBASE/proteins/defaults/vdw.siz (Van der Waals radii for qnifft)
```

```
$DOCKBASE/proteins/defaults/prot.table.ambcrg.ambH (atom typing for
chemgrid)
```

```
$DOCKBASE/proteins/defaults/vdw.parms.amb.mindock (Van der Waals
parameters for minimizer)
```

In our experience, most protein input structures can be converted into complete docking grids with the default parameters provided in DOCKBASE. For some modifications such as capped termini or structural waters, parameters are included in the provided files (e.g., `amb.crg.oxt`) but may use slightly different atom names compared with default names from modeling programs such as Chimera, PyMol or Maestro. In these cases, we suggest adapting the corresponding atom names in the input protein structure files (`rec.pdb`, `rec.crg.pdb`) rather than adding more atom types to the default parameter files. Common naming errors occur for disulfide bonded cysteines (`CYX` in `prot.table.ambcrg.ambH`) and water atoms (`HOH, TIP, WAT` and `SPC` in `prot.table.ambcrg.ambH`).

The qnifft program may crash if too many atoms (>50,000) are present in `rec.crg.pdb`. While we do rarely encounter this problem, particularly large proteins or other molecular complexes may have to be reduced in atom number (e.g., removing fusion proteins or distal monomers). Typically, protein segments >20 Å away from the binding pocket are unlikely to influence the resulting docking grids.

In certain cases, blastermaster may be able to calculate all grids, even though some parameters might be missing. For example, if nonstandard amino acids are used, the protonation state as well as partial charges or VDW surfaces may not be computed correctly, but will not cause blastermaster to crash.

To check if all force field parameters were assigned to the protein structure correctly, we suggest visual inspection of docking grids (Chimera). The scoring grids (`vdw.vdw`, `trim.electrostatics.phi`, as well as `ligand.desolv.heavy` and `ligand.desolv.hydrogen`) are found in the `dockfiles` directory. The following scripts can be run in the `dockfiles` directory to convert the grids into dx files for visualization.

Convert VDW grid:

```
python $DOCKBASE/proteins/grid_visualization/create_VDW_DX.py
```

Input: `vdw.vdw, vdw.bump` (default)

Output: `vdw.dx` (default)

Convert electrostatics grid:

```
python $DOCKBASE/proteins/grid_visualization/create_ES_DX.py
trim.electrostatics.phi trim.electrostatics.dx
```

Input: `trim.electrostatics.phi`

Output: `trim.electrostatics.dx`

Convert desolvation grid:

```
python $DOCKBASE/proteins/grid_visualization/create_LigDeSolv_DX.py
```

Input: `ligand.desolv.heavy` (default)

Input: `ligdesolv.dx` (default)

To visualize the grids, first open the `rec.crg.pdb` file in Chimera. The dx files can be opened with the Volume Viewer application. The `vdw.dx` file should resemble the surface of the receptor. The `ligdesolv.dx` file demonstrates various solvation levels of the pocket (the grid itself is a continuum). By changing the level of the representation, the volume should fill the pocket illustrating the ligand moving from a less solvated state to the most solvated state. For the `trim.electrostatics.dx` file, it is best to set the minimum and maximum levels to −100 and 100, respectively. Then, set the colors to red for −100 and blue for 100. This now shows regions of positive partial charges (blue) and negative partial charges (red). For all grids, ensure that the representations match what is expected for the binding pocket and that there are no regions of missing grid points. If there are obvious holes in any of the grids, in close proximity to the ligand, certain residue types were not identified correctly and corresponding parameters could not be assigned.

A list of all partial charges assigned to `rec.crg.pdb` is given in `working/qnifft.atm` (column 11). If the total charge of the system does not sum up to an integer value, certain partial charges may not have been found in `$DOCKBASE/proteins/defaults/amb.crg.oxt`. Furthermore, if many partial charges for a given residue (or other structural components such as metals or water molecules) are 0, proper parameters were likely not assigned. Specific errors in partial charge assignment can be found in `working/OUTPARM`.

Docking

Section 3, Step 38A(ii)—The DOCK executable (`$DOCKBASE/docking/DOCK/bin/dock64`) will only read a file called INDOCK.

Section 4, Step 64—If ligands dock in poses far from the binding pocket, visualize the matching spheres to ensure they occupy the same area as the `xtal-lig`. The

`matching_spheres.sph` file in the `dockfiles` directory contains the set of matching spheres. To view this file in a protein visualization application, they first must be converted to a pdb file.

```
csh $DOCKBASE/proteins/showsphere/doshowsph.csh matching_spheres.sph
1 matching_spheres.pdb
```

Input: `matching_spheres.sph`

Output: `matching_spheres.pdb`

The output file, `matching_spheres.pdb`, can be visualized with the receptor and ligand in either Chimera or PyMOL. The matching spheres should align to the ligand's heavy atoms, and additional random spheres should be scattered around the ligand as in Fig. 7a.

If many ligands fail to dock (>50%), a number of issues may be at fault. It is common for 10–20% of ligands to fail to dock in retrospective calculations because of mismatches between the pocket and ligand properties. In the OUTDOCK file, two common statements may indicate these issues: 'Grids too small' and 'Skip size'. The 'Grids too small' statement indicates that the ligands do not fit in the binding pocket. The 'Skip size' statement is used when the number of atoms in the ligand exceeds the atom_maximum value in the INDOCK file. Adjusting this number to a higher value will ensure that larger ligands will be docked and scored. If the OUTDOCK file shows many compounds getting scored but the poses are not being saved, the mol2_maximum_cutoff value may be too stringent. Relaxing this term will save more poses, but be careful to not go too high as the size of the output files will correspondingly increase and may be an issue for disk storage capacity. Additional INDOCK parameters can be adjusted according to the INDOCK Guide available in the Supplementary Information.

Anticipated results

At the end of this protocol, a receptor will have been converted into a dock-readable binding pocket, the system optimized on retrospective control calculations, and the system screened prospectively against a large chemical library. While this protocol has been specific to a single target that yielded successful results[13], we have equally applied this protocol to a number of targets with general success demonstrating its broad applicability[12,83,118]. We have attempted to curate all of the relevant steps, though expert intuition brought by each user for their target will result in a different selection of final hits for purchase. For these intuition-based steps, we recommend multiple people review the data before making the final decision regarding which compounds to buy.

If none of the purchased compounds demonstrates activity at the target of interest as determined experimentally, there remain several reasons for failure, including:

- The calculations were performed using the wrong conformation of the binding pocket

- The compound library did not contain enough examples of the class of compound most likely to dock

- Retrospective optimization was not possible due to lack of known chemical matter

- The experimental structure contained ambiguities inside chain or loop placement due to poor electron density or refinement errors

- Incorrect or low-quality homology model

For example, targets that bind dianions or dications will suffer from much smaller library sizes, while targets that bind large protein ligands may be difficult to modulate with small molecules. As in experimental biology, no set of retrospective controls will ever guarantee prospective success, and the many approximations in docking ensure that we still measure success by hit rates.

Nevertheless, these controls and optimization steps can reduce obvious sources of failure, and allow one to better design a subsequent campaign. The experience of the field by now suggests that the approach is likely enough to succeed to be worth the investment, while the novel ligands that result can bring genuinely new biological insight to a field, a longstanding goal of the structure-based enterprise.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

An example set of files used in this protocol, including ligand and decoy sets, default docking grids and optimized docking grids, can be downloaded from http://files.docking.org/dock/mt1_protocol.tar.gz. The example dataset uses the MT_1 structure (PDB: 6ME3) co-crystallized with 2-phenylmelatonin.

Software availability

DOCK3.7 can be downloaded after applying for a license from http://dock.docking.org/Online_Licensing/index.htm. Licenses are free for nonprofit research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Box 1 |

Control experiments to identify false positives among tested compounds

Mechanism	Structural alert	Control experiments
Covalent Useful if sought, but if unintended are an artifact	Presence of an electrophile (e.g., Michael acceptor, α-halo-ketone, activated nitrile)	Incubation of ligand with the target typically increases activity. For soluble proteins, pre-incubate ligand and target at a concentration higher than the EC_{50}, and then dilute to below the EC_{50}. If irreversible, activity will reflect the pre-incubation concentration. If reversible, ligand activity will diminish over time
Redox cycling Almost always an artifact	Several chemotypes (e.g., rhodanines)	Test in the presence and absence of a reductant or radical trap, like β-mercaptoenthanol or ascorbic acid
Spectroscopic interference Assay disruption via light absorption or fluorescence[137-140], or inhibition of a reporter enzyme, like luciferase[141]	Multiple chemotypes, often highly conjugated	Spectroscopic interference will change linearly with compound concentration, following Beer's law, rather than sigmoidally as in a binding isotherm. Molecules that might be inhibiting a reporter enzyme used in the assay (e.g., luciferase) may be tested for inhibition against it
Colloidal aggregation Likely the most common single mechanism of artifact in early discovery	Multiple chemotypes. It is difficult to predict which compounds will aggregate. LogP > 3.5 or similarity to known aggregators can capture some of these, but many will be missed (http://advisor.bkslab.org/)	Run the assay in the presence and absence of a non-ionic detergent like Triton X-100 (for soluble proteins) or Tween-80 (for cellular assays). Right shifts of two- to threefold or greater in EC_{50} on detergent addition suggest compound aggregation. Detergent solutions should be made fresh daily. Control for the effect of the detergent on a well-behaved, nonaggregating ligand, which should be unaffected[93,142,143] Test for the formation of particles >30 nm in radii by dynamic light scattering[92,144] For assays that will not tolerate detergent, an inert protein can be added to prophylactically coat the colloids, protecting the assayed target from adsorption. BSA is commonly used, though most inert soluble proteins will work. The inert protein should be present in mg/mL concentrations[92,95] Colloids are liquid droplets of pure compound, and can typically be pelleted in a microfuge after a 20 min spin at low temperature. Draw off the supernatant and test for activity—if it is much reduced, the compound is likely an aggregator[32,96] As with covalent inhibitors, pre-incubation typically increases the activity of a colloidal aggregator. Unlike covalent inhibitors, incubation at higher enzyme concentrations will typically slow the onset of aggregate-based inhibition, eventually eliminating it almost entirely[92] Counter-screen against unrelated off-target model enzymes, with and without detergent. Common enzymes include malate dehydrogenase (commercially available) and AmpC β-lactamase (freely available from the authors)[145]
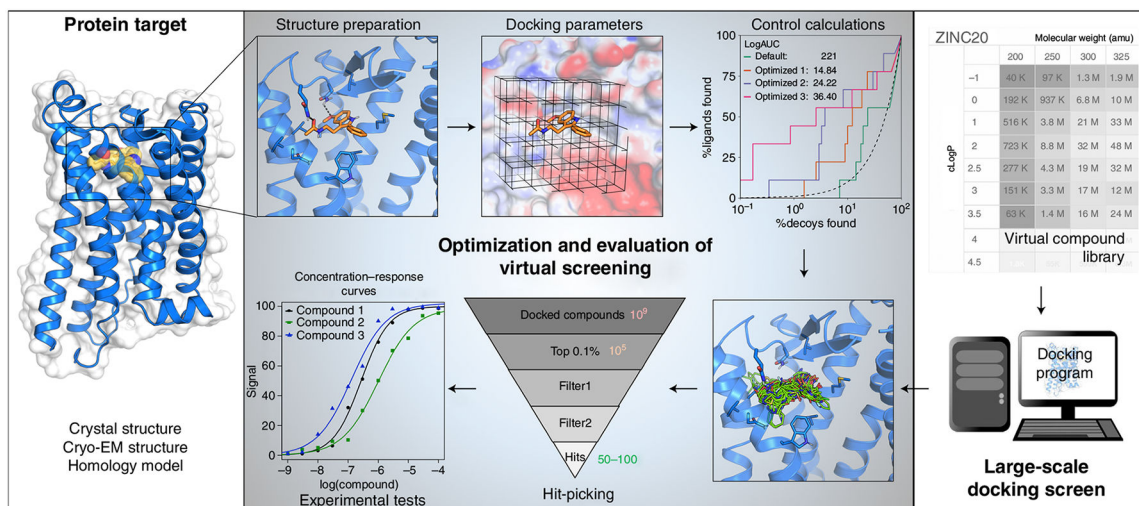
Fig. 1 |. Large library docking workflow.

The two required inputs for such a screen are the target structure and a screening database. Prior to using the database, the target structure must be converted into a representation used by the docking software and the pocket should be optimized with control calculations using retrospective analysis on known actives. After the prospective library has been docked, top-ranked hits can be filtered and selected for experiment. Multiple assays and controls are typically necessary to confirm activity.
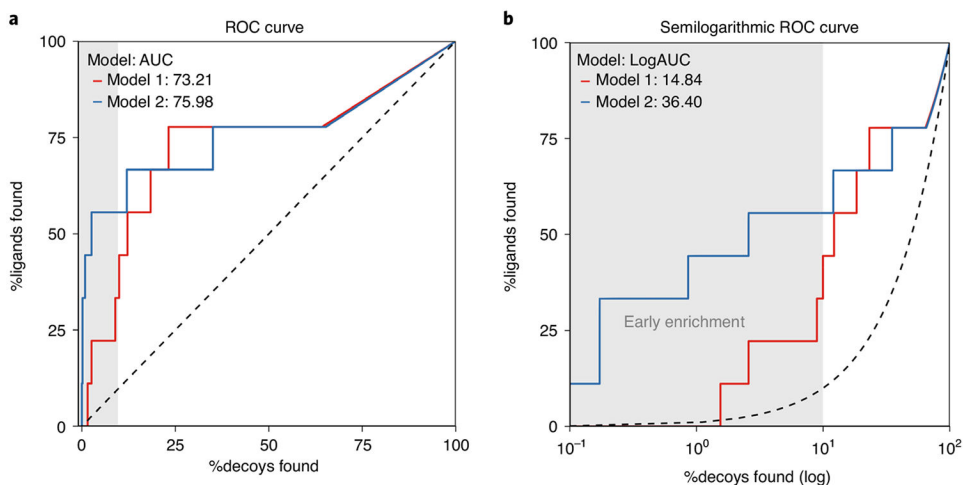
Fig. 2 |. Enrichment of actives against decoys.
a, ROC curves for two models used for retrospective docking screens plotting the rate of true positives found against decoys found. The AUC can be used to describe the ability of the models to identify true positive, known ligands against a background of decoys. In this format, the two models have similar AUCs, suggesting similar performance. **b**, Semilogarithmic ROC curves focus on the early enrichment, i.e., determine if true positives are identified within the e.g. top 10% (gray area) of docked decoys. The LogAUC is calculated as the difference between the semilogarithmic AUC of the model and the random semilogarithmic AUC (dashed line). In this format, it is clear that model 2 outperforms model 1 in early enrichment with a LogAUC value more than double of model 1.

Decoy set	MWT	LogP	Charge	# HBA/HBD	Topology	# of decoys	Goal
Property-matched decoys (DUDE-Z)	Matched to actives	Matched to actives	Matched to actives	Matched to actives	Unmatched	50/active	Measure enrichment
In-stock set	Matched to actives	Matched to actives	Matched to actives	Any	Any	~10^6	Simulate performance in prospective screen
Extrema set	Matched to actives	Matched to actives	−2,−1,0,+1,+2	Any	Any	10^3–10^6	Test for bias
Physical screen inactives	Any					~10^3	Measure real-world performance

Fig. 3 |. Control sets for retrospective docking calculations.
For DUDE-Z decoys, properties of the decoys are either forced to match (green) or be different (red). Properties that are neither selected for or against are highlighted in yellow. In the Extrema set, the charge state is explicitly sampled.
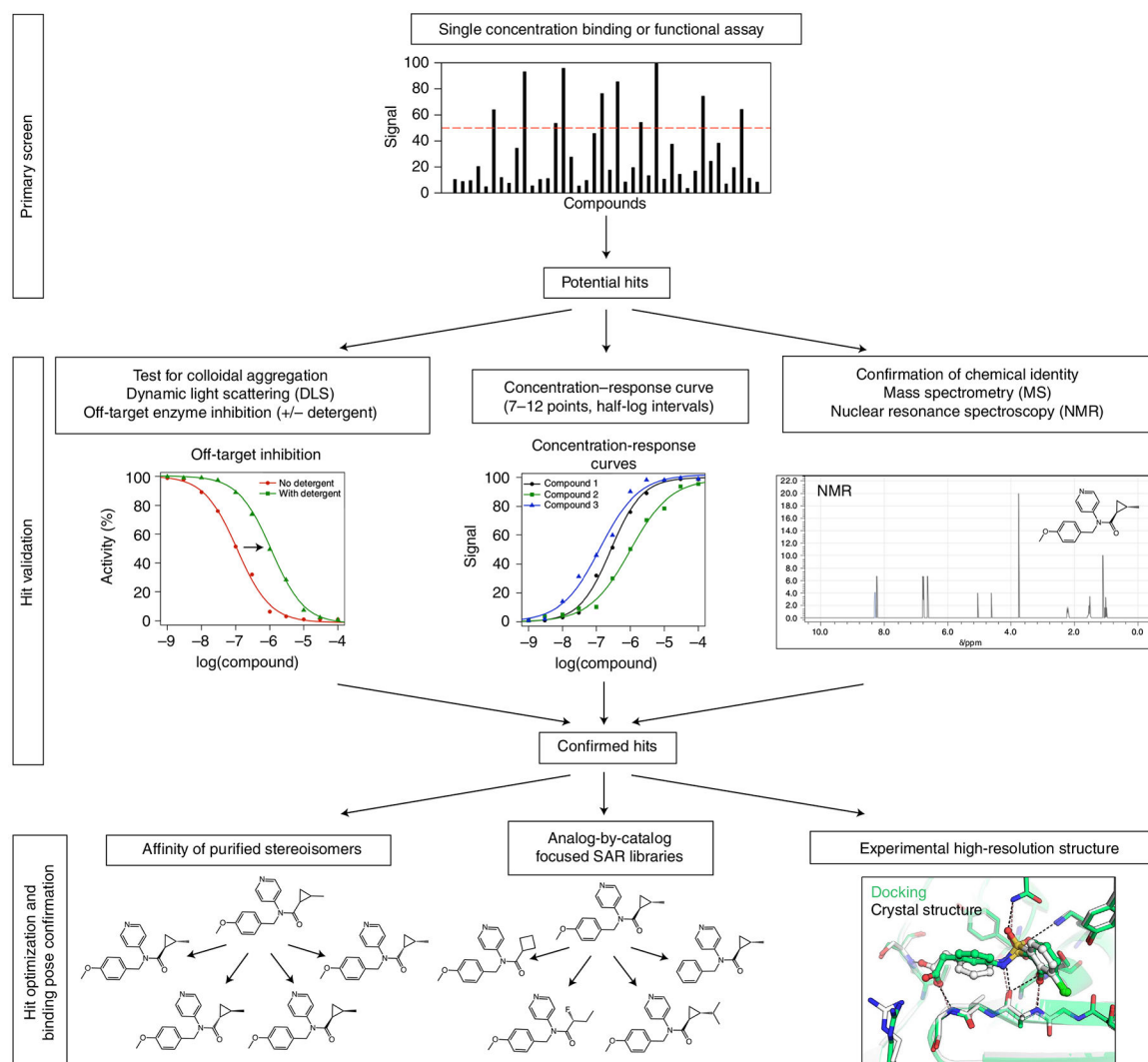
Fig. 4 |. Suggested experimental validation of docking hits.

In general, a primary screen will use a limited number of compound concentrations to test for activity at a target. Compounds that pass a set threshold of activity in the primary screen will be moved forward to secondary confirmation of activity that is not attributed to colloidal aggregation. Identity of the compound should be confirmed if it passes these stages and before proceeding to optimization by stereoisomer purification, selection of analogs and/or experimental structure determination.
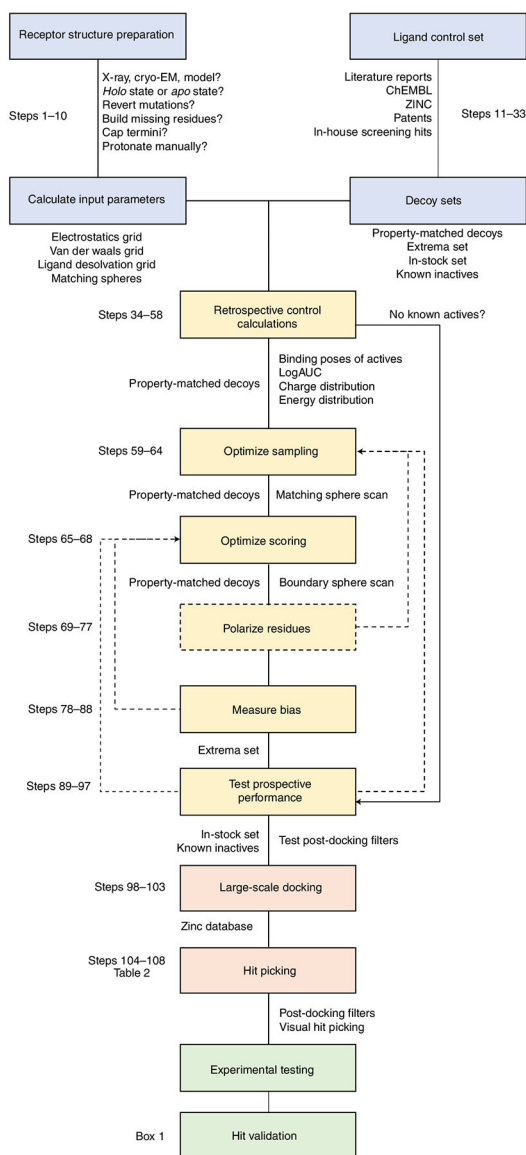
Fig. 5 |. Outline of the procedure for DOCK3.7 virtual ligand discovery campaigns.
Collecting and preparing materials (blue) requires obtaining a structure or model and
ligand control sets and setting them up for retrospective control calculations (yellow).
In each control calculation, modifications may demand returning to a previous step and
reoptimizing. In the absence of known actives for robust retrospective analysis, one may
jump to testing the prospective performance with a small library. With a final setup, large-
scale prospective screening (orange) can proceed, followed by in vitro testing of docking
hits (green). The numbers refer to steps described in the Procedure.
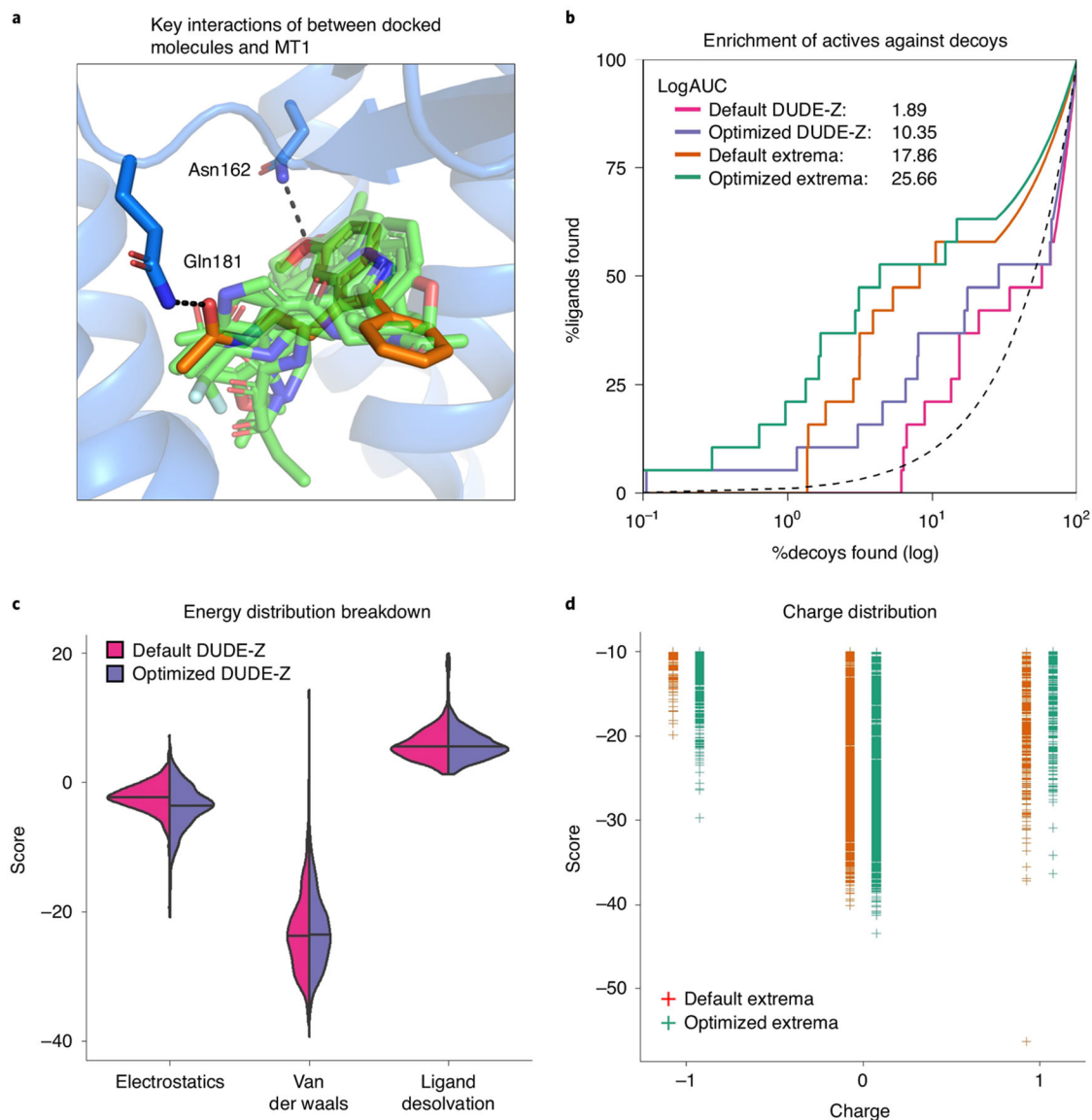
Fig. 6 |. Controls for docking optimization.
a, The receptor (blue) is shown with the crystallized ligand (orange). Docked control actives are shown in green and yield similar poses and interactions as the crystal ligand. The two residues, Asn162 and Gln181, that have their dipoles artificially increased ('polarized') to enhance the weight of polar interactions are shown hydrogen bonding to the crystal ligand. **b**, A log-transformed ROC plot is shown comparing the rate of identifying ligands versus decoys. A random selection would follow the dashed black line. The area under this dashed line is subtracted from the values reported for LogAUC such that a curve above the line would have a positive LogAUC, a curve below the line would have a negative LogAUC, and a curve following the dashed line would yield a LogAUC value of zero. Shown are the curves for the default settings and optimized settings for either the DUDE-Z control set and the Extrema control set. In both cases, the overall LogAUC value increases and the early enrichment improves. **c**, The energy distribution breakdown shows the individual score

terms for each scored molecule in the docked setup. Based on this breakdown, it is clear that VDW interactions primarily drive ligand recognition. However, in the optimized setup in which electrostatic spheres with a radius of 1.9 that extend the dielectric boundary are used, the electrostatic score term shifts to more negative values. The desolvation spheres at the dielectric boundary in the optimized setup, with a radius of 0.1, have only minor effects on the ligand desolvation score term. **d**, In the Extrema challenge, the top-ranking ligands are plotted by their charge and DOCK score. In the Default settings, there is a preference for neutral ligands followed closely by monocations. The Optimized settings enhance the preference for neutrals.
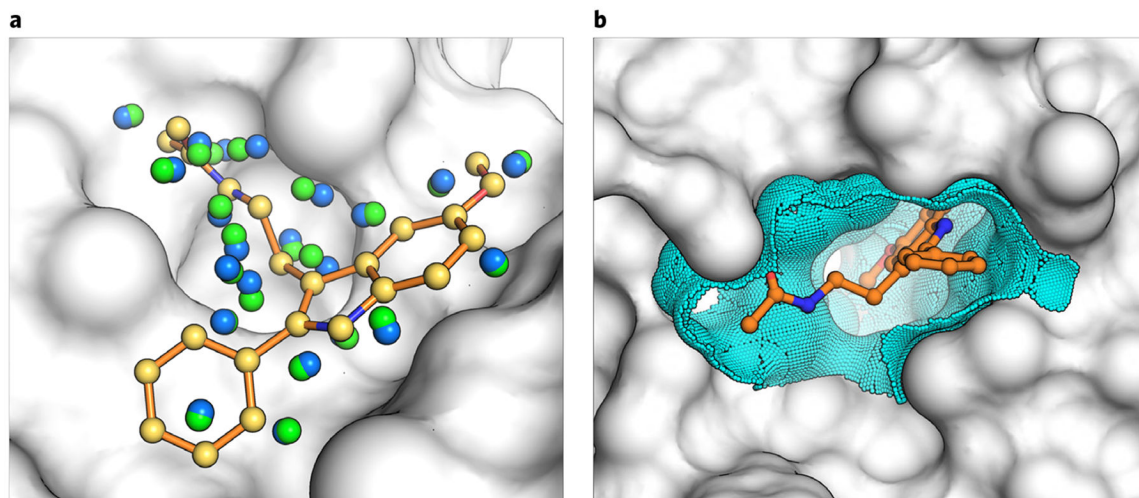
Fig. 7 |. Matching and dielectric boundary spheres drive changes to sampling and scoring in DOCK3.7.

a, the crystal ligand is shown as orange sticks in the receptor pocket (gray). Matching spheres derived from the coordinates of the crystal ligand are shown in yellow and remain fixed during sphere perturbation. Random spheres (blue) are calculated with the program SphGen, and a set of spheres are selected that are near the crystal ligand. In a matching sphere scan, only the random spheres are perturbed and a new set is obtained (green). **b**, the crystal ligand (orange) is again shown in the context of the receptor binding pocket (gray). Dielectric boundary spheres (cyan) cover the binding surface around the crystal ligand to alter the electrostatic or desolvation potentials at the boundary between solvent and protein.

N	ASN	−0.520	8	ASPARAGINE	N	ASM	−0.520	8	ASPARAGINE polarized
CA	ASN	0.217	4		CA	ASM	0.217	4	
C	ASN	0.526	1		C	ASM	0.526	1	
O	ASN	−0.500	11		O	ASM	−0.500	11	
CB	ASN	0.003	3		CB	ASM	0.003	3	
ND2	ASN	−0.867	8		ND2	ASM	−0.867	8	
CG	ASN	0.675	1		CG	ASM	0.675	1	
OD1	ASN	−0.470	11		OD1	ASM	−0.870	11	
H	ASN	0.248	6		H	ASM	0.248	6	
HD21	ASN	0.344	6		HD21	ASM	0.544	6	
HD22	ASN	0.344	6		HD22	ASM	0.544	6	
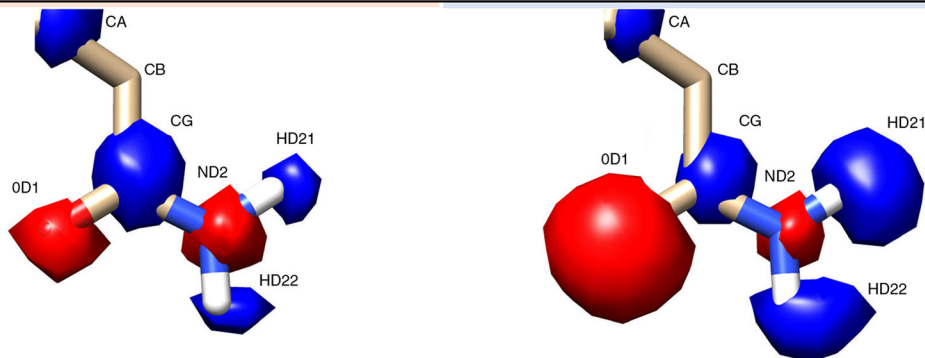
Fig. 8 |. Polarizing effects specific atoms' electrostatic potential.
In contrast to global modifications to the electrostatic potential with the incorporation of thin spheres, polarizing allows for very specific modifications to a residue's charge status. A canonical asparagine (ASN) from the `prot.table.ambcrg.ambH` file is shown with its polarized version ASM in which the carbonyl becomes more electronegative while the amide hydrogens become more electropositive to maintain the overall charge. The electrostatic potential corresponding to each atom is shown as spheres, with red corresponding to negative charge and blue corresponding to positive charge.
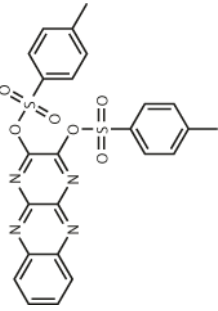
Fig. 9 |. Navigating the ZINC20 Tranche Viewer.
Several options are available at http://zinc20.docking.org/tranches/home/ for selecting
different subsets of ligands for virtual screening. Important criteria such as selecting
between 2D/3D, purchasability, charge, molecular weight and logP are highlighted. To
download compounds, different methods such as downloading as an index file or directly
downloading with cURL and WGET are shown.

Table 1 |

Some recent large-scale docking campaigns

Screen target	Library size/type	Best hit	Docking rank	Docking program	Computing time	Hit rate (hits/molecules tested)
D_4 dopamine receptors[12,37]	138 million (make-on-demand)	ZINC621433144 EC50 = 180 pM	Top 0.07%	DOCK3.7	4.2×10^4 core h (1 s/molecule)	24% (58/238)
	600,000 (in-stock)	Compound 9 EC50 = 213 nM	Top 0.33%	DOCK3.7	Not reported	20% (2/10)
AmpC β-lactamase[12,119]	99 million (make-on-demand)	ZINC339204163 Ki = 1.3 μM	Top 0.00001%	DOCK3.7[79]	4.1×10^4 core h	11% (5/44)
	69,000 (HTS library)	Compound 3 Ki = 37 μM	Not reported	DOCK3.5[120]	Not reported	12.5% (2/16)
MT_1 Melatonin receptor[13]	150 million (make-on-demand)	ZINC442850041 EC50 = 470 pM	Top 0.005%	DOCK3.7[79]	45,020 core h	39% (15/38)
KEAP1[14]	1 billion (make-on-demand)	iKeap1 Kd = 114 nM	Top 0.0001%	QuickVina 2[121] (initial screen) Smina Vinardo[122] and	30 s/molecule	11.7% (69/590)

Screen target	Library size/type	Best hit	Docking rank	Docking program	Computing time	Hit rate (hits/molecules tested)
				AutoDock Vina[23] (secondary screen)		
A2 adenosine receptor[38]	4 million (commercially available)	Compound 9 Ki = 32 nM	Within top 0.05%	ICM docking and Screening[123]	30 s/molecule	41% (23/56)
HisG[124]	500,000 (commercially available)	Compound 4 Ki = 2 μM	Within top 0.2%	GOLD[125] (initial screen) FLEXX[24] (secondary screen of top 1%)	~60 h on 350 networked PCs	14% (7/50)
Dipeptidyl peptidase IV (DPP4)[126]	190,000 (commercially available)	Compound 1 IC50 = 5.77 μM	Within top 0.05%	GLIDE25 (initial screen) AutoDock4[26] (secondary screen of top 2%)	Not reported	15% (15/99)

Docking rank indicates how highly ranked the best hit was among all virtually screened compounds. Hit rate denotes the fraction of docking hits confirmed in physical experiments.

Table 2 |

Hit picking criteria

Docking score	Docking scores are approximations of interaction energies and therefore cannot be used for absolute ranking of hits. Its main purpose is to identify compounds that are likely to bind from the large background of compounds that are unlikely to bind
Broken molecules	Errors in 3D building can result in incorrectly built compounds. Common errors include improper tautomerization or protonation states owing to the difficulty in predicting pK_a. Such 'broken' molecules should be deprioritized during visual inspection
Internal strain	Often internal strain is not included in energy functions but is an important concern since a ligand may score highly if it adopts a strained conformation that increases its contacts with the receptor (e.g., nonplanar amide). Visual examination can catch these strained compounds, but various tools have been developed to systematically analyze hundreds or thousands of compounds making them useful in this setting[127-129]. We recently proposed a computational tool that estimates torsion strain energies based on experimentally determined torsional populations in the Cambridge Structural Dataset[129,130]
Interaction patterns	Specific interactions to key residues are main features of promising candidates. Hydrogen bonds between ligands and side chains can be computationally identified by simple distance cut-offs (<3.5 Å). For example, hydrogen bonding to kinase hinge residues[131], or salt bridges to the conserved aspartic acid D3.32 in aminergic GPCRs[132], offer reasonable anchor points for interaction filters. In a similar fashion, a pharmacophore filter can be used to search for any atoms in an unexplored subpocket within the larger binding pocket. Molecules that score well but form only one key interaction may be deprioritized. 'Floating' molecules that may bury an energetically frustrated water should also be eliminated
Unsatisfied hydrogen bond donors and acceptors	Molecules with unsatisfied hydrogen bond donors or acceptors, especially in hydrophobic pockets of the site, often pay a high desolvation cost; not all scoring functions are designed to filter these out explicitly. Therefore, we suggest keeping the number of unsatisfied acceptors below 3 and the number of unsatisfied donors to 1 or below, as burying a donor can incur a greater penalty than burying an acceptor[133]
Novelty filter	One of the advantages of large-scale docking with make-on-demand chemical libraries is the ability to screen for novel chemistry. Therefore, if the goal of the project is to identify novel scaffolds for hits, it is useful to filter out docking hits that resemble known actives. This can be accomplished using any number of computational methods that compare the 2D or 3D topologies of hit compounds. In our hands, we typically remove hits that have an ECFP4 Tanimoto cutoff of 0.35 or higher to any known active[12,13]
Scaffold clustering	From the initial docking screen, a set of diverse scaffolds should be selected for experimental testing to cover a larger subset of chemical space. Clustering metrics to identify diverse hits may include ECFP4[134] fingerprints, Bemis-Murcko[88] scaffolds, bcl::Cluster similarity[135] or Atom Counts[136]