Docking Screens for Novel Ligands Conferring New Biology

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Abstract

It is now plausible to dock libraries of 10 million molecules against targets over several days or weeks. When the molecules screened are commercially available, they may be rapidly tested to find new leads. Although docking retains important liabilities (it cannot calculate affinities accurately nor even reliably rank order high-scoring molecules), it can often can distinguish likely from unlikely ligands, often with hit rates above 10%. Here we summarize the improvements in libraries, target quality, and methods that have supported these advances, and the open access resources that make docking accessible. Recent docking screens for new ligands are sketched, as are the binding, crystallographic, and in vivo assays that support them. Like any technique, controls are crucial, and key experimental ones are reviewed. With such controls, docking campaigns can find ligands with new chemotypes, often revealing the new biology that may be docking's greatest impact over the next few years.

Graphical Abstract
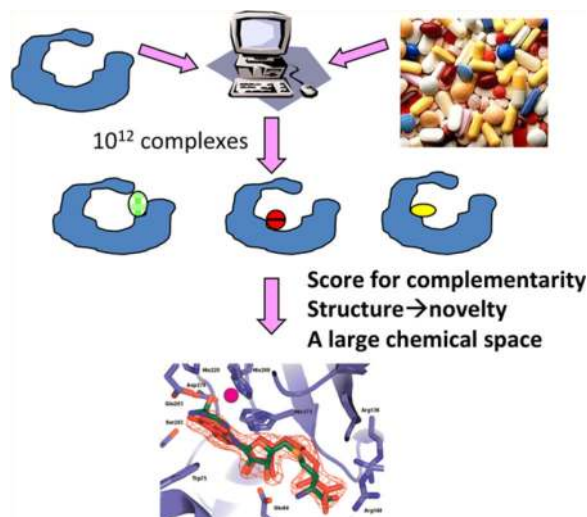
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STRUCTURE-BASED LIGAND DISCOVERY

Beginning in the 1970s, simulations of protein structures were promoted for structure-based or "rational" drug design.[1,2] The techniques struggled to meet their initial promise, and early discovery continues to be dominated by empirical methods. Still, in the past decade, drugs for which structure and computation were genuinely pivotal have begun to appear; close to 20 drugs, with clear links to structure-based discovery or design, are now in clinical use.[3–7] As few as they are, they may exceed those deriving from another widely heralded approach, high-throughput screening (HTS).[8]

DOCKING AND ITS DISCONTENTS

A central technique in structure-based discovery is molecular docking.[9] In docking screens, libraries of about 10^7 molecules can be interrogated for those that complement a protein structure (Figure 1). For each molecule in the library, hundreds to thousands of orientations may be sampled in the protein site, and for every orientation there are hundreds of conformations; increasingly, alternative protein conformations are also considered. Overall, 10^{12}–10^{13} ligand complexes may be calculated in a large library screen, each ranked using scoring functions that consider several polar, nonpolar, and solvent-dependent scoring terms, all approximate.

There are several first-principle reasons why docking screens might never work. Among them, docking's emphasis on throughput ensures that it undersamples states, principally protein conformations and ordered water networks. Even for ligand conformations, which are heavily sampled, the energy weighting among them is poorly treated or ignored. Docking complexes are rarely fully relaxed, leading to what amounts to irreversible work. Ligand and protein desolvation energies are at best approximate and are often entangled with docking interaction energy scores. These scores ignore important terms (among others: cation-π interactions and polarizability), and even the terms that are included are rarely well-parametrized for the diverse molecules in docking screening decks. The docking scoring energies are calculated all-at-once rather than via small perturbations that are central to techniques, such as free energy perturbation and alchemical thermodynamic integration, that can at least pretend to the calculation of relative and absolute affinities of binding. Over a calculation that samples 10^7 candidate ligands in 10^{13} configurations, any one of these terms could ensure the failure of a docking screen for plausible ligands.

PRAGMATIC SUCCESS: DOCKING AS A SCREENING TECHNIQUE

Despite what would seem to be eviscerating weaknesses, docking screens have been successfully prosecuted, discovering new ligands (Table 1)[10–65] that are increasingly confirmed with detailed biophysics, from concentration–response curves to K_i and K_d values of binding, to the comparison of subsequent crystal structures to the docking predictions (Figure 2). In blind, prospective comparisons with HTS against enzymes like HSP90,[66] PTP-1B,[67] β-lactamase,[68,48] and cruzain,[50,69] the docking "hit rates" (actives/tested) were 2–3 orders of magnitude better than for the empirical screen, and while HTS found docking false negatives, docking in its turn found false negatives from HTS.

How can these successes be reconciled with the weaknesses of the approach? Like other large-scale screens, docking seeks new observations, in its case new ligands, unanticipated from previous series, with the hope of conferring new biology. It emphasizes throughput and the polling of many possibilities and tolerates both false positives and false negatives as long as enough experimentally confirmed positives are found. Whereas docking can fail for any of the reasons sketched above, it happens that, crude as they are, the docking scores successfully reduce the many millions of molecules screened to a relatively small number of plausible candidates. As these candidates are available commercially or, for industrial users, from an in-house library, the cost of failure is cheap. Even a 10% success rate is often more than sufficient to justify the enterprise.

Here we describe practical strategies for successful docking campaigns and the resources that allow for their prosecution by a wide community, not just specialists. Because the enterprise depends on a close integration of the docking screen and the experimental testing of a few candidates, we will consider strategies to pick hits for testing and for the critical evaluation of the experimental tests. We will only summarize recent methods developments in docking, which have been well reviewed elsewhere.[70–74] We will close with a discussion of the major contribution of docking to early discovery in medicinal chemistry and chemical biology, which is the illumination of genuinely new chemotypes for targets of active biological interest.

STEPS IN A DOCKING CAMPAIGN

Docking is increasingly enabled for nonspecialists by Web tools (Table 2) and available compound databases (Table 3). Still, the approach integrates disparate methods, and there are several places it can go wrong if attention is not paid to maddening details. Among these are the protein structures on which docking depends, whether from X-ray crystallography, NMR, comparative modeling,[75–77] and soon electron microscopy. Binding sites can have incomplete, flipped, or ambiguous side chains, and some residues may occupy more than one position in the structure. Some binding site metals will lack parameters. Structural waters,[78] which can play a critical role in recognition, must be treated as part of the site, taken out, or treated as displaceable if the method can do so.

We find it useful to conduct retrospective sanity checks before undertaking a new, prospective docking campaign. When possible, annotated ligands may be gathered as positive controls, including any ligand-bound crystal structures if available. Large ligand sets may often be found in ChEMBL, and if so, they are likely also to be in ZINC, where they exist in a ready-to-dock form (Table 3). These sanity check calculations work best if the ligands are combined with property-matched decoys, which physically resemble the ligands but are topologically dissimilar.[79,80] We typically use about 30–50 decoy molecules for each ligand in these sanity-check screens, asking if the calculation can enrich the ligands at the top of the docking-ranked list, in sensible poses, compared to the decoys. Parameters may be modified to improve ligand enrichment, but overfitting will bias the ultimate prospective screens toward precedented chemotypes, defeating the purpose. Decoys can be calculated readily using DUD-E[81] (Table 2).

In prospective screens, large libraries of molecules are docked for those that complement the binding site. A focus on commercially available molecules (Table 3), for academic laboratories or on in-house libraries, for pharmaceutical research organizations, ensures that the molecules may be readily acquired and tested, which is crucial for the success of an enterprise that anticipates a 90% failure rate. ZINC incorporates many of these, amounting to over 100 million commercially available "druglike" compounds represented in precalculated, ready-to-dock formats. ZINC is easily subdivided by physical properties corresponding to current opinion in the field, such as "fragment-like"[82] and "lead-like"[83] subsets. Especially for those programs optimized for the parallel execution that docking allows and not limited by seat licenses, a full screen may be completed in a week or two of wall time on a typical academic cluster. What emerges at the end of the calculation is a list of molecules, rank-ordered by complementarity to the binding site by the docking scoring function in one or more high-scoring configurations in the binding site. These may be visualized individually and prioritized for purchasing and testing.

HIT PICKING PARTIES

Docking ignores or approximates key energy terms and over a screen of 10^7 compounds simply misrepresents molecules; strained conformations of ligands may be docked, pK_a values may be miscalculated or high-energy tautomers sampled, among other potential problems. Even if these errors affect only a small percentage of the docked library, the scum often rises to the top (for instance, high-energy ligand conformations and mischarged molecules can make interactions that low-energy states cannot, resulting in artificially favorable scores). Like other high-throughput methods, rapid filters for artifacts are crucial, as are chemoinformatic controls for similarity to known chemotypes. Since the purpose of a docking screen is often the discovery of new chemical matter, complementing a binding site but dissimilar to molecules that could be found by QSAR, for instance, it is sensible to eliminate those docking hits that would have been found by topological similarity alone (this control is insisted on by some journals, including this one).

In addition to automated, typically postdocking filters, visual evaluation of docked, high-ranking candidates can bring an integrated knowledge of the system and of physical chemistry. As an evaluation of HTS campaigns,[84] "hit-picking parties", involving two to eight scientists from a broader team, can help select a final set of high-ranking molecules to acquire and physically test; these molecules, though always from the very top scoring molecules from a screen, are rarely the top ranked by raw docking score. Rather, the molecules may be selected for diversity and for favored interactions, for instance, the prioritization of a key hydrogen bond. More important than prioritizing favored interactions, which after all can bias the ligands toward known chemotypes, is the deprioritization of ligands that have unfavorable features for which the docking scoring function does not properly penalize. As mentioned above, common examples include ligand conformational strain, improper tautomerization, and improper protonation of ionizable groups. Each of these can be a challenging problem in molecular representation, requiring relatively high level calculations for any given molecule,[85] and it is little surprise that in over 10 million molecules, errors are made. Sometimes errors that slip through the automated library creation pipeline can be caught by an experienced medicinal chemist or biophysicist and

deprioritized. Thus, it is often the case that a molecule that is ranked below 250th will be prioritized over a molecule that is ranked better than 10th. These hit-picking parties have a secondary benefit of training a wider team in molecular interactions, medicinal chemistry, and target biology.

With docking's current accuracy, we often find that acquiring and testing 25–50 new molecules is sufficient to find two to five new ligand families. Sourcing this many compounds from commercial vendors is typically economically feasible even for academic laboratories (many more may be considered by pharmaceutical research organizations), and this number of compounds is often suited to the low throughput, careful assays in which docking hits are tested. For targets with better binding sites, like aminergic G protein coupled receptors (GPCRs), the number of compounds to acquire might be lower, while for targets with more solvent accessible sites, like proteases or β-lactamase, the number of compounds to acquire will be higher because the success rate will be lower. Improvements in docking methods, compound accessibility, and ease of testing will change these numbers, but the logic of testing enough molecules to ensure a sufficient number of plausible new lead chemotypes, given an expected hit rate, will remain valid.

CRITICAL EVALUATION OF EXPERIMENTAL RESULTS

Whereas there remain docking campaigns where no molecules[86–118] are tested whatsoever, the most interesting are those that lead to experiments. To the delight (and sometimes the surprise) of the dockers, they often reveal "hits", molecules that are active in a binding or functional assay. As with other screening techniques, these initial experimental hits must initially be viewed with suspicion, as they can be pock-marked with artifact.

The origins of these experimental artifacts have been[119–122] extensively reviewed: they can be broadly divided into molecules with pan assay interference (PAINS) chemotypes, which are promiscuous hitters,[120] covalent acting molecules[123] (but see below), molecules that interfere with assay spectroscopy,[124–126] and molecules that form colloidal aggregates[127–129] (Figure 3). Hits with PAINS chemotypes[130–133] are not always pathological (over 60 FDA-approved and worldwide drugs contain PAINS chemotypes) but often are and should be flagged for detailed follow-up if not simply discarded. Many PAINS chemotypes can be rapidly identified using publicly available filters (e.g., http://zinc15.docking.org/patterns/home, Appendix 1). Sharing no single mechanism, they fall to no single set of controls but can be interrogated for classical dose–response curves, lack of incubation effects, imperviousness to mild reductants, and specificity versus counterscreening enzymes (all favorable features, arguing against artifactual activity). Covalent and spectroscopic interference molecules do have specific physical mechanisms for which they can be controlled (Appendix 1). By far the most common artifact from virtual and high-throughput screening campaigns is colloidal aggregation. Between 1% and 3% of the molecules in most screening libraries will aggregate at relevant concentrations, and the colloids that they form sequester and artifactually inhibit,[68,69,129] and occasionally activate,[134] proteins. Fortunately, this mechanism is readily controlled (Appendix 1). Studies that omit controls for these experimental artifacts may waste person years of effort as hopeless compounds are progressed.[135–138,133,139]

Detailed biophysical testing of new inhibitors for mechanism is always illuminating, irrespective of whether one suspects artifacts. There is an understandable tendency to fall in love with early hits, but hard experience[127] suggests that doing so can obscure well-behaved, optimizable molecules that one would find if not distracted by the false positives. Measuring, and publishing, full concentration–response curves is a simple but crucial step toward this; much can be learned from the steepness of the curve and how well it is sampled (we recommend sampling every half-log in concentration and low and high enough in concentration to establish upper and lower baselines). A step further is to measure the full binding constant, through enzymological K_i, radioligand displacement or by reporter-free methods such as isothermal titration calorimetry, surface plasmon resonance, or related techniques. Here too, full curves should be measured and reported.

IMPROVEMENTS IN DOCKING

Investigators have long sought fundamental breakthroughs that would address docking's core liabilities. Perhaps because of its entangled approximations, docking has resisted dramatic improvements and has had to be content with incremental progress. Still, over time, these have made noticeable improvements. Methods have added new polar interaction terms, flexible waters,[78,146–151] metal coordination,[152,153] protein preparation tools,[154] parallelization,[155–157] combinatorial library docking,[158] new sampling methods, and treatments ordered waters, often using a version of inhomogeneous solvation theory.[159–161] Just as important as these improvements to docking itself have been improvements in the quality and extent of the chemical libraries. Among the most widely used is the ZINC database, which has grown from about 750 000 commercially available molecules a decade ago[162] to over 100 million for which three-dimensional, parametrized, dockable structures are now available.[163] The quality of the molecules represented has also improved, drawing on improved treatments of molecular protonation and tautomerization,[164,165] as well as conformational libraries that are more likely to sample the experimentally observed form.[166] Finally, the druggability of docking targets has improved. Over 60% of drugs act on membrane proteins, but 10 years ago the structures of few pharmacologically relevant membrane proteins had been determined, something that has changed dramatically in the past decade. These receptors, with their well-formed ligand binding sites, are often well-suited to complementarity-based approaches like docking and have afforded hit rates that are almost a log-order better than those experienced with the more open sites typical of soluble proteins, and hits that can be 2–3 log-orders better in affinity. As unglamorous as it seems, incremental improvements in docking methods, libraries, and target selection will continue to be central to progress in the field over the next few years.

What would a big breakthrough in docking look like? If one could accurately calculate binding free energies or even accurately rank-order among the top 0.1% of docking-prioritized hit-list (about 10 000 molecules), the impact would be enormous and would justify substantial investment. In principle, the biophysical bases for calculating accurate binding constants are in hand via alchemical methods and thermodynamic integration,[167,168] though these methods are only beginning to be tested prospectively.[169,170] Even with recent advances in long molecular dynamics simulations,[171–173] however, these approaches remain far too slow to be practical on the scale of a large library screen. Calculating the K_d values of

a 10 million molecule, diverse library would take decades, even with the increases in computer power likely in the next several years. Meanwhile, lack of proper parametrization of the library molecules ensures large errors, even for these high-end methods. Where the more accurate biophysical methods may have impact on a large scale screen, in the next few years, is in rescoring a top tranche of a docking hit-list, helping to prioritize the final few that will get tested. They can also improve the accuracy of docked geometries, which is crucial for the enterprise and is where docking can add the most once campaigns have progressed from initial discovery to optimization. Such goals for the high-end biophysical methods may seem modest, but if successful, they would have great impact.

FRAGMENT AND COVALENT DOCKING

Fragment-based discovery addresses the chemical space problem by screening molecules typically smaller than ~17 non-hydrogen atoms (about 250 amu). Just as chemotype possibilities combinatorially explode as molecules grow in size, they collapse as they shrink, and fragment screens span a far greater percentage of chemotypes than screens of the druglike (up to 500 amu) or even leadlike (up to 350 amu) molecules. As powerful as fragment screens have been for chemotype discovery, empirical fragment libraries are rarely more than 10 000 molecules, owing to the low throughput of the biophysical methods used to assay them. This is less than needed to cover even known biorelevant chemotypes,[174] and the empirical libraries are dwarfed by the 2.4 million fragments that are simply commercially available. Screening the commercially available fragments is beyond biophysical screening methods used in fragment-based discovery but would take a few days on a decent academic cluster by docking.

The concern in fragment docking has been that the methods have been optimized for larger molecules, and docking would lead to promiscuous prediction of fragment poses. This worry has merit, as newly discovered docked fragments often have less fidelity to X-ray structures than do those of larger molecules; many in the field believe that fragment docking remains risky. Still, many predicted fragment geometries are fairly accurate, and the hit-rates in fragment docking can be a log-order better than leadlike or druglike screens.[51,52] With all their potential liabilities, fragment docking can fill chemotype holes in empirical libraries, sometimes leading to hits with much higher affinity and ligand efficiency than empirical screens alone.[174]

The gap in our libraries for covalent probes is more compelling still. From bitter experience with false positives, most empirical screening libraries have been scrubbed clean of hot electrophiles, making it hard to intentionally screen a large library for covalent ligands. Conversely, electrophiles remain common among commercial libraries; among leadlike molecules in ZINC, over 600 000 can act covalently as Michael acceptors, epoxides, boronic acids, nitriles, and other war-heads[175] (http://covalent.docking.org). Recently, several methods have been introduced to intentionally screen such libraries for covalent ligands[175–181] typically by modifying an existing noncovalent scoring function to sample the close bond approaches and to discount the van der Waals clashes implied by the formation of the covalent adduct. In favorable circumstances, these methods predict poses with high fidelity to subsequent crystal structures and have prospective hit rates substantially higher

than experienced with noncovalent docking, while retaining decent specificity against off-targets.[175] This is an area where new tools are rapidly being introduced[175–177,182–185] and in which there is widespread[98,186–188] interest.

EXPANSION TO COMPARATIVE MODELS

With all the errors in docking, targeting homology models might seem risky. On the other hand, the number of targets with experimental structures is outstripped by several log-orders by those for which plausible comparative models may be calculated. Modeling from templates with sufficient sequence identity and, where possible, fortified by other constraints,[77,189] the technique can provide useful structures for ligand discovery.[190,191] Indeed, in a prospective, head-to-head study of docking against a comparative model and then, subsequently, a crystal structure of the same receptor, about the same hit rate (20–23%) and hit affinity (0.2–3 μM) was returned for both structures. Multiple docking screens against homology models have been reported for ligand, substrate,[192–194] and deorphanization studies,[188] and indeed for one of the approved drugs where structure-based design played a pivotal role, aliskiren, homology modeling of lead complexes long predated crystal structures.[195] Even with all the errors in docking and protein structure modeling, targeting comparative models can greatly expand dockable targets.

NEW CHEMOTYPES FROM DOCKING

What structure-based screens can reliably return are novel chemotypes unrelated to previously known ligands for a target. There are multiple ways a protein structure can recognize any given ligand chemotype,[196] and a particular binding site can recognize multiple, unrelated scaffolds. Even for well-precedented targets, structure-based screens can find new ligands without antecedents in previous medicinal chemistry. Though there is no strong logic for why new ligand chemistry should lead to new biology, it often does. This ability to break out of medicinal chemistry boxes and to discover new chemotypes is where docking screens may find some of their most important contributions over the next few years. This may be illustrated by example.

New β-Lactamase Inhibitors That Do Not Up-Regulate Enzyme Expression

Class C β-lactamases are bacterial enzymes that are the major source of resistance to β-lactam antibiotics like penicillins and cephalosporins in the clinic. Whereas β-lactam-based inhibitors, such as clavulanate, are well-known, they have grave liabilities for the class C enzymes: their IC_{50} values are modest, and worse still they up-regulate the expression of the very enzyme that they are meant to inhibit, actually increasing resistance. A docking screen for new inhibitors of the class C β-lactamase, AmpC, discovered a family of thiophene-carboxylate sulfonamides. These inhibitors were dissimilar to the known β-lactams, including clavulanate, but they were found by crystallography to bind in the same site[47] and to inhibit the enzyme with about 1 μM potency.[197] Unlike clavulanate, the new inhibitors simply inhibited the enzyme in bacterial cell culture; they did not up-regulate its expression. Thus, they, unlike classic β-lactamase inhibitors, were synergistic, not antagonistic, with primary β-lactam antibiotics such as ceftazidime and piperacillin (Figure 5a).

Receptor Deorphanization

Tm0936 was an orphan enzyme from *Thermatoga maritime* whose structure had been determined as part of a structural genomics initiative. Whereas by sequence and structure Tm0936 could be readily assigned to the amidohyrolase superfamily, its function was unknown and automated assignment misannotated it as a cytosine deaminase. A docking screen of 14 000 metabolites, represented as high-energy intermediates, against the enzyme's structure revealed several analogs of adenosine as top-ranked hits, suggesting that they went through a deamination reaction on the purine ring (revealed by the high-energy intermediate form docked). Among these was *S*-adenosyl homocysteine, which on testing was found to be an excellent substrate for Tm0936, with a second order rate constant of 10^5 mol^{-1} s^{-1}. Subsequent crystallography confirmed the product of the predicted deamination, *S*-inosyl homocysteine, to superpose well with the docked pose (Figure 2I). The conversion of *S*-adenosine homocysteine to the inosyl derivative suggested a previously unknown *S*-adenosyl homocysteine degradation pathway in bacteria.[198]

A similar strategy has recently illuminated the function of the orphan GPR68 (also known as OGR1) in learning and memory.[256] This GPCR was screened in general function assays against a small drug library, and the benzodiazepine lorazepam was found to be a positive allosteric modulator (PAM) of it. Because of its binding to its therapeutic target, the GABA ion channel, lorazepam itself was not useful as a biological probe for GPR68's function. Accordingly, thousands of 3D models of GPR68 were constructed using MOD-ELLER[199,200] and prioritized by their ability to highly rank lorazepam versus the known decoy molecules from the initial empirical screen. A subsequent series of docking screens, testing, and optimization led to a PAM with about 30-fold improved efficacy over lorazepam. Unlike lorazepam, the new molecule had no measurable activity against the GABA channel and was specific for GPR68 versus sequence-related orphan receptors, such as GPR4 and GPR65. This molecule, dubbed ogerin (for OGR1 ligand), was advanced into in vivo functional studies. In a screen of mouse behavioral assays, ogerin reduced fear-conditioning in a context-based learning assay, consistent with its high expression in the hippocampus, which is associated with this modality. Conversely, ogerin had no effect on cue-based learning, associated with the amygdala, nor did it have any effect on even context-based learning in GPR68 knockout mice. A close analog of ogerin that had little effect in vitro also had little effect in vivo. These observations support the on-target effect of ogerin on GPR68 in vivo and by extension a function of this formally "dark" receptor in learning and memory.

Targeting the Serotonin Receptor

Recent studies have suggested that the 5-HT$_{2B}$ GPCR plays a role in the development of irritable bowel syndrome and may be a target for new therapeutics. Currently, only a few selective antagonists of the receptor have been reported, due to the high degree of homology with its closely related protein family members. Huang and co-workers recently used a crystal structure of 5-HT$_{2B}$ with an agonist bound to discover novel and selective antagonist chemotypes for the receptor.[201] They created induced-fit models using antagonists and docked an in-house collection of over 100 000 diverse compounds against it. Several new compounds emerged including a chemotype that contained a protonated triazine ring instead

of the conventional tertiary aliphatic amine. This novel chemotype led to subtype selective compounds for the 5-HT$_{2B}$ receptor. Tested in vivo, these selective antagonists substantially attenuated visceral hypersensitivity in irritable bowel syndrome rat models. This scaffold represents a new avenue for the development of selective 5-HT$_{2B}$ antagonists for the treatment of 5-HT$_{2B}$-related diseases.

Targeting Ras-like Kinases

Ras and related soluble GTP-based kinases are perhaps the proteins most implicated in cancer but have long resisted modulation because of their high affinity of GDP and GTP. Theodorescu and Meroueh used molecular docking screens to discover inhibitors to modulate the GDP-form of the Ras-like kinases RalA and RalB.[202] These inhibitors were active not only in vitro but also in Ral-mediated cell spreading in murine fibroblasts and anchorage-independent growth of human cancer cell lines. Two of the compounds were selective for Ral versus Ras or Rho kinases and inhibited tumor growth in a xenograph mouse model at a level similar to depletion of Ral by siRNA. These results illustrate the ability of a structure-based approach to discover chemotypes that have long eluded purely empirical approaches.

FORWARD LOOKING GUIDANCE

In the past decade docking has advanced by incremental improvement, not least in the size and quality of docking libraries. A true breakthrough in the rapid calculation of binding affinity or the ranking of diverse compounds, even if occurring postdocking, on a list of 1000–10 000 prioritized molecules would change the field. Even without such a breakthrough, steady optimization has brought the field to where it often can find new lead matter for biologically relevant targets. A great opportunity for docking is its application to the discovery of genuinely new molecules for both precedented and unprecedented targets. Whereas there is no strong reason why new chemotypes should lead to new biology, they often do, even for well-studied targets. The opportunity to use docking screens for new chemotype discovery is increasingly available not only to specialists but to the community, via open access tools (Tables 2–4). The size of the chemical libraries routinely used by these structure-based screens has, moreover, grown steadily since they were first introduced 20 years ago, from 55 000 in the early 1990s to 250 000 in the early 2000s, to 750 000 by 2005, to now approaching 10 million and with another log-order expansion in sight. They far exceed most empirical libraries except perhaps those that are DNA-encoded. These days, a conservative "leadlike" docking campaign might screen six million molecules, most available from vendors within a couple of weeks. Were one willing to wait a couple of months, this number would grow to 60 million leadlike molecules. Naturally, such large libraries are interesting only if the structure-based methods can prioritize the tiny percentage of plausible ligands from among the vast number of decoys, and there is always a concern that adding more library molecules will simply drown the signal in noise. Still, results over the past several years, on multiple targets from multiple laboratories, suggest that docking, for all its problems, can distinguish plausible from implausible molecules, at least for well-formed binding sites. It also suggests that larger libraries have led to more novel and often more potent chemotypes, though admittedly this has not been investigated in a controlled

way. In the next several years, docking's greatest impact may be in its pragmatic application to biomedical problems where new chemical matter can confer new biology.

Acknowledgments

APPENDIX 1

CONTROLS FOR ARTIFACTUAL ASSAY ACTIVITY

(a) Irreversible Inhibitors

Some noncovalent docking hits act as covalent artifacts, often via a reactive center present on the docked molecule or on an impurity. A rapid counterscreen for irreversible inhibition is to incubate the target protein and the hit at 5× their apparent IC_{50} and then dilute them 10-fold (other IC_{50} ratios may of course be chosen). If inhibition is rapidly reversible, the inhibition on dilution should drop to about 33% of full inhibition at this ratio. If dilution changes the inhibition little, it supports covalent activity. Legitimate, slow off-rate inhibition is another alternative, but such molecules are rare among initial screening hits. This experiment will only work for soluble proteins, but related experiments to measure off-rate may be adapted for membrane proteins.

(b) PAINS Molecules

The chemotypes represented in these molecules often occur among promiscuous molecules that fail to progress. Several tools are available to rapidly detect these chemotypes, including at http://zinc15.docking.org/patterns/home. Just because a hit has PAINS functionality does not invalidate it, but it does make careful testing for mechanism important. We recommend counterscreening the molecule against unrelated targets, determining whether it competes with a ligand known for the site and whether its concentration-response curves are well-behaved (Figure 4a, red curve).

(c) Spectroscopic Interference Compounds

Compounds that are absorb light or that fluoresce in a region used to measure activity can look like hits via assay interference,[124–126,140] as can molecules that inhibit a reporter enzyme, like luciferase.[141] Spectroscopic interference should change linearly with concentration, following Beer's law, rather than log-linearly as in a single site isotherm. Inhibitors of the reporter may be counterscreened against it.

(d) Active Impurities

Sometimes compounds are not what they pretend to be or contain reactive impurities. Active molecules should be tested for purity. Anything below 95% should be further purified. ACS journals often request purity of 98% or better.

(e) Colloidal Aggregation

Perhaps the largest single source of artifacts in early discovery is colloidal aggregation by small molecules.[68,69,127] These particles, between 50 and 1000 nm in radius, adsorb protein without specificity, partially denaturing them. About 1–2% of molecules in a typical screening deck will aggregate at relevant concentrations, ensuring hits reflecting colloid-formation dominate screens, virtual and empirical, that do not control for them. Fortunately, this mechanism of inhibition may be readily controlled:

i. If inhibition can be attenuated by small amounts of nonionic detergent, the compound is likely an aggregator. We typically use 0.01% v/v freshly prepared Triton-X 100 or 0.025% v/v Tween-80[142–144] for membrane or cell assays.

ii. Direct observation of particles in the 50–1000 nm size range by dynamic light scattering (DLS). Formation of particles does not guarantee promiscuous inhibition, but it is a worrying sign.

iii. For cell-based assays, colloidal particles can be precipitated by centrifugation before the assay is run, from assay media. If the compound is much more effective before spin down than after, it suggests colloidal aggregation.

iv. Noncompetitive inhibition with high Hill slopes. There are classical reasons for noncompetitive inhibition and for cooperative binding, but the latter is rare in early discovery and the two together suggest aggregation.

v. Attenuation of inhibition by increasing target concentration. Except when the receptor concentration to K_i ratio is high,[145] increasing receptor concentration should not affect inhibition for well-behaved inhibitors, but it will be much reduced for colloidal aggregators and increase the steepness of the response curve (Figure 4b). This experiment is only accessible for soluble proteins.

Biographies

John J. Irwin trained in organic chemistry and crystallography with Jack Dunitz at ETH Zurich (1988–1991) and in macromolecular crystallography software development with Gerard Bricgone at the MRC Laboratory of Molecular Biology (1994–1998). He developed the ZINC database and is part of the team behind the DUD and DUD-E docking benchmarks, the DOCK Blaster Web tool for public access molecular docking, and the similarity ensemble approach for associating proteins by the ligands they bind. His work is supported by the NIH and the FDA.

Brian K. Shoichet trained in molecular biophysics with Tack Kuntz at University of California—San Francisco (1986–1992), where he developed new docking methods and applied them to the first screens of readily available molecules. In his postdoctoral work he trained in protein structure with Brian Matthews at the Institute of Molecular Biology (1993–1996), where he investigated trade-offs between protein stability and ligand recognition. A theme of his lab has been a cycle of methods development and experimental testing. This has led to the discovery of colloidal aggregation, chemoinformatics methods for

target identification, structure-based methods for ligand discovery, and the application of both to GPCRs. The work is supported by the NIH and by the FDA.

ABBREVIATIONS USED

3CLpro	SARS-CoV 3-chymotrypsin-like protease
5-HT$_{2B}$	serotonin G protein coupled receptor 2B
ACS	American Chemical Society
AR	androgen receptor
ChEMBL	The EMBL Medicinal Chemistry database
DLS	dynamic light scattering
DUD-E	A Directory of Useful Decoys—Enhanced
EGFR ED	epidermal growth factor receptor extracellular domain
FDA	Food and Drug Administration
G4	G-quadruplex
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GLR	glucagon receptor
GPCR	G protein coupled receptor
GPB	glycogen phosphorylase B
GPR4	G protein coupled receptor 4
GPR65	psychosine receptor
GPR68	ovarian cancer G-protein coupled receptor 1
GTP	guanosine triphosphate
HTS	high-throughput screening
HCoV-OC43	human coronavirus nucleocapsid protein
HisG	*Mycobacterium tuberculosis* ATP phosphoribosyl transferase
HSP90	heat shock protein 90
OGR1	ovarian cancer G-protein coupled receptor 1
MELK	maternal embryonic leucine zipper kinase
NRPS	nonribosomal peptide synthetase
PAINS	pan assay interference
PAM	positive allosteric modulator
pgMDD	meso-2,6-diaminopimelate dehydrogenase

PKR1	prokineticin receptor 1
PTP-1B	tyrosine-protein phosphatase 1B
PA-Nter	influenza virus PA endonuclease
PTPσ	protein tyrosine phosphatase σ; RalA, Ras-related protein Ral-Al
RalB	Ras-related protein Ral-B
TGT	tRNA-guanine transglycosylase
siRNA	small interfering RNA
SLC1A5	alanine–serine–cysteine transporter ASCT2
VHZP	VHZ phosphatase
ZINC	"ZINC is not commercial", a database of compounds for virtual screening

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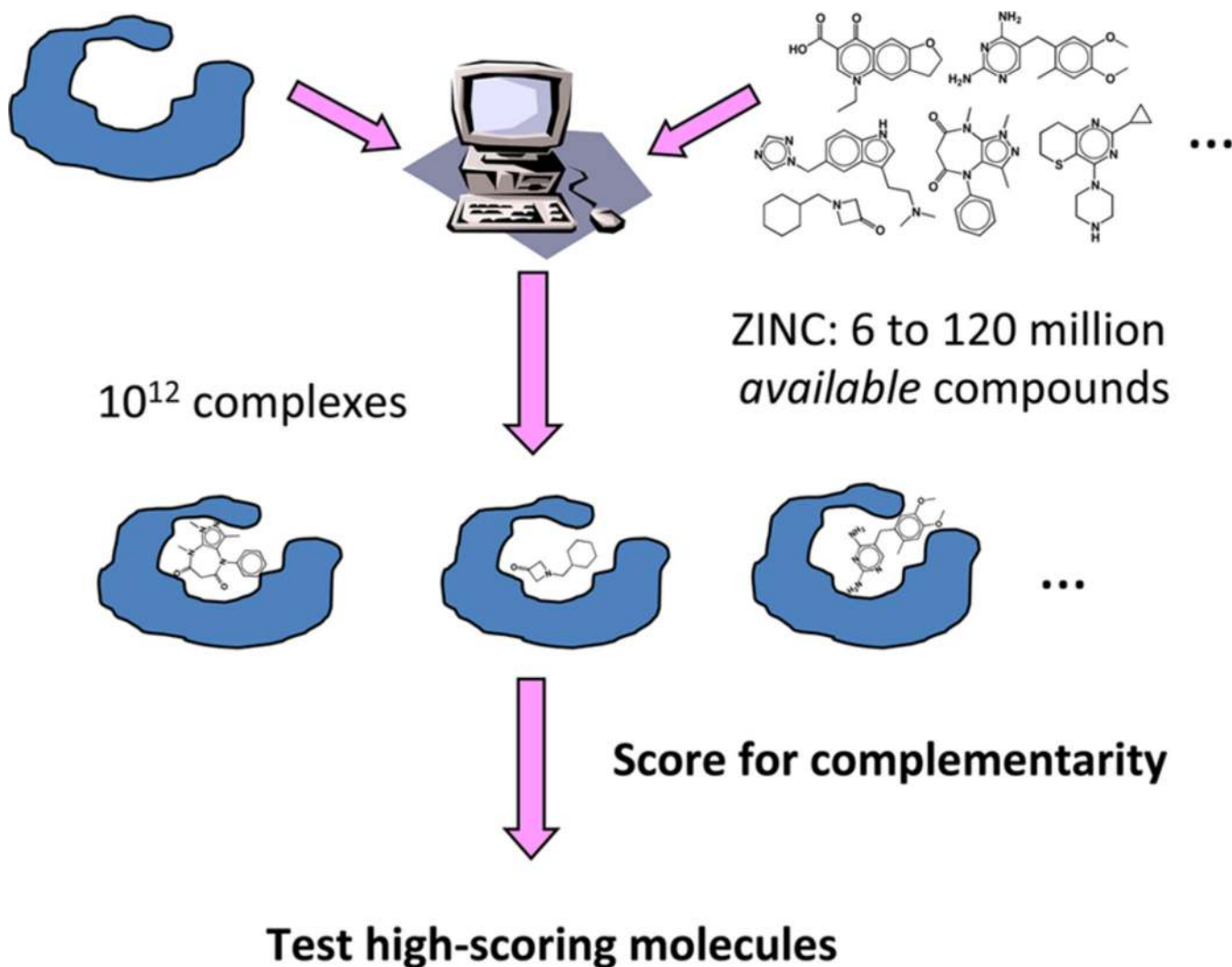
Figure 1.
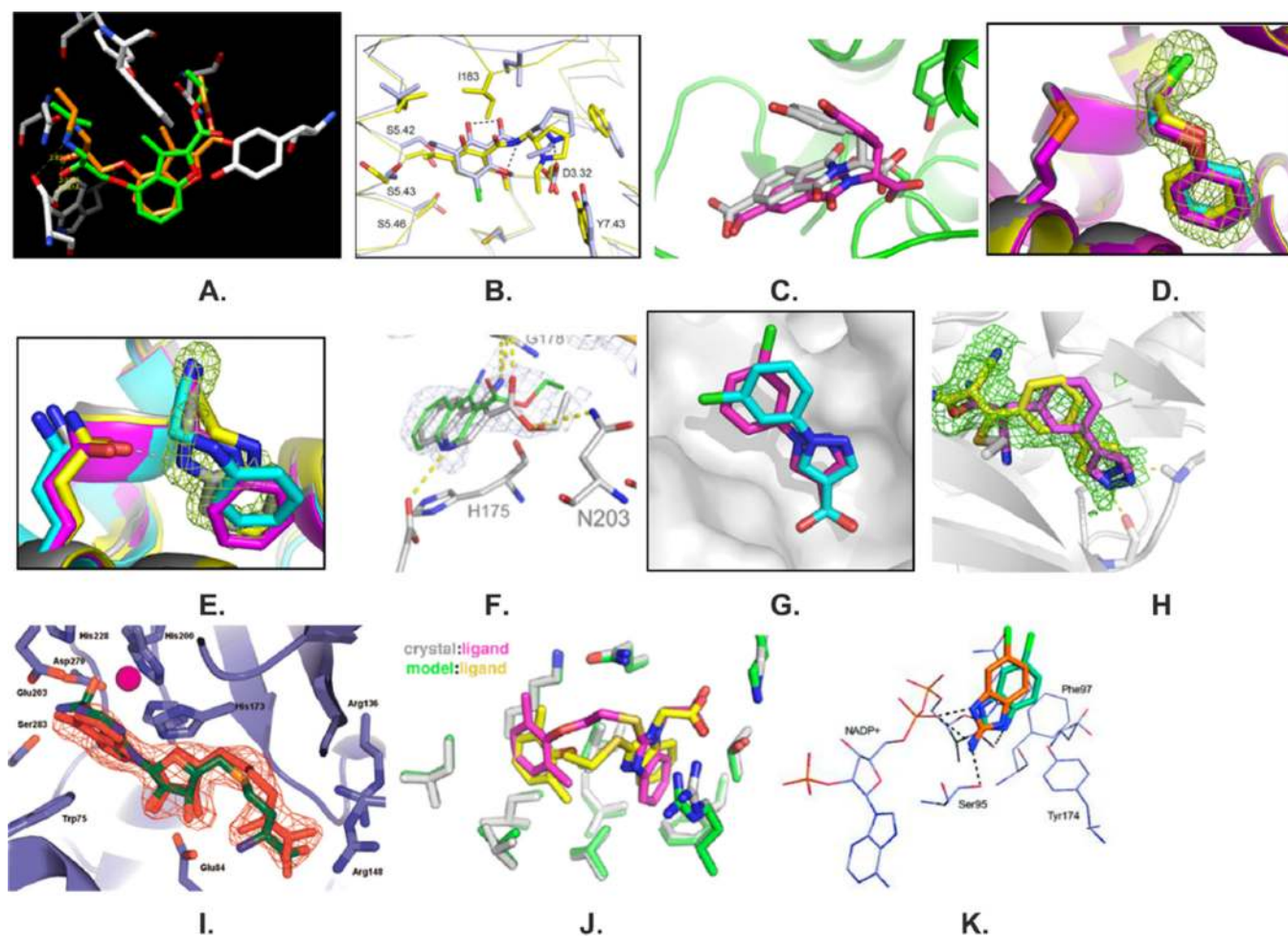Docking for new chemotypes from large libraries.

Figure 2.

Superposition of docking-predicted and subsequently determined crystal structures. (A) A 9 nM inverse agonist of the β2 adrenergic receptor discovered by docking (orange) superposed on the crystallographic result (green).[49] (B) Docking-predicted structure of eticlopride (light blue) superposed on the crystallographic result (yellow) in the dopamine D3 receptor. Reproduced with permission from *Nature Chemical Biology* (Carlsson, J.; et al. Ligand discovery from a dopamine D3 receptor homology model and crystal structure; **2011**, 7, 769–778);[213] Copyright 2011 Macmillan Publishers Ltd. (C) A 30 μM inhibitor of β-lactamase discovered by docking (purple) superposed on its crystallographic structure (white).[48] (D, E) Two crystal structures (gray carbons, electron density in wire mesh) superposed on the docking predicted ligands (yellow carbons) and poses refined by postdocking rescoring programs AMBERDOCK (cyan carbons) and PLOP (magenta carbons) for the nonpolar T4 lysozyme L99A and L99A/M102Q model cavities. Reproduced with permission from *Journal of Molecular Biology* (Graves, A. P.; et al. Rescoring docking hit lists for model cavity sites: predictions and experimental testing; **2008**, 377, 914–934);[245] Copyright 2008 Elsevier, Ltd. (F) A docked ligand superposed on the crystallographic result from a screen against the model anion cavity in cytochrome c peroxidase W191G/gateless. Reproduced with permission from *Nature Chemistry* (Fischer, M.; et al. Incorporation of

protein flexibility and conformational energy penalties in docking screens to improve ligand discovery; **2014**, 6, 575–583);[243] Copyright 2014 Macmillan Publishers Ltd. (G) A 40 nM boronic acid inhibitor of AmpC predicted by covalent docking (cyan carbons) superposed on its crystal structure with the enzyme.[175] (H) A 370 nM inhibitor of RSK2 kinase predicted by covalent docking (magenta carbons) superposed on its crystal structure with the enzyme (yellow carbons, with $F_o - F_c$ electron density shown in green mesh.[175] (I) The docked structure (green carbons) of the high-energy intermediate of S-adenosyl homocysteine superposed on the crystallographic structure of the product, S-inosyl homocysteine (red carbons) in complex with Tm0936. Reproduced with permission from *Nature* (Hermann, J. C.; et al. Structure-based activity prediction for an enzyme of unknown function; **2007**, 448, 775–779);[198] Copyright 2007 Macmillan Publishers Ltd. (J) An 8 µM inhibitor of PDK1 showing the docking pose (yellow carbons) superposed on the subsequently determined crystal structure (magenta carbons).[234] (K) Modeled binding mode of a 10.6 µM fragment (orange carbons) superimposed with the dominant binding mode determined crystallographically (green carbon atoms).[251]
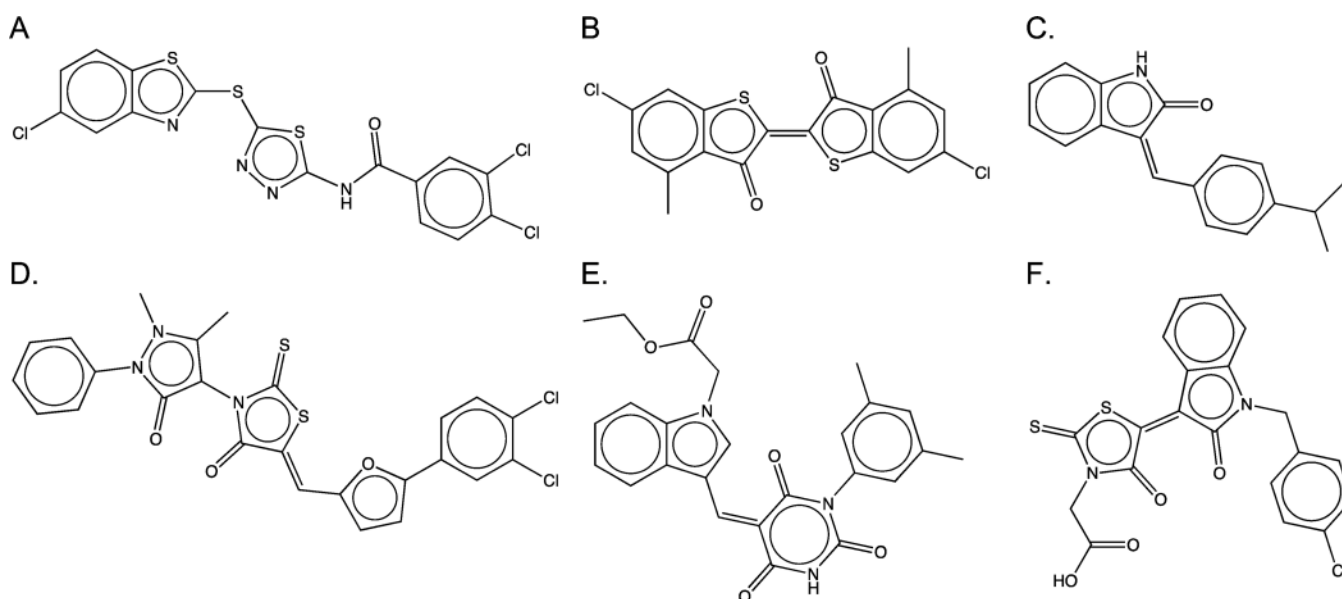
Figure 3.
Aggregators (first row) and PAINS[120] (second row) identified by docking: (A) 0.5 μM inhibitor of β-lactamase;[127] (B) 7 μM inhibitor of DHFR;[252] (C) 5 μM inhibitor of VEGF (and 10 μM IGF-1);[253] (D) 14 μM inhibitor of tyrosine phosphatase;[131] (E) 30 μM inhibitor of PTPRQ;[130] (F) 3 μM inhibitor of DHFR.[254]

Figure 4.
Classical and aggregation-based concentration–response curves. (a) The well-behaved, competitive inhibitor BZBTH2B (■, red curve) and the aggregator rottlerin (●, black curve), against the enzyme β-lactamase (adapted from ref 255, used with permission). (b) As the concentration of β-lactamase is increased, the inhibition curve shifts right and steepens for colloidal aggregators like rottlerin.
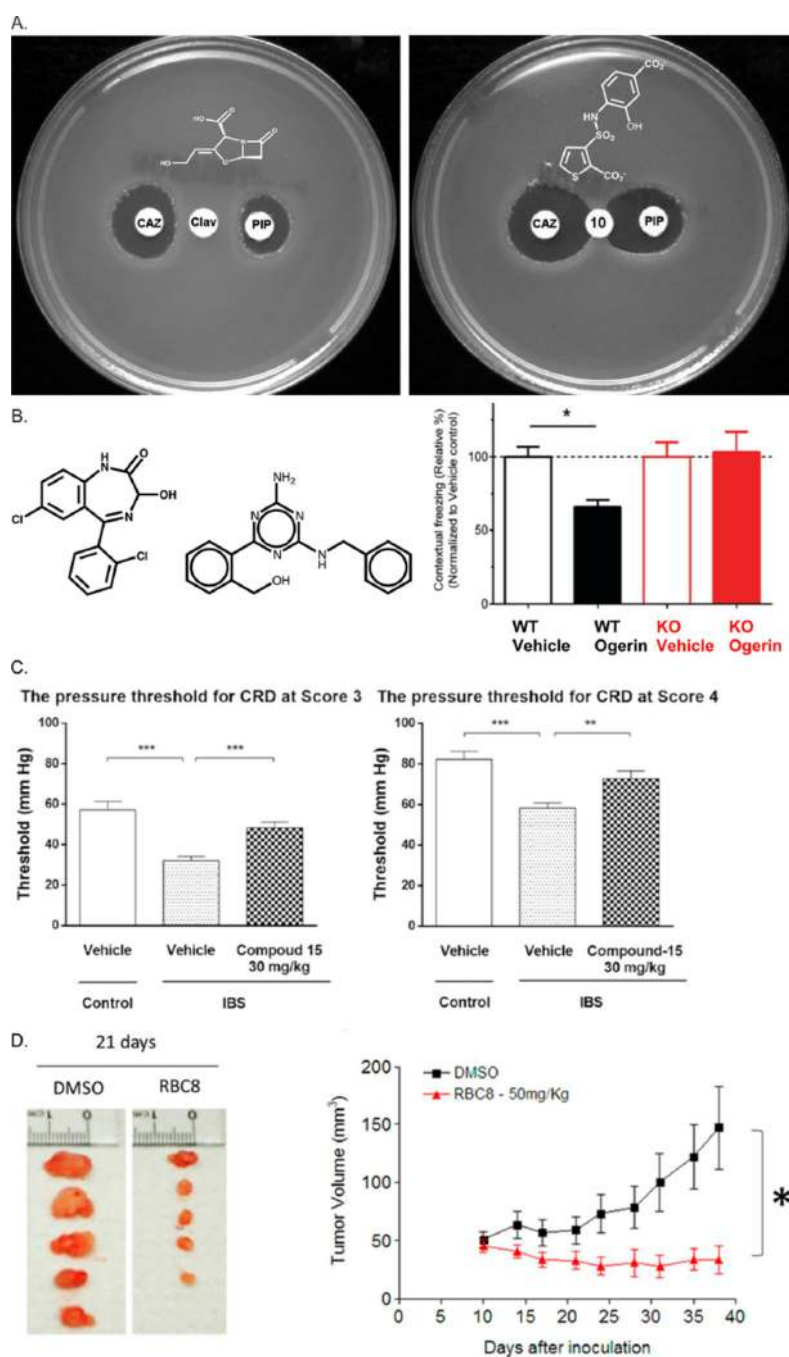
Figure 5.

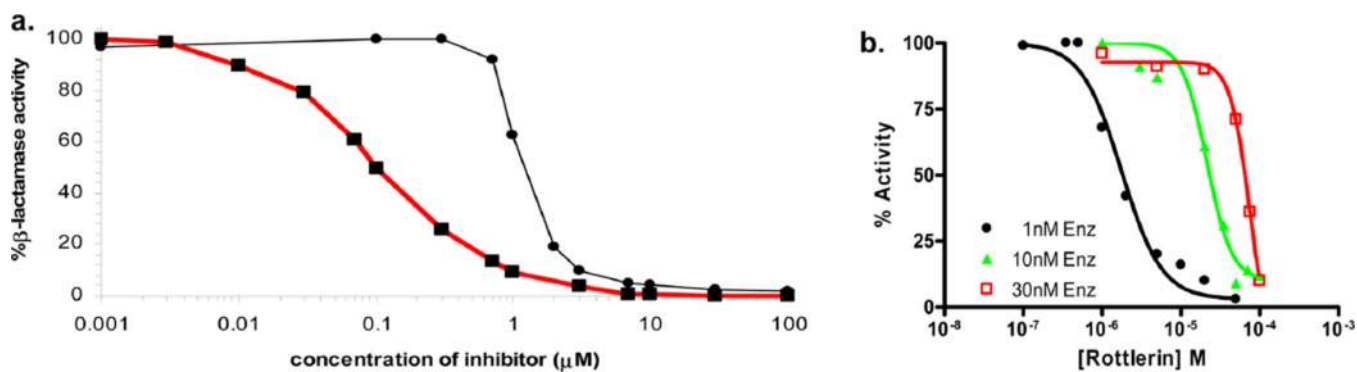New functions conferred by new chemotypes discovered by docking. (A) The β-lactam inhibitor clavulanic acid up-regulates the expression of β-lactamase in *E. cloacae*, antagonizing the efficacy of the primary β-lactam antibiotics ceftazidime (CAZ) and piperacillin (PIP) in a disk diffusion assay (left).[47,197] In contrast, the docking-discovered inhibitors simply inhibit the enzyme without up-regulating it, synergizing with the same primary antibiotics (right). (B) Lorazepam (left) and ogerin (middle) are both modeled to bind at the same site on GPR68. Ogerin, however, is more potent and specific than

lorazepam. In wild-type mice, ogerin reduces fear conditioning in a contextual freezing assay compared to knockout mice, where it has no measurable effect (right). Reproduced with permission from *Nature* (Huang, X. P.; et al. Allosteric ligands for the pharmacologically dark receptors GPR68 and GPR65; 2015, 527, 477–483);[256] Copyright 2015 Macmillan Publishers Ltd. (C) Inhibition of visceral hypersensitivity in IBS rat model. Therapeutic effects of compound 15 in IBS rats. The pain threshold decreased in response to CRD (at score 3 (left) and score 4 (right)).[201] (D) Left panel. Xenograft tumor growth of human lung cancer cell line H2122, 50 mg kg^{-1} day^{-1} RBC8 initiated 24 h after inoculation inhibited tumor growth. Typical tumor appearance at 21 days is shown. Right panel. Effect of RBC8 on H358 xenograft models. RBC8 treatment (50 mg kg^{-1} day^{-1}) initiated 24 h after inoculation inhibited xenograft tumor growth of human lung cancer cell line H358. Data represent the mean ± SEM of six mice. Tumor volume in the treatment group was statistically different from controls as determined by the Student's *t* test. Reproduced with permission from *Nature* (Yan, C.; et al. Discovery and characterization of small molecules that target the GTPase Ral; 2014, *515*, 443–447);[202] Copyright 2014 Macmillan Publishers Ltd.

Table 1

Selected Examples of Ligand Discovery by Molecular Docking

Target	hit rate (actives/tested)	best hit (μM)[a]	structure determined?	biology investigated?
Carbonic anhydrase II[203]		0.0008	yes	
H1 histamine GPCR[204]	73% (19/26)	0.006	yes	
β2 adrenergic[49,205]	24% (6/25)	0.009	yes	
A2a adenosine GPCR[206]	45% (9/20)	0.015		
SLC1A5[207]	50% (7/14)	0.02		cell culture
A2a adenosine GPCR[208,209]	41% (23/56)	0.032		
PTPσ[210]	4.7% (7/147)	0.1		
CD40-TRAF6[211]	4.5% (7/150)	0.1		mouse
DDX3 helicase[212]	40% (10/25)	0.2		
dopamine D3 GPCR X-ray[213]	20% (5/25)	0.3	yes	
dopamine D3 GPCR model[213]	23% (6/26)	0.2	yes	
LSD1[214]	7% (9/121)	0.2		cell culture
MELK[215]	19% (3/16)	0.37		
NQO2[216]	14% (35/250)	0.4	yes	mouse
NRPs[217]	0.2% (56/3000)	0.6		mouse
P38 MAPK[218]	6% (6/98)	0.7		
NEDD8[185]	37% (3/8)	0.85		
PA-Nter[219]	20% (3/15)	0.94		influenza virus
G4[220]	16.6% (3/18)	1		cell culture
DYRK1A[221]	3.5% (6/168)	1.5		cell culture
GLR[222]	8.5% (2/23)	1.9		cells/functional
Crm1[223]	8% (33/402)	2		
HCoV-OC43[224]	12% (1/8)	2	yes	
HisG[225]	14% (7/50)	2		Mycobacterium
AR[226]	11% (1/9)	2.7		cell culture
cruzain[227]	52% (12/23)	3		trypanosomes
VHZP[228]	5% (7/142)	3.5		
SET7[229]	5% (7/127)	4.9		
GPB[230]	57% (4/7)	5	yes	hepatocytes
DPP-IV[231]	15% (15/99)	5		
11β-HSD1[232]	10% (4/39)	5.3		
RSV[233]	66% (20/30)	6		
PDK1[234]	20% (3/15)	8	yes	
TGT[235]	50% (3/6)	8.3[a]	yes	
EGFR ED[236]	13% (14/109)	10		cell culture
PKR1[237]	10% (1/10)	10		mouse
mGlu1[238]	14% (5/35)	10.2		

Target	hit rate (actives/tested)	best hit (μM)[a]	structure determined?	biology investigated?
3CLpro[239]	4% (3/74)	11		
BRD4(1)[240]	3.2% (5/153)	13	yes	
RSV[241]	10% (5/50)	20	yes	virus
AmpC β-lactamase[47]	5% (3/5)	26[a]	yes	
CDK4[26]	5% (18/382)	0.04	yes	
HIVgp41[135]	1.7% (2/120)	56		cell fusion
CcP W191G cavity[242]	94%(15/16)	20[a]	yes	
CcP W191G/gateless cavity[243]	60% (9/15)	7[a]	yes	
T4 lysozyme L99A cavity[244]	100% (7/7)	56[a]	yes	
L99A/M102Q cavity[245]	70% (23/33)	ND[b]	yes	
L99A/M102H cavity[53]	74% (21/29)	3[a]	yes	
DPP-IV[246]	50% (3/6)	0.72		rat
MOR42-3[247]	55% (22/40)	100[a]		frog
TLR2[248]	20% (1/5)	11		cell culture
pgMDD[249]	36% (4/11)	157[a]		cell culture
RSK kinase (covalent)[175]	62% (5/8)	0.43	yes	cell culture
iNOS[250]	12.5% (1/8)	2.5		zebrafish

[a] Fragment docking.

[b] ND = not determined (binding, no K_d values).

Table 2

Web Resources Widely Used for Docking Screens

Name	Web site	compounds and cost	used for
ChemNavigator	http://www.chemnavigator.com	9.2×10^7, commercial	Procurement service
ChemSpider	http://chemspider.com	3.5×10^7, free	Chemical structure database with integrated information
DOCK Blaster	http://blaster.docking.org	Free	Noncovalent docking
Docking Server	http://www.dockingserver.com	Free and commercial	Noncovalent docking
DOCKovalent	http://covalent.docking.org	Free	Covalent docking
DUDE	http://dude.docking.org	Free	Decoy database and builder
eMolecules	http://emolecules.com	7×10^7, free/commercial	Procurement service
iScreen	http://iscreen.cmu.edu.tw	Free	Noncovalent docking with a TCM focus
Modbase	http://salilab.org	3.5×10^7, free	Database of comparative models for docking
MolPort	http://molport.com	2.6×10^7, free	Procurement service
PDB	http://www.pdb.org	1.1×10^5, free	Database of crystal structures for docking
ZINC	http://zinc15.docking.org	1.3×10^8, free	Commercially available compounds for docking

Table 3

Databases Widely Used for Docking Screens

name	Web site	compounds and cost	used for
ACD	http://accelrys.com	7×10^6, commercial	Screening and building blocks
ChEMBL	http://www.ebi.ac.uk/chembldb	1.3×10^6, free	The curated medicinal chemistry literature
MDDR	http://accelrys.com	2×10^5, commercial	Biological active compounds compiled from patents
Open NCI	http://cactus.nci.nih.gov	3×10^5, free[a]	Free samples for testing in cancer research[a]
TCM@Taiwan	http://tcm.cmu.edu.tw	3.7×10^4, free	Compounds from Traditional Chinese Medicine
WOMBATZINC	http://www.sunsetmolecular.com	3×10^5, commercial	Bioactivity databases for lead and drug discovery
ZINC	http://zinc15.docking.org	1.3×10^8, free	Commercially available compounds for docking

[a] Free net of shipping, available for cancer-focused projects only.

Table 4

Widely Available Docking Software

docking program	Web site	terms
AutoDock	http://autodock.scripps.edu	Free
AutoDock Vina	http://vina.scripps.edu	Free
DiscoveryStudio	http://www.accelrys.com	Commercial
DOCK	http://dock.compbio.ucsf.edu	Free to Academics
FlexX	http://www.biosolveit.de/FlexX/	Commercial
FRED	http://www.eyesopen.com	Free to qualified academics
Glide XP/SP	http://www.schrodinger.com	Commercial
GOLD	http://www.ccdc.cam.ac.uk	Commercial
ICM-Pro	http://www.molsoft.com	Commercial
MOE	https://www.chemcomp.com	Commercial
rDOCK	http://rdock.sourceforge.net	Free
Surflex-Dock/Sybyl-X	http://www.tripos.com/surflex	Commercial
CovDOCK	http://www.schrodinger.com	Commercial