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From laptop to benchtop to bedside: Structure-based Drug Design on Protein Targets

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Abstract

As an important aspect of computer-aided drug design, structure-based drug design brought a new horizon to pharmaceutical development. This *in silico* method permeates all aspects of drug discovery today, including lead identification, lead optimization, ADMET prediction and drug repurposing. Structure-based drug design has resulted in fruitful successes drug discovery targeting protein-ligand and protein-protein interactions. Meanwhile, challenges, noted by low accuracy and combinatoric issues, may also cause failures. In this review, state-of-the-art techniques for protein modeling (e.g. structure prediction, modeling protein flexibility, etc.), hit identification/optimization (e.g. molecular docking, focused library design, fragment-based design, molecular dynamic, etc.), and polypharmacology design will be discussed. We will explore how structure-based techniques can facilitate the drug discovery process and interplay with other experimental approaches.

Keywords

Structure-based drug design; protein modeling; focused library design; pharmacophore; flexible docking; high-throughput virtual screening; *de novo* design; protein-protein interaction; polypharmacology

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Modern computational-aided drug design established a novel platform by which researchers perform in-depth *in silico* simulation prior to labor-extensive wet-lab validation [1]. It comprises of two major parts corresponding to the information of molecular source it utilizes: structure-based (or receptor-based) drug design and ligand-based drug design. Structure-based drug design, which relies on the knowledge of biological target structures, aims to discover small molecules/peptides leads with desired chemistry properties, and orchestrate the following experimental validation and lead optimization. Structure-based approach provides mechanism-based basis, where potential ligands are excavated using receptor-dependent parameters, while ligand-based approaches bypass the consideration of complex biomolecular "black box" in a living cell. This *in silico* method permeates all aspects of drug discovery today [2], and we expect it will draw more attentions with the unprecedented advances of computational power and modeling accuracy in this decade.

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Conflict of interest

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In this review, we will overview the state-of-the-art structure-based drug discovery techniques ranging from receptor modeling to lead identification and optimization. In each topic, we will also highlight the fruitful successes as well as challenges that obstructs or cause the direct failure in both preclinical and clinical stages.

Protein modeling

The development of techniques for macromolecular determination and the wide availability of these structures in repositories such as the PDB have provided essential knowledge for drug design. Rapid advances in structural biology, *e.g.* macromolecular crystallography, NMR and Cryo-EM, consequence a boosting of structural data deposited in Protein Data Bank (PDB), which contains 72104 structures nowadays. However, there are many macromolecules which are not amenable to structure determination, or have incompletely determined structures. As an example, membrane proteins are an important class of macromolecules which serve as important drug targets, but present technical challenges for structure determination [3]. Moreover, many structures are not necessarily static as they are determined. It is becoming increasingly acknowledged that the native state of a protein is not a static structure, but consists of an ensemble of structures which interconvert in a dynamic equilibrium. This dynamic equilibrium has functional importance, participating in such processes as the diffusion of oxygen into the heme cavity of myoglobin, the diffusion of ions across ion channels, substrate binding, and ion channel gating [4]. Here, we first highlighted the contribution of *in silico* receptor modeling, a critical process that aims to ease one of the major bottlenecks of structure-based drug discovery: limited availability of experimental protein structures [5, 6]. Despite the lower resolution than experimental counterparts, using protein models as the starting point has already achieved great successes in current pharmaceutical industry. They predict three dimensional protein models either by using such comparative protein structure prediction approaches, such as homology modeling, or sequence information in conjunction with physical principles (*ab initio* modeling). Second, we concentrated on the methods to model flexible proteins.

Homology modeling

Homology modeling is an established and widely documented process [7], a method based on the assumptions that proteins that possess similar sequences share similar three-dimensional structures, and only a limited number of protein folds exist in nature [8, 9]. Homology modeling has been stated as the best structure prediction method of homologous protein so far, and it was widely used in structure-based drug discovery projects [10]. In brief, homology modeling consists of 1) suitable homologous template selection, optimal target-template sequence alignment, crude model building stage and 2) model evaluation, refinement and validation stage. Continual research is advancing methods available for each stage. Methods for sequence alignment and target identification include bioinformatics-based approaches (MUSCLE [11], DIALIGN [12, 13], MuSiC [14], MANGO [15]), machine learning methods (hidden Markov models [16, 17], neural networks [18], support vector machines [19]), and threading methods where target sequence is compared and matched against known three dimensional folds [20]. Model construction includes the techniques based on satisfaction of spatial restraints (Modeller [21]), Cα positions from conserved segments in template structure(s) [22, 23]. Highly critical loop modeling part remains ambiguous, despite the approach such as matching protein structure segments from PDB and using them as templates [24]. Conformational search of unmatched sequence segments using energy-based selection [25, 26] and *ab initio* modeling [27] has shown promising results. However, the imperfection of the constructed models may limit their applicability in structure-based design methods. Several methods have been reported to refine crude homology models or at least the side-chain conformations of the crucial binding

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pocket residues [28–34]. Interested readers are encouraged to refer [10] for a comprehensive list of homology modeling programs and servers.

In the field of drug discovery, modeling ligand-bound structure is more crucial than modeling *apo* structure because complex structure offers a more reliable starting point for later virtual screening. Ligand-supported/ligand-steered homology modeling method shows exciting prospects in reproducing active site by introducing "induced-fit" theory [29]. By extending the basis of homology modeling that evolutionary-related proteins share similar conformations, the hypothesis that pertinent proteins should also bind similar ligands which share a common core substructure has been widely accepted [35, 36]. James and Richard recently developed two automated protein-ligand homology modeling approaches, both of which used 3D-Coffee for template alignment, Modeller for modeling construction, Omega for ligand conformer generation and LigMatch/Rocs for ligand superposition, in order to tackle the modeling of homologous protein with distinct but related native ligands. According to their assessment, both hybrid-template strategy (exchange ligands first) and original-template strategy (exchange ligands after homology modeling) outperformed Fred docking in absence of the information of new ligand by 1–2Å RMSD [36]. Instead of inputting new ligand by structural alignment, docking-based global energy minimization via stochastic side chain movement, force fields or user-defined scoring function has become another refinement routine. This "ligand-centric" approach has been widely employed in identification of novel inhibitors against GPCR targets including MCH-R1, mGluR and CCR2 [29, 34, 37–39]. Other applications include protein structural analysis and function prediction [40–45], understanding of protein-ligand interactions [46–53] and compound optimization [54–56]. Successful stories for drug design starting from homology models are listed in Table 4.

Ab initio modeling

Ab initio structure prediction, or *de novo* structure prediction, is an energy-based method that predicts the 3D structure of protein from sequence alone. *Ab initio* approach was conceptualized based on the assumption that protein of specific sequence folds to a native/native-like conformation or ensemble that is close to/at the global free-energy minimum [57]. This protein structure modeling approach provides flexibility as well as uncertainty in absence of template as reference. The power of *ab initio* approach has been significantly improved within this decade under the community-based assessments by Critical Assessment of Structure Prediction (CASP), a blind-test platform for the evaluation of structure prediction methods. While it has been widely used in fold recognition or small protein modeling, *ab initio* modeling of large protein, especially proteins with over 150 amino acids, remains hard to cope with due to the exponentially growing computational expenses and accumulated errors.

There are two long-standing challenges involved: scoring function in solvent and vast conformational space of searching [58]. These can be severely problematic when applied to proteins with biologically relevant length (>150 aa) [59]. Rosetta *ab initio* module is the most famous and cited program, which employed Monte Carlo annealing algorithm for structural sampling [60, 61]. However, one of the bottlenecks of Rosetta was the limitation in sampling critical "linchpin" feature, which is characterized by irregular edge of β-strand frequently occurred in functional regions, which was evaluation by Baker's group [62]. To overcome the ruggedness of energy potential surface in traditional Monte Carlo annealing algorithm, several advanced generalized ensemble Monte Carlo methods were developed, which includes Temperautre Replica Exchange Monte Carlo (TREM) and Hamiltonian Replica Exchange Monte Carlo (HREM) [63, 64]. According to Alena's assessment, HREM outperforms other algorithms in its wide range of energy landscape and prediction accuracy when using Rosetta full atom predictive scoring function [59]. As for scoring function,

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Rosetta full-atom knowledge-based force field has shown encouraging results in CASPs [60, 61]. In addition, SimFold (solvent induced multibody force field) considered solvent-induced effects by introducing terms like residue hydrophobicity and preference of phi/psi dihedral angles, which were defined distinctly in Rosetta. SimFold achieved identical success rate (12/38 within RMSD 6.5Å) as Rosetta, and well-performed in CASP6 (http://predictioncenter.org/casp6) [65]. Interestingly, both Rosetta and SimFold groups indicated a significant contribution of hydrophobic effects upon protein folding, which was related to the bad performances on β-strand-only proteins. Readers interested in *de novo* scoring functions are recommended to read Shing-Chung's review [66].

Since *ab initio* approach is computational expensive, to develop better algorithms and to run these algorithms on faster computing architecture are urgent requirements. Recent development in high-performance computing, such as supercomputers (i.e. Blue Gene), distributed computing projects (i.e. Rosetta@home) and crowdsourcing (i.e. Foldit) may make this approach applicable and computational affordable in nowadays translational research. TOUCHSTONE II is similar to Rosetta in conformational sampling and energy function but uses a new lattice representation of protein structure [67]. Cα, Cβ and side-chain center of mass are used so that this program explicitly reduced the computational cost. However, the accuracy of TOUCHSTONE II is still limited by the size of protein, which is less than 200 amino acids [67]. In addition, a number of established webservers, such as Robetta [68], provided user-friendly testing platform that can help improve the structural prediction algorithm. Even though there are few cases of drug discovery based on *ab initio* models, the algorithms and scoring functions developed by such template-free methods have undoubtedly exhibited great potentials in systems biology [69] and template-based model refinement [70]. Another study carried out recently observed a significant improvement in threading approach through employing *ab initio* energy function, in which 68 out of 127 were greatly improved in Prosup benchmark [71]. Christopher and Yu reported a rational combination of Rosetta *ab inito* structure prediction with I-SITES library of sequence motifs and HMMSTR model. In brief, a fragment moveset was generated by I-SITES, and then submitted to three separate HMMSTR models, which predicted backbone angles, secondary fold and supersecondary structure, respectively. These restraints were later applied in Rosetta Monte Carlo Metropolis conformational sampling [72]. I-SITES-HMMSTR-Rosetta approach resulted in 61% topologically corrected residues among 31 proteins in CASP4. Finally, *de novo* scoring functions may provide insights to guide polypeptide and antibody engineering in the future.

Modeling flexible active site

One of the earliest, and simplest, models of ligand binding is the lock-and-key paradigm [73], which views ligand-receptor binding as a rather rigid shape-matching process, akin to assembling a jigsaw puzzle. Modern models, such as the induced fit or conformational selection models, generally include flexibility in both ligand and substrate molecules [74]. While it has become common sense that biological receptors may subject to conformational rearrangements upon ligand binding [75], to model the bound-complex (or *holo* form) from unbound-receptor (or *apo* form) still remains quite challenging for years. Two distinct strategies offer potential solutions to this issue: direct modeling *holo* receptor prior to docking or ligand-dependent flexible docking. Recent developments have provided insights to modeling protein mobility by introducing receptor rearrangements in process of or prior to virtual screening. Several reviews are available in the previous literature [76–78]. In this section, we will briefly introduce the rationale to fulfill receptor flexibility and its related application (Table 1).

Since the majority of movement upon ligand binding involves side-chain and loop rearrangement, flexible docking is a cost-effective means to consider small degrees of

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receptor flexibility during docking. Minor side-chain movement in protein can be addressed by soft potentials, which put "softer" penalties on non-polar van der Waals clashes [79]. Due to its relative computational efficiency, soft potential options have been routinely added in a large variety of docking packages [80–82]. Library-based rotamer exploration is another knowledge-based approach to explore side-chain variations. This strategy is most widely utilized to treat moderate side-chain and minor backbone movement. The rationale of using rotamer library is to model continuous rotametric changes by discrete amino acid side-chain rotamer libraries and circumvent computational-prohibitive exhaustive searching. Commercially available software, such as GOLD, ICM and Glide, implemented their own robust side-chain rotamer libraries. As for open-source ones, RosettaLigand developed by Backer's lab aimed to save computational expenses by simultaneously searching protein side-chain, backbone and ligand rotamers using predetermined discrete rotamer libraries by Monte Carlo minimization [83, 84]. More recently, MedusaDock used stochastic rotamer library of ligands (STROLL) and was validated to sample ligand conformers in a more comprehensive fashion [85]. However, secondary structure rearrangements and domain movement still remain significantly challenging so far. As an efficient solution, normal-mode analysis based on protein elastic network model is able to estimate *apo-holo* transformation in which Cα movements are involved [86]. Still, it should be aware that flexible docking may offers both risks and prosperities in structure-based drug discovery [87], and expert knowledge on receptor and docking parameters are the bottom requirement.

Molecular dynamics (MD) can be used in various ways to model receptor flexibility. One of the most straightforward ways is to simply perform a simulation of the receptor structure prior to docking, and form an ensemble of structures by taking snapshots of structures during the simulation. This is the ensemble docking-based approach carried out by Sivanesan *et al.*, who generated a 51-member ensemble of structures by performing 3ns of MD simulations of human estrogen receptor α [88]. These structures were used for screening a 3500 compound library, successfully identifying a handful of promising compounds. To sample large protein rearrangement that leads to *apo-holo* transition, including secondary structure/domain movement, normal modes or low modes are frequently used to model concerted motions observed in molecular dynamics. For instance, "low mode", employed by MacroModel (Schrödinger), helped to validate novel inhibitors against such targets as farnesyltransferase [89] and Galectin-3 [90]. A slightly more sophisticated use of molecular dynamics is the research by McCammon and coworkers, who have shown that incorporating a short molecular dynamics refinement step into an iterative docking method gives improved results and can find alternative docking poses which otherwise would be missed [91]. However, due to the computational expense of standard molecular dynamics, its practical use in high-throughput virtual screening is limited to detailed studies of a limited number of selected compounds, late in the screening pipeline [92]. This may change in the future with improvements in computing hardware.

Standard molecular dynamics simulations remain too computationally expensive for virtual screening against a large compound database, but modifications to molecular dynamics have been developed to increase its efficiency. One such technique is the temperature replica exchange method [93], in which several replicas of the system of interest are run in parallel using molecular dynamics at different temperatures. Periodic exchanges are performed between replicas such that the correct sampling is achieved at each temperature. This allows for a faster search of configurational space. The parallelization of this algorithm is well-suited to modern cluster computing architecture. Examples of the application of replica exchange molecular dynamics include the work by Gerek and Ozkan who, in a ligand-binding replica exchange simulation study of PDZ domains, used dihedral restraints on the receptor to induce a bias towards binding site conformations [94], further reducing the simulation time, while still allowing for binding site flexibility. Of course, replica exchange

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can also be used to efficiently sample ligand populations as well [95]. Conceptually similar but more computational efficient than ensemble docking, in which compounds were docked iteratively to multiple receptor conformers (MRC), Abagyan's group developed ICM four-dimensional docking (4D docking) on the basis of the idea that receptor flexibility can be reflected as the 4th discrete dimension on the docking grids [96]. 4D docking approach was tested against 99 proteins and 300 diverse ligands and achieved comparable accuracy in the benchmark (77.3%), but 4-fold efficiency in ligand conformational sampling compared to traditional MRC docking [96]. This innovative methodology, combined with receptor conformer sampling, may elicit promising speed and accuracy in flexible docking study of unfamiliar targets.

Interestingly, a ligand-based strategy may ease the flexible docking against some well-defined targets. Quantitative structure-induced conformation relationship (QSiCR) model [97, 98], sharing similar concept to structure-activity relationship (QSAR), predicts the displacements of key residues by statistical analysis of ligand-encoded properties [78]. In both CDK2 [97] and p38 MAP kinase [98] systems, QSiCR models derived from 2D descriptors of co-crystallized ligands were found sufficient to predict the variation in active sites. Take CDK2 as an example, the *trans* side-chain position in Lys33 was often induced by bulky negatively charged group, whereas Lys89 side-chain conformation was determined by the hydrophobicity of ligand [97]. This multivariate statistical method circumvents the combinatoric issue possessing by library-based rotamer searching and MD approaches, but the predictive power of QSiCR for non-cognate molecule still remains to be tested.

Basic methods for protein inhibitor design

The spirit of structure-based drug identification and optimization is to identify biologically active compounds from "compound forests" with higher efficiency and hit enrichment by utilizing the information from protein structures. This can be realized through different strategies. First, lead compounds or scaffolds can be identified from diversified compound pool and accelerated screening, such as high-throughput virtual screening (HTVS). Second, instead of making random screening pool, we build up a focused library based on the knowledge of bio-target, such as structure-based library design followed by screening. Third, we rationally shrink the screening pool based on the activity-associated patterns, such as structure-based/ligand-based pharmacophore modeling. Fourth, "conceptualize" the active compounds and search inversely for the compounds that may exist or synthesizable in the nature, such as *de novo* design/fragment-based design. Finally, we can augment the hit enrichment by directly estimating the binding free energy in a cost effective manner. We will discuss each strategy by the following part in more detail.

Molecular docking-based virtual screening

Generally speaking, molecular docking is a computational approach that predicts the orientation of a small molecule (ligand) in stable complex with a macromolecular target (receptor) using specific scoring functions. It has become the basis of structure-based virtual screening and has shown to be influential in current pharmaceutical design which is more economic than experimental screening. Docking is overly a "standard" procedure when protein-ligand complex structure is available. The advantages of structure-based virtual screening are obvious: quick and minimal manual interventions. Docking-based virtual screening has played pronounced roles in the lead identification and optimization in the history.

As summarized by Elizabeth *et al.*, the general issues in molecular docking that currently need to be addressed consist of: 1. bioinformatics and chemoinformatics of receptor and ligand representations (side-chain and backbone flexibility, protonation, structural water,

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tautomerism, rotamerism, efficiency of sampling, *etc.*) 2. sophisticated scoring function [100]. Besides, performing high-throughput virtual screening is faced with other critical obstructs such as combinatoric complexity and limited availability of computing resources. We made a list of popular docking software along with their respective algorithm in Table 2. Open-source software, such as Autodock (The Scripps Research Institute) [99], DOCK (UCSF) [101], RosettaLigand [83, 84] are widely used and exploited in several studies. The expandability of source code enables researches to understand, customize, improve and redistribute more robust docking packages [102, 103]. Commercial packages, such as Glide (Schrödinger) [81, 82], ICM (MolSoft LLC.) [104], GOLD (CCDC) [80], Surflex [105], FlexX (BioSolveIT) [106], Fred (OpenEye Inc.) [107], eHiTS (SimBioSys Inc.) [108] and LigandFit (Accelry Inc.) [109], are often considered more advanced. They usually outperform open-source competitors in atom typing, pocket representation, docking restraints setup, *etc*. Nonetheless, they are still far from perfection [110].

Here arises the paradox of using docking as an accelerator of drug discovery: predictor or liar?

Interestingly, although experimental high-throughput screening is known to considerably expensive and unreachable by most academic laboratories, the cost of establishment and maintenance of *in silico* virtual screening, including program licensing and purchase of high-performance computing facilities, are also considered significant [111]. It calls for the need to re-evaluate the docking accuracy before HTVS.

Conformational sampling and scoring are two sub-problems in docking programs, whereas accurate scoring remains more problematic than sampling [112]. A recent comprehensive comparison of 7 docking packages was carried out on a large dataset containing over 1300 protein-ligand complexes by Plewczynski *et al.* [113]. They proposed a general ranking regarding the pose prediction capability to be: GOLD ~ eHiTS > Surflex > Glide > LigandFit > FlexX ~ Autodock, yet the scoring functions for all 7 packages is less than satisfactory, with the best RMSD-rank correlations merely achieving 0.32 [113]. As to ranking and scoring decoys, stand-alone scoring packages, such as X-Score and DrugScore^{CSD}, are found to have better Spearman correlation around 0.66 based on another study using 195 diverse dataset [114]. According to another study carried out by Wang *et al.*, 14 scoring functions were extensively evaluated on an 800 PBDbind database. X-Score, DrugScore, Sybyl::ChemScore and Cenius::PLP exhibited robustness based on consensus results [115]. Perola *et al.* found that GOLD and ICM are more binding site-dependent and docked poorer at the binding site where hydrophobic interactions are predominant, whereas Glide shares consistent performance across various pockets [110]. Rescoring poses by other knowledge-based scoring functions, such as ENTess which is based on interfacial atomic quadruplets and electronegativity, may offer solution to address this issue [116]. Moreover, the starting conformation of ligand may significantly impact the docking results in such software as Fred and AutoDock. However, since the full mastery of any docking package requires a high level of user intervention, these comparative studies may be only offered as a guideline for external users due to the differences in program settings and target characteristics. Stand-alone scoring function may also encounter overfitting issue. Therefore, to solve these behind-the-screen issues, one may need knowledge of the binding mode, multiple starting conformations, cross docking evaluation and consensus scoring.

Does docking-based virtual screening actually accelerate the drug discovery cycle? Honestly, yes, but only wisely programmed. Computer may also experience embarrassments as we human beings when faced with a screening task that involves millions of compounds. High-throughput technology has become a requirement for computer-aided drug discovery nowadays. Docking through parallelization or distributed computing experiences unprecedentedly advances than ever before. Parallel docking mode employed by such

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software as DOCK, GOLD and Glide has significantly enhanced the screening efficiency. As for open-source software, DOVIS, a HTVS program using AutoDock4 docking engine, enables researchers to perform massive virtual screening efficiently on Linux-based high performance clusters [102, 103]. Hyper-extensive file operation issue was optimized by automating load balancing. This software has been successfully employed during the development of PHT-427 which targets AKT1 pleckstrin homology (PH) domain [117, 118]. VSDocker, which is also relied on AutoDock engine, was the first high-performance virtual screening software under Microsoft Windows system [119]. In addition, several volunteer computing projects have been carried out recently that utilize unused computing resources to accelerate molecular discovery cycle [120]. For instance, developers of Rosetta Docking@home [121] and Docking@GRID aim to perform precise and effective docking; more importantly, they tried to explore the optimal searching pathway, maximize the simplicity of searching, and optimize distributed algorithms via metaheuristics methods or community sharing.

Structure-based ligand library design

Computational screening of large compound libraries have been characterized by shortfalls such as limited hit rates [122], poor biochemical quality of hits [123], high costs [124, 125] and quality of this process [126, 127]. One way of addressing the above mentioned shortcomings without compromising on success rates is the development of structure-based library design methods [128, 129]. Recent advances in the availability of protein structural information [130, 131], combinatorial chemistry [132] and computational methods (e.g. docking) have increased the feasibility of library design and enrichment methods. Fragment-based design and diversity oriented synthesis (DOS) [133] are two widely used approaches for library design and are discussed elsewhere [134–138]. On the other hand, ligand-based library design methods, e.g. using pharmacophores [139, 140] and QSAR [141], are also used and applied.

A protein-ligand complex structure serves as the foundation for subsequent structure-based library design. Protein-ligand interaction patterns (e.g. hydrogen bonds) are determined, and the compound core is retained. Next, either a library of compounds is generated by attaching relevant fragments satisfying the interaction patterns to the core, and then sequentially docked to identify top hits; or the core is placed in the binding pocket with fragments attached to generate target-specific library based on the docking scores. Docking programs e.g. CombiDock [142], DREAM++ [143], BUILDER v.2 [144], OptiDock [145], CombiGlide (Schrödinger), and CombiSMoG [146] have been specifically developed for structure-based library design. Here, we highlight a small set of applications for structure-based library design (Table 3).

For example, the complex structure consisting of a hit obtained from high-throughput screening and Hepatitis C NS5B polymerase was used to design a compound library for rational drug discovery [147]. The scaffold of this hit was used to intuitively design another compound to maximize interactions within the binding pocket while retaining a similar binding mode as the original hit. A focused library of 1175 compounds were docked and filtered based on the pharmacophore and revised scaffold, resulting in a 3μM hit amongst the 10 top ranking molecules. This compound laid the foundation of further focused library generation and low micromolar hit identification.

A group from Pfizer applied a structure-based library design methodology to identify hits for soluble epoxide hydrolase (sEH), a potential target against cardiovascular dysfunction and renal damage [148]. The crystal structure complex of sEH with a benzoxazole template compound was used to identify 2000 reagents. The compound library was docked and filtered using the presence of critical enzyme-compound hydrogen bonds in lieu of docking

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scores. 383 of the total of 591 compounds were synthesized. 102 compounds showed nanomolar activity (~26%) and 192 had submicromolar activity.

The hyperactivation of receptor tyrosine kinase EphB4 is responsible in many types of cancer [149]. Kolb *et al.* developed an anchor-based library tailoring (ALTA) approach to develop a structure-based focused library to identify inhibitors against EphB4 [150]. In brief, 35513 rigid fragments were identified from three commercial compound libraries (~782K). The fragments were rigidly docked against two homology models of EphB4 differing in the orientation of the hydroxyl group of Thr693, filtered by their ability to have a) a rigid moiety and b) hydrogen bonds with the residues Glu694 and Met696 in the hinge region, and finally ranked according to their binding energy. 21418 compounds containing the top ranked 1205 fragments were docked by keeping the fragment as an anchor in the two models and ranked using linear interaction energy with continuum electrostatics model. Top 40 hits were experimentally tested and 8 of them showed low micromolar activity in a florescence-based enzymatic assay.

Shah *et al.* developed a focused cysteine protease inhibitor library using a two-step process of substructure-search and pharmacophore based filter, prior to screening for anti-malarial inhibitors [151]. In step 1, compounds in 6 commercial libraries were shortlisted if they possessed soft electrophile substructures of α-heteroatom-substituted ketones and amides, azetidinones, α-keto amide, α-keto acid, and α-keto ester. A receptor-based pharmacophore was developed using the docked complex of the peptidic vinyl sulfone and Falcipain-2 (PDB ID: 2GHU), a related papain family cysteine protease. Two hydrophobic features corresponding to the S2 and S3 regions of the binding pocket, a hydrogen bond donor and hydrogen bond acceptor feature with Gly83 and Cys42 and Gln36 respectively formed the pharmacophore. Only those compounds from step 1 that satisfied three or four pharmacophore features were selected for the docking process. Subsequent docking, selection and experimental testing led to the discovery of 21 diverse non-peptidic inhibitors of FP-2 with a hit rate of 42%.

Structure-based pharmacophore modeling

A pharmacophore is defined as a spatial arrangement of functional groups and substructures common to active molecules and essential to biological activities [152]. The majority of pharmacophore models relied on the concept of “molecular similarity” of small molecules are derived from a series of active compounds and inactive ones [153]. Ligand-based 3D pharmacophore modeling, which is often used in the absence of target structure, has been reviewed in many publications [154–158]. On the other hand, various receptor-based pharmacophore approaches have been explored by probing predisposed interactions in the protein active site [159, 160]. The concept of structure-based pharmacophore has been widely introduced not only in drug discovery stage, but implemented in lead optimization and *in silico* ADME prediction, e.g. MetaSite [161], as well. The advantages of using pharmacophoric models over conventional docking scoring functions are that pharmacophore provides versatility for any type of protein of interest and options to accentuate target-specific interactions, such as π-orbital interactions and entropy effects [162].

LigandScout utilizes six types of chemical features and volume constraints to build pharmacophore models from ligand and its surrounding amino acids as the representation of protein-ligand interactions [152]. LigandScout was frequently used for *in silico* virtual screening against large-scale compound database [163]. This tool was first validated in context of human rhinovirus serotype 16 (HRV16) inhibitors [152]. Three dimensional pharmacophores were automatically created from three complex structures of HRV16, from which a common pharmacophore model was derived by applying the overlay algorithm of

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LigandScout. The combined HRV16 pharmacophore model was taken to search PDB and Maybridge database, which yielded four distinct hits, including such capsid-binding agents as pleconaril, all of which have been reported to be HRV16 hull protein binding agents [164]. More recent publications also implied the profound potentials of LigandScout in studying substrate binding specificity (e.g. Factor Xa), structure-guided pharmacophore-based virtual screening (e.g. novel sub-micromolar HIV-1 reverse transcriptase inhibitor and 11β-HSD1 inhibitors) [165, 166], and developing inhibitors targeting protein-protein interactions (e.g. CHIBA-3003 which interrupts HIV-1 Integrase-LEDGF/p75 interaction) [167].

Mason *et al.* developed a four-point pharmacophore method to perform molecular similarity search, which can be used for the design of combinatorial libraries [168]. This method calculated all potential pharmacophores/pharmacophoric shapes for a ligand and complementary site points in the binding site of a target protein. In combination with the ChemDiverse/Chem-X software, this pharmacophore method can be customized to enable a new measure of pharmacophore diversity. For example, Mason applied his method to design combinatorial libraries for 7-transmembrane GPCR targets, where “privileged” substructures were used as special features to internally referenced the pharmacophoric shapes [169]. The Mason’s method was used to consider up to 7 features and 15 distance ranges, which gave up to 350 million potential 4-point 3D pharmacophores/molecules. Subsequently, the resultant pharmacophore “key” served as a powerful measure for diversity or similarity, and provided a consistent frame of reference for comparing molecules, sets of molecules and protein sites.

FLAP (Fingerprints for Ligands And Proteins) uses a common reference framework of four-point pharmacophore fingerprints derived from GRID analysis and a molecular-cavity shape to describe small molecules and protein structures [170]. The complementary description of the target and ligand generated by FLAP can firstly be implemented for selectivity or similarity analysis of macromolecules in a superimposition-free manner; secondly be used for ligand/structure-based virtual screening; and thirdly calculate chemometrics analysis and initiate a docking exercise [170]. Compare with DOCK and FieldScreen, better chemotype enrichment was achieved by both ligand-based and receptor-based screening by FLAP in an assessment using Directory of Useful Decoys (DUD) dataset [171]. Similar concept is embodied in MetaSite program, a robust software predicting the cytochrome P450 (CYP)-mediated metabolic site of given compound (Fig. 1a). Specifically, CYP-based GRID flexible molecular interaction fields (MIFs) are used to model the involved cytochrome, whereas GRID probe pharmacophore recognition is developed to characterize the substrate chemotypes [161]. The site of metabolism is predicted based on the hypothesis that the distance between the reactive center (e.g. oxene atom in protoporphyrin) on CYP and GRID-MIF points should correlate with the distance between the reactive center of the substrate and the positions of different chemotypes in the substrate. This state-of-the-art methodology facilitated the accurate prediction of the primary site of metabolism in 85% of the cases.

Weil and Rognan developed a novel protein-ligand fingerprint (PLFP) method to mine chemogenomic space (Fig. 1b) [172]. Their method combines standard fingerprint representations of ligand with a protein “cavity fingerprint (CavFP)” representing pharmacophore features of protein binding site residues. This concept was tested on GPCR family whose members share a homogeneous cavity description. GPCR’s CavFP is a composition of pharmacophoric properties retrieved from 30 cavity-lining residues, whereas ligand fingerprints are generated by combining MACCS, SHED descriptors and charge [172]. PLFP model was trained by known protein-ligand pairs, and in GPCR case, this combined model outperformed other corresponding ligand-fingerprint-only models in mining chemogenomic space. While ligand prediction (ligand screening) performed better

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than receptor prediction (target profiling), PLFP was already robust in terms of the high recall and precision, short hit list size and low hit rank in ligand screening [172]. This approach can be utilized in future polypharmacology design to directly predict chemogenomic protein-ligand pairs. Furthermore, Sato *et al.* reported a new interaction fingerprint (IF) based on ligand pharmacophore (Pharm-IF) (Fig. 1c) [162]. Similar to residue-based PLIF developed by MOE, Pharm-IF is calculated from the distances of pairs of pharmacophore features which form interactions with protein [162]. Trained with machine learning techniques, i.e. SVM, Pharm-IF can be used to post-process docking poses generated by Glide during virtual screening. Pharm-IF-SVM model was tested in various protein contexts (PKA, SRC, cathepsin K, carbonic anhydrase II and HIV-1 protease), and they demonstrated that Pharm-IF-SVM model achieved a higher enrichment factor at 10% (5.7) than those of Glide score (4.2) and PLIF (4.3). This structure-based pharmacophore modeling offers extra restraints on the interaction patterns so that it can effectively enhance the enrichment of active compound during virtual screening against well-known protein targets.

Fragment-based drug design (FBDD)

FBDD aims to discover novel chemical leads with expected pharmacological properties, namely tight binder, from small “efficient” binder [173]. FBDD approach has been successfully utilized to guide pharmaceutical design inhibitors against a variety of targets, such as CDK4 [174], estrogen receptor [175, 176], factor Xa [177], HIV protease [178], to name just a few [138]. Besides lead identification, FBDD also plays a significant role in lead optimization. Raf-1 kinase inhibitor, sorafenib, as a successful example, was derived from a 318Da fragment-like lead by FBDD [138, 179]. Building blocks in FBDD are typically the substructures from drug-like molecules in 140–400Da range [138]. To ensure a higher likelihood of obtaining chemicals with proper physiochemical properties, “Rule of 3” was introduced during the construction of fragment database [180]. Binding modes can be determined via either experimental (e.g. structure-activity relationship by NMR (SAR by NMR), X-ray crystallography and tethering) or *in silico* techniques (e.g. molecular docking). For instance, SAR by NMR was utilized to guide the synthesis of ABT-737 and SP-4206 [181, 182]. Higher-affinity binders are designed by either linking fragments bound in distinct parts of the binding pockets or growing initial scaffold based upon the concept of combinatorial chemistry [128]. From a computational perspective, initiating from the lead fragment, “seed and grow” method iteratively assemble suitable fragments binding site before onto its suitable sites pre-docked seed. In contrast, “dock and link” approach is to dock substituent groups based on the chemical environment, and then link based on chemistry constraints [128]. “Seed and grow” involves more combinatorics issues than “dock and link”. This issue has been partially addressed by a number of *in silico* tools that were developed to optimize the “privileged” fragment enumeration process. These computational tools have been reviewed [138, 173]. In brief, LEGEND [183], RASSE [184] and F-DycoBlock [185] were developed based on force field-based scoring functions. Other programs as LUDI [186, 187], LigBuilder [188], SPROUT [189] and CONCERTS [190] employed empirical scoring functions. SMoG implemented its own knowledge-based potential grounded from statistical analysis of ligand-protein interactions [146, 191]. Several programs developed combined scoring functions. GANDI scoring function, for example, is a weighted sum of force-field term and two knowledge-based similarity terms against known CDK2 inhibitors [192]. SYNOPSIS utilizes a hybrid scoring function which combines empirical scoring with force fields [178].

However, a number of challenges have been characterized in FBDD. Synthetic accessibility issue, for example, has been addressed by assigning restrictions on growing/linking site or penalties during fragment assembly. SYNOPSIS established “*in silico* reaction” that grow

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compounds by simulating organic synthesis steps [178]. Similarly, LigBuilder 2.0 also employed a synthesis accessibility analysis module which checks the synthetic feasibility and selects reasonable synthetic routes [193]. PRO_SELECT was implemented with template-substituent idea from combinatory chemistry when pre-docked seed and proposed fragment were linked. Of note, PRO_SELECT achieved great success in designing factor Xa protease inhibitor, LY-517717 by Eli Lilly [177]. Second, the binding mode of compound is not necessarily in a modular fashion as desired. High internal energy, abundance of abnormal torsions, unstable chemical bonds and tautomers may impede the designed molecule to maintain the proposed interactions. Third, since computational FBDD still largely depends on molecular docking, it inevitably inherits such unsolved issues as protein flexibility and solvation. The only report regarding incorporating residue rearrangement function was made by Ian *et al.* [194]. In summary, FBDD is a paradigm-shifting methodology which allows “privileged” chemicals to target “difficult” pockets and exercise lead optimization. Other than worldwide scoring function issue, more stringent ligand-based constraints await further developments.

Free energy calculation by molecular mechanics

Scoring function can be perceived as the representation of relative binding free energy, based on the assumption that the free energy difference upon different ligands binding is predominantly contributed by protein-ligand interactions as predicted in the end-point complex. From a recent perspective, formulating computational-efficient scoring functions by focusing on static molecular interactions may misleadingly neglect a large part of contribution of thermodynamics involved in binding free energy: solvation, long-range interactions and conformational changes [195]. Moreover, interaction pattern such as hydrogen bonding may not contribute equally under different chemical environments [196]. Protein-ligand binding energy is now perceived as a nonadditive effect, which is protein context-related, solvent-sensitive and involves protein-ligand cooperative dynamic processes [195, 197]. Therefore, a more sophisticated and context-flexible approach is essential in structure-based drug design.

Molecular dynamics simulations take on a vital role in calculation of binding free energy [2, 198–200]. Recent advances in coarse-graining techniques have allowed the probing of dynamics of large membrane-bound protein systems on the microsecond time scale [201]. Methods for deriving more accurate force fields are an intense area of current research involved in drug discovery, such as “polarizable” force fields [202, 203], in which atomic parameters (e.g. charge) may change in response to changes in environment. This can be expected to improve predictions of binding free energy, where the molecular environment of a ligand may change greatly during binding. For the interested reader, more details of the development of force fields and other aspects of molecular dynamics can be found in various references [204–206]. These knowledges will immensely facilitate free energy calculation. The difference in free energy between different states (such as the free and bound forms of a receptor-ligand system) or along a reaction path (such as the path for ligand binding) can be related to experimentally measurable quantities, such as dissociation constants. The methods for binding free energy computation are well-known [207, 208], but implementation has been hindered by the computational expense of accurate calculations. Nevertheless, binding free energy calculations are available in molecular dynamics packages such as GROMACS [209]. Arguably the most common type of free energy calculation involving molecular dynamics is the free energy perturbation (FEP) method. This method is often used to calculate relative binding free energy for two similar ligands to the same receptor. This method takes advantage of the possibility of simulating unphysical alchemical transformations of one ligand into another, and the free energy of binding is reconstructed using a thermodynamic cycle [209]. The transformation of ligands is accomplished by

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discretizing the path from one to the other. At each discrete point along the path, the system is simulating using a potential which is a combination of the potentials for the two different ligands. This can become very computationally intensive, especially if the ligands are very different. This limits the ligands studied to be a small set of chemically similar ligands, which lessens the attractiveness for use in high-throughput screening. Use of sheer computation is feasible if we limit ourselves to only a handful of system. Examples include the determination of the free energies of binding for eight ligands to FKBP using a massively parallel computing platform [210], or the calculation of free energies of binding for several sparso-mycin analogs to the bacterial ribosome [211]. The computational difficulty lies in the fact that free energies cannot be calculated from a single static structure, but requires sampling of a large configurational space.

As a result, for virtual screening of large numbers of ligands, binding free energy prediction during docking usually relies on fast approximation techniques, such as the use of simple empirical or knowledge-based scoring functions [212]. These approximations generally cannot accurately rank ligands in order of binding affinity, as we have discussed above [213, 214]. An attempt to quantify the errors introduced by various levels of approximation in calculating binding free energies was performed by Mitomo and coworkers [215], who used three methods, namely, in order of decreasing computational expense: explicit water molecular dynamics, an implicit solvent molecular mechanics generalized born surface area (MM-GBSA) model, and a docking scoring function. The test systems used were streptavidin and biotin. As expected results from the explicit water simulations most closely matched experimental measurements (error of 1.8 kcal/mol), with the MM-GBSA model overestimating (error of 17 kcal/mol) and the docking scoring function underestimating (error of 9.6 kcal/mol) the binding strength. Moreover, Noriaki *et al.* combined MM-PBSA with molecular docking in a virtual screening exercise, and yielded an improvement of 1.6–4.0 times that of enrichment performance with molecular docking alone [216]. This points the importance of correctly incorporating the solvent degrees of freedom, which are unfortunately often the most computationally expensive component of explicit solvent simulations. An attempt to generate an intermediate method which is faster than FEP but more accurate than simple scoring functions has been developed [217], called the linear interaction energy (LIE) model. Also, improvements in computational efficiency of free energy calculations can be obtained by using continuum solvation models, which treat water only implicitly, combined with molecular dynamics [218]. Research into fast implicit solvent methods which can match the accuracy of explicit solvent simulations is one of the most pressing problems which, if solved, would greatly enhance the effectiveness of computational drug design. High-throughput version of MM-PBSA, developed by Brown *et al.* from Abbott Laboratories, speeded up the relative binding free energy calculation to an order of 100 CPU min per structure by a rational combination of GB implicit solvation model and Zap (OpenEye) on an enterprise grid [219, 220]. To perform calculation more efficiently, Huang *et al.* incorporated the upstream docking (DOCK and Glide) to the free energy calculation, followed by energy minimization in GB implicit solvent, instead of commonly used MD conformer sampling. The speed has reached as fast as 1 min per structure [199]. Yet, this method may appear to be inaccurate because it bypassed the time-consuming entropy losses calculation and fully relied on GB semi-analytical approximation which contained documented drawbacks [221].

In conclusion, as computational costs continue to decline, we may expect molecular dynamics to play an increasing role in drug design. The power of molecular dynamics lies in its versatility. It is a general technique which can be readily adapted to specific needs, such as docking and free energy calculation. Concepts from molecular dynamics force field development have shown their influence in current pharmacology.

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Targeting protein-ligand interaction

The discovery of a series of HIV-1 protease inhibitors, such as Ritonavir (Abbott laboratory), Indinavir (Merck) and Nelfi-navir (Agouron Pharmaceuticals, acquired by Pfizer), opened up an era of structure-based drug design scenario (Fig. 2) [222]. Previous successful development of protein inhibitors using free virtual screening packages, mainly AutoDock and DOCK, have been reviewed by Bruno *et al.* in their review [223]. Tanaji *et al.* reviewed the development of 12 small molecules that have entered late clinical trials or final approved by FDA for therapeutic use as direct consequences of structure-based drug design [224]. These drugs include Aliskiren (Tekturna®), NVP-AUY922 (Novartis), LY-517717 (Eli Lilly), etc. Van Montfort *et al.* discussed a subset of cancer-related drugs in which structure-based concept was involved in their development [225]. These targets involve oncogenic kinases (i.e. EGFR, Akt/PKB, PI3K, B-Raf, *etc.*), epigenomic target (i.e. HDACs), DNA repair target (i.e. PARP) and chaperone (i.e. Hsp90). *In silico* methods are frequently used in late lead optimization when target structure is accidentally available; however, in some cases, such as Aliskiren and LY-517717, computer models initiated the early lead identification. Here, combined with our lab's experience in structure-based drug design, we picked up several typical successful stories in this field.

From existing structures

The discovery of LY-517717 (Eli Lilly) is a direct consequence of computational *de novo* drug design approach. LY-517717 is an indole derivative (Fig. 2) in which indole replaces benzamidine moiety of 3a lead, and this inhibitor has just finished the phase II clinical trial in prevention of venous thromboembolism in patients with hip or knee replacement surgery (http://clinicaltrials.gov/ct2/show/NCT00074828). In identification of 3a, template-substituent idea of combinatorial chemistry was used by *in silico* program PRO_SELECT. Starting from benzamidine (K_i>200μM) as a seed, a series of factor Xa structure-based focused-libraries were iteratively constructed. The best benzamidine-based lead synthesized after virtual screening, 3a, obtained K_i 16nM [177]. To improve the oral bioavailability and other pharmacokinetic properties, benzamidine moiety was replaced by indole, and it resulted in LY-517717, which achieved K_i value at 5nM [226]. The success of LY-517717 in clinical trials is a proof-of-principle of *de novo* drug design and accentuates the importance of addressing synthetic issue in *de novo* design and library design.

Poly-ADP ribose polymerase-1 (PARP-1) involves in DNA single strand break pathway, which facilitates DNA damage repair by conjugating ADP-ribose to DNA and other DNA repair machineries [227]. PARP-1 inhibitor has been widely used in treatment of cancer, Parkinson's disease and stroke [225]. Early developments of nicotinamide-based inhibitors were unsuccessful because poor specificity and pharmacokinetics were observed in spite of the high potency [227]. The breakthrough started from the development of tricyclic novel PARP-1 inhibitor, based on the crystal structure of conventional nicotinamide-based PARP inhibitors and chicken PARP complex [228]. To reach the pocket formed by two coplanar Tyr907 and Tyr896, amide edge was extended to [5,6,6]-tricyclic indole lactam or [5,6,7]-tricyclic indole lactam. Their model predicted a high binding affinity with [5,6,7]-tricyclic indole lactam, and later crystal structure showed that this crucial amide is locked in a *cis* conformation in this seven-member lactam ring, thus strengthening the interaction. In addition, the 2-phenyl group interacted with Tyr907 and Tyr896 via π-π stacking, as the design proposed [227]. Later minor optimizations led to Rucaparib (AG-014699), a potent and selective PARP-1 inhibitor with K_i <5nM [229]. Rucaparib has finished Phase I trial and submitted Phase II clinical trial in treatment of cancer with BRAC-1/2 mutation to achieve synthetic lethality (ClincialTrials.gov ID: NCT00664781) (Fig. 2).

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A typical high-throughput docking-supported virtual screening was conducted in our lab which leaded to several promising sulfonamide inhibitors that targets AKT1 PH domain. AKT is known as oncogenic serine/threonine protein kinase that plays a key role in mediating signals for cell survival, proliferation, apoptosis, transcription and glucose metabolism [230]. AKT comprises three highly conserved isoforms, all of which have N-terminal pleckstrin homology (PH) domain, serine/threonine kinase domain and C-terminal hydrophobic motif [231]. PH domain is crucial for the translocation to the cellular membrane by attaching phosphatidylinositol (3,4,5)-triphosphate (PIP3). A ligand-based pharmacophore model identified 4 active compounds from a three million combined database. AutoDock was combined with GOLD to identify leads which contained sulfonamide moiety. After lead optimization and structure-activity relationship (SAR) investigations, PHT-427 was synthesized and exhibited K_i 2.7μm activity against AKT1 PH domain [117, 118, 232, 233] (Fig. 2). As we will further discuss in polypharmacology part, PHT-427, as a results of docking-based strategy, was also found to target phosphatidylinositide-dependent protein kinase 1 (PDPK1) PH domain at a slightly higher K_i (5.2μM) [118].

Starting from protein models

Strikingly, protein models, especially homology models, are widely used in structure-based drug discovery projects [10]. Some applications include protein structural analysis and function prediction [40–45], understanding of protein-ligand interactions [46–53] and compound optimization [54–56]. In order to compensate from the low-resolution models, additional target-specific constraints and/or more accurate scoring function are often applied. Here we will briefly discuss some successful hit identification stories starting from homology models (Table 4).

Renin is an aspartic peptidase which is able to cleave angiotensionogen and therefore trigger agiontensin II pathway, is an attractive target for the treatment of hypertension [234]. Homology model of renin played a critical role in developing Aliskiren (Tekturna®), an FDA approved drug in 2007 [235]. Aliskiren is the 3rd generation renin inhibitor, whereas both 1st generation peptide inhibitor CGP29287 and 2nd generation peptidomimetic inhibitor CGP38560 experienced disastrous failure in the clinical trials. The fact that CGP38560 exhibited very poor oral bioavailability (<1%) and half life (<7min) aroused the interests to develop small-molecule drugs that had better pharmacokinetic properties. Since no crystal structure available at that time, a homology model of renin completely orchestrated the structure-based drug designing project. The critical residues of binding were predicted by extensive docking of 2nd generation peptidomimetics, and four interaction pharmacophores (P1, P2, P3, P4) were determined. Then, a number of potent leads (<5nM) were rationally designed, including tetrahydroquinoline (THQ), phenoxy, indole and salicylamide, based on predicted docking poses and binding free energy. Aliskiren is a direct descendant of a phenxy lead extended by methoxyl-*tert*-butyl group, which has potent IC_{50} in a human cell line (0.6nM) (Fig. 2). The approval of Aliskiren raises two amazing facts: 1. The crystal structure for renin in complex with CGP38560 was solved in 1991, and this binding poses have already predicted in 1987 by homolog modeling, and 2. This 4-year-ahead advantage competed other pharmaceutical companies, such as Merck, Abbott, Roche, Pfizer, *etc*., in clinical trials and marketing of the drug targeting renin-angiotensin system (RAS) pathway [235].

Inhibiting uncontrolled cycles of endocytosis as in epileptic seizures represents a significant ($15 billion market) and largely unmet medical need [236]. Dynamin receptors are responsible for this disease. Lack of crystal structures for human dynamin has impeded rational drug discovery efforts, resulting in promiscuous compounds with varying side effects. Odell *et al.* have attempted to address this problem by developing a rational drug

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design study using homology modeling and docking-based protocol to identify selective dynamin I inhibitors. Two models of the GTPase domain of dynamin I were developed using the *D. discoidum* GDP-bound dynamin A GTPase domain, which were validated by their ability to reproduce low RMSD docking poses (<1Å) of GDP. The models were used in a virtual screening study against ~80K compounds and post experimental testing. A new class, namely pthaladyn, was identified to have micromolar potency. This hit and its protein-ligand interaction details were exploited to design several other potent GTP-competitive analogues [237].

Pharmacophore modeling can be used to promote the hit identification rate of homology model-based drug discovery. In an interesting approach involving homology models, researchers at Sanofi-Aventis developed sequence derived 3D pharmacophore models for class A GPCRs. A multi-step modeling and docking approach using known ligand-protein experimental data as restraints was used to model three-dimensional protein-ligand complexes. Key protein-ligand interactions (e.g. Asp332 interaction with positively charged nitrogen of ligands) were encoded as 2D chemoprints and were mapped to 35 single feature structure-based pharmacophores. As an application, the sequence of C3AR1 receptor identified four 2D chemoprints. Using the corresponding 4 pharmacophores, a ligand-based virtual screening identified the first small molecule inhibitors for C3AR1 [246]. A similar receptor-based pharmacophore was successful applied by Sala *et al.* to a virtual screening study against the homology model of hIKK-2 protein to increase the identification rate of novel chemotype inhibitor [239].

Targeting protein-protein interaction

In order to function properly, most proteins do not act as isolated units; they often form complexes with other macromolecules, usually proteins. The formation of these complexes relies on the protein interactions (PPI). PPIs are ubiquitous in all biological processes, and cellular dysfunction via faulty protein-protein interactions is the root cause of a plethora of diseases, including cancer and neurological disorders [247, 248]. Similar to protein-ligand interaction, recent structural analyses of protein-protein surfaces refined the definition of PPI as locally-optimized and stable protein-protein association, dominantly contributed by clustered, networked, highly packed, cooperative and structurally conserved residues [249]. However, in contrast to classic protein inhibitors that target well-defined small molecule's binding grooves or channels [250, 251], PPIs are usually mediated by flat and large interfaces. The rapidly increasing knowledge of thermodynamic basis of PPI as well as the quality and diversity of lead compound have allowed multiple studies of drugs indeed targeting interfaces [252, 253]. Hence, this new class of novel, druggable interactions greatly expands the target space available for a myriad of diseases.

Hot spots identification and prediction

It has been well documented that for all PPI interface, the energy distribution is not uniform across a particular interaction; a small set of residues contribute more significantly to the binding free energy than other residues, so-called hot spots [254–261]. Bogan and Thorn defined a hot spot as a residue whose mutation to alanine results in a binding free energy change of at least 2.0 kcal/mol [262]. As one might expect, the residues at protein interfaces [263] and functional sites [264] are mutating at a slower rate when compared to other surface residues. Furthermore, hot-spot residues are complementary, interfacial residues that are usually surrounded by weaker interactions providing specificity [265].

Experimental identification of hot spot residues in PPIs is primarily performed by alanine scanning, whereby hot spot is defined as the residue that, when mutated to an alanine, results in a remarkable drop in the binding constant, typically tenfold or higher [266]. Alternatively,

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we can consider a hot spot mutation as a mutation that destabilizes the bound state ensemble relative to the unbound one if we consider the insights of free-energy surfaces and conformational ensembles from protein folding studies [267, 268]. Alanine scanning allows one to determine the specific contribution of residues to the stability of the ensemble, and protein's resulting function. Results from alanine scanning are deposited in the Alanine Scanning Energetics Database (ASEdb), and the Binding Interface Database (BID) contains experimentally verified hot spots from the literature [262, 269]. These repositories, while useful, may have drawbacks such as limited complexes [266]. Experimental mutagenesis of target proteins for elucidation of hot spots is not applicable on a large scale since it is very time consuming and expensive. People are seeking effective ways to accelerate the laborious alanine scanning, such as using reflectometric interference spectroscopy [270], "shotgun scanning" [271] and *in silico* prediction. There are many computational tools available to the user that can accelerate this process. Three primary types of predictive approaches exist for computational hot spot prediction: energy-based, machine learning-based and empirical-based methods. Energy-based method estimates the energetic contribution of each residue to the total binding energy via either virtual alanine scanning (e.g. FOLDEF [272], Robetta [68, 273], PP_SITE [274]) or MD simulations (e.g. snapshot-based $\Delta\Delta G_{binding}$ estimation [275], anchor residue analysis [276, 277]). Machine learning-based methods can overcome the blockroad of energy-based method-high computational cost-through both pure machine-learning models (e.g. decision tree-based KFC and SVM-based model [278]) and hybrid models where energetic terms are partially involved (e.g. KFCA [279] and HSPred [280]). While machine learning methods hold great promise, simpler empirical models may better their predictive power with the introduction of additional terms, such as residue conservation. For example, both HotPoint webserver [281] and HotSprint database [282] were established based on empirical models which combined residue conservation term with other terms such as buried/complex accessible surface area and knowledge-based pairwise residue potentials of the interface residues.

Towards potent PPI inhibitors

PPI networks have been studied extensively in multiple species, and their complexity and diversity are unrivaled among other macromolecular interactions [283–286]. The hub proteins (ones with multiple binding partners) in disease-related pathways, such as p53, have proved to be crucial to signaling networks; these proteins gain much interests as they can interact with multiple binding partners through the same interface (promiscuous proteins), as well as simultaneously through different binding regions [287–289]. A number of studies have been carried out through targeting these critical PPIs (Table 5).

The most successful PPI inhibition target up till now is Mdm2-p53. Mdm2, a ubiquitin E3 ligase, targets p53 for 26S pro-teosome-mediated proteolytic pathway. The overexpression of Mdm2 in some tumor cell line was found correlated with the inactivation of p53-dependent apoptosis, and thereby "awakening" p53 by inhibiting Mdm2 binding has been highlighted in previous researches [290]. Mdm2 binds to a 15-residue α-helical region in the transactivation domain of p53, where three contact points (Leu26, Trp23 and Phe19) on this helix confer most of the binding energy (Fig. 3a) [291, 292]. Three classes of inhibitors have been reported as small-molecule potent inhibitors against Mdm2-mediated ubiquitylation: Nutlins [293], benzodiazepine-based inhibitors [294] and spiro-oxindole based inhibitors [295]. Of note, Nutlins and benzodiazepine derivatives were discovered through high-throughput screening, whereas spiro-oxindole scaffold through structure-guided *de novo* design. Seeded with Trp23 residue, a number of spiro-oxindole scaffold containing natural compounds, such as Alstonisine and spirotryprostatin A, were discovered. Further *de novo* lead optimization was performed and guided by GOLD docking conformations to achieve maximal binding affinities. A series of spiro-oxindole based low nanomolar inhibitors,

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namely MI-63 (K_i=3nM) [296] and a more bioavailable derivative MI-219 (K_i=5nM) (Fig. 3a) [297], were designed by expanding the binding pocket to the proximal Leu22. Particularly, MI-219 was fully capable of inhibiting cancer cell proliferation by induction of p53-dependent apoptosis both *in vitro* and *in vivo*. Both Nutlin and MI-219 had entered phase I clinical trial for safety testing [298]. Other active compounds with novel chemical cores, such as NSC66811, were recently identified by a combination of pharmacophore filtering and structure-based virtual screening against NCI database [299]. The resulted NSC66811 acquired binding affinity K_i 120nM that was 3.3 times less potent than Nutlin-3 (Fig. 3a) (36nM), yet exhibited functional restoration of p53 and p21 in HCT-116 $p53^{+/+}$ colon cancer cell lines [299].

B-cell lymphoma 2 (Bcl-2) family of proteins are pronounced regulators of apoptotic cell death, which include both anti-apoptotic factors, such as Bcl-2 and Bcl-X_L, and death agonists such as Bak, Bax, Bim, Bad, etc. [300, 301]. These proteins can form homodimers and heterodimers with other family members, particularly the pro-apoptotic molecule Bak containing BH3 domain (Bcl-2-antagonist/killer) [302]. Many research groups have produced α-helical mimics targeting the hydrophobic binding pocket which mediates Bcl-2/Bak interaction [303–306]. The most distinguished of these are ABT-737 and ABT-263 (Fig. 3b) (Abbott Laboratories), inhibitors of Bcl-2, Bcl-X_L and Bcl-w that were discovered by NMR-based high-throughput screening of a chemical library [307]. These molecules mimics the Bak-derived peptide in binding region on Bcl-X_L, and binds deeper in cavities with more puckered grooves at subnanomolar level [307]. ABT-737 exhibits profound inhibitory potency against lymphoma and leukemia cell lines, and synergizes with other cytotoxic chemotherapeutic agents. ABT-737 has been in phase I clinical trial [308]. Bioavailable ABT-263 (Navitoclax) was developed upon ABT-737 lead and is currently in Phase I/II clinical trial [309]. Besides ABT-737 derivatives, Jia-Lun *et al.* identified a nonpeptidic Bcl-2 inhibitor, HA14-1, after a virtual screening exercise using Bcl-2 homology model and DOCK for screening MDL/ACD database (Fig. 3b). This compound achieved ~9μM IC_{50} against Flu-BakBH3 and exhibited *in vitro* pro-apoptotic efficacy in an Apaf-1-dependent manner [310]. Other research groups, such as, applied similar approaches and identified hits against this target from NCI 3D database. 7 compounds out of 35 selected hits were tested to obtain anti-proliferative effects varying from IC_{50} 1.6 to 14.0 μM. Michele *et al.* conducted structure-based virtual screening targeting BH3 binding pocket using the crystal structure of Bcl-X_L in complex with Bak derived peptide. A total of 320 hits, selected from Maybridge according to FlexX docking poses and H-bonding to Bcl-X_L, [311]. J042 was identified from ZINC database using a combination of receptor-based pharmacophore filtering and cross-docking, and achieved 2.58 μM activity [312]. All of the above active compounds were assessed based on several apoptosis-related parameters, such as swelling, $\Delta\Psi$m loss and cytochrome c release, in which ABT-737 still outperformed other compounds that were designed computationally [313].

As another promising prosurvival target, XIAP (X-linked inhibitor of apoptosis), was demonstrated to bind and specifically inhibit caspase-9 activity via BIR3 domain [314]. XIAP-caspase-9 interaction also shows its direct role in resistance to radiation therapy in cancer cells by blocking both intrinsic and extrinsic apoptosis pathway. Furthermore, the existence of endogenous XIAP inhibitors, such as SMAC and HTRA2, provides proof-of-principle to exploit XIAP as a novel therapeutic target. An incomplete list of XIAP inhibitor is available in Nature Cell and Differentiation Review [314]. As an example of XIAP inhibitors screened from Traditional Chinese Medicinal herbs 3D database, embelin was first discovered by a computational virtual screening using DOCK program [315]. Embelin, along with its derivatives [316], has been proved to be robust apoptotic-inducing agents at low micromolar IC_{50} by overcoming protective effects of XIAP overexpression [315]. The other strategy of targeting XIAP is to block its interaction with caspase-9 by the compounds

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mimicking SMAC-XIAP N-terminal interactive motif, NH_2-AVPI [317]. Frederick *et al.* performed an *in silico* optimization by CAVEAT program on ML-IAP-binding peptide, ALP-2,2-diphenethylamine. They discovered bioavailable azabicyclooctane derivatives as a novel antagonist of apoptosis inhibitor that suppressed BIR3 domain binding at Ki 140nM [318]. Starting from NH_2-AVPI peptide, Huang *et al.* introduced a fragment-based approach by replacing amino acids by drug-like and synthetically accessible scaffolds [319]. Optimizing the lead compound guided by docking, they discovered and synthesized cell-permeable inhibitor BI-75D2, which has anti-IAP cellular activity at IC_{50} 16.4 μM.

Recent anti-HIV researches shed more spotlights on targeting early stage of HIV-1 infection, such as fusion and entry, other than maturation. Two critical protein, gp120 and gp41, were found to mediate the $CD4^+$ cell recognition and membrane fusion, in which gp41 forms a six-helix bundle core upon surface gp120 binding to CD4 and co-receptors (e.g. CCR5 and CXCR4). Massive efforts have been exerted on CCR5 targets in order to block gp120/CCR5 interactions. Maraviroc (Pfizer) defeated aplaviroc (GlaxoSmithKline) and vicriviroc (Schering-Plough) for final approval after racing for the first CCR5-targeted entry inhibitor. As to gp41 target, the stabilization of six-helical bundle requires the antiparallel packing of three C-terminal heptad repeats (HR1-3) into the hydrophobic grooves on the central trimeric core (Fig. 3c), making such hydrophobic cavity attractive as a potential drug-binding pocket [320, 321]. Although great successes have been made by Enfuvirtide [322], the first polypeptidic fusion inhibitor derived from HR2 and approved by FDA, people are still seeking more bioavail-able drugs that circumvents the frequent intravenous injection. The first nonpeptidic gp41 inhibitor identified by structure-based method was ADS-J1 and ADS-J2 (Fig. 3c), two large and hydrophobic compounds that suppressed the fusion-active gp41 core at IC_{50} 4.95 μM and 21.85 μM respectively [323]. XTT formazan has similar anti-fusion activity against gp41 according to their ELISA and docking results [324]. Further drug-like inhibitors explorations, including the primary identification of NB-2 and NB-64 micromolar leads [325] and docking-guided optimization to derivatives of 2-aryl 5-(4-oxo-3-phenethyl-2-thioxothiazolidinylidenemethyl) furan (Fig. 3c), have been carried out recently by the same group [326, 327]. The most potent inhibitors synthesized so far acquired 14nM IC_{50} against the formation of gp41.

Ras GTPase family proteins are well-known notorious in its oncogenic mutations that lead to constitutively active growth signals and mutants-associated drug-resistances [328]. Efforts have been done to silence Ras (or other oncogenic Ras family members e.g. Rho and Rac) signaling by inhibiting 1) post-translational modification, such as farnesylation 2) Ras-effector interaction 3) Ras-GEF interaction 4) GTPase activity restoration [329]. However, little progress was made in designing potent PPI inhibitors probably due to the chemical property of Ras-binding pocket and its associated binding modes. In sharp contrast to other targets discussed above, the interactions observed from Ras-effector complex are mainly composed of antiparallel β-strands hydrogen bonds and electrostatic attractions. Early developments of Ras-Raf1 inhibitor elicited some high micromolar hits, such as MCP derivatives [330], radicicol [331], and effector-derived synthetic peptides [332, 333]. Yuan *et al.* made breakthrough on Rac1-GEF interaction by targeting Trp56 region from comparative and mutagenesis analysis. NSC23766 was identified by structure-based virtual screening using UNITY (Tripos Associtates) [334]. Similar docking-based virtual screening was utilized by Nicola *et al.* in their study, and the best inhibitor they discovered was ZINC08010136 with IC_{50} 12.2 μM, which was slightly better than NSC23766 [335]. Other less potent allosteric inhibitor (e.g. cyclen-metal) that can stabilize the weak binding state for downstream effectors is still in early stage development [336]. In summary, hot topic as Ras-associated disease is nowadays, silencing mutant Ras signaling by interrupting Ras-effector interactions still confronts great challenges.

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It is worthwhile to notice that most successful pockets of PPI inhibitor development are characterized with massive hydrophobicity. The core scaffolds that target Bcl-X_L-Bad, gp41, Mdm2-p53, XIAP-caspase-9, CD4-MHC II [337] and IL-2-IL-2Rα interactions [338, 339] are overwhelmingly hydrophobic. In contrast, such interactions as Ras-effector and PTEN-MAGI3 [340, 341] are mainly mediated by β-strands hydrogen bonds and electrostatic attractions, and those targets have unavoidably experienced obstacles to achieve low IC_{50}. These facts imply the poorly-understood but pivotal roles of entropy and desolvation effects that trigger protein-protein association. Furthermore, targeting PPI also experiences common challenges such as inherent flexibility. From the lesson of developing IL-2 inhibitors, we know that Ro26-4550 (Roche) induced significant conformational changes compared to unbound IL-2 [339]. Hence, novel insights are needed for future drug design and scoring function improvement.

Polypharmacology: Selectivity or promiscuity? Or both?

Modern drug development projects primarily aim to deliver target-specific active compounds. The genesis of this approach was the "one disease, one target and one drug" Mendelian model. However, retrospective analysis proved that approved drugs are often promiscuous and bind to several protein targets [342, 343]. This property of an active compound binding to multiple proteins is termed as polypharmacology. Polypharmacology of bioactive compounds elicit either beneficial (e.g. Sorafenib: A Raf inhibitor originally developed against lung or pancreatic cancer [344] proved effective against renal cell cancer by its action on VEGFR2 receptors [345]) or adverse effects (e.g. Paxil, a serotonin uptake inhibitor, also binds to beta-adrenergic receptors thus offering plausible explanation for increased heart rate [346]). Several other examples can be found are available in previous review [347]. Moreover, increasing evidence proving the complexity of diseases that void the Mendelian model (e.g. multiple targets implicated for one disease state [342]) and the robust or compensatory behaviour of biological systems [348] suggest that designing *selectively promiscuous* compounds may offer better therapeutic outcomes [349]. Likewise, prediction of compound polypharmacology has the potential to identify possible adverse effects [350, 351] which are implicated in about 30% of drug failures.

Several methods have been developed for the computational prediction of compound polypharmacology. Network-based methods integrate chemoinformatics, bioinformatics, and systems biology techniques to investigate compound polypharmacology by mapping drug-target associations [352–355]. Our laboratory developed a network that allows users to intuitively interrogate drug-target relationships based on functional annotation (e.g. biological targets, reported side effects, toxicities or metabolism) and chemical structural similarities [352]. At present over 5000 drugs, ~10 million virtual library compounds, and ~56K biological macromolecules are available for exploration via a web-portal which will be publicly accessible in the near future. Our novel network-based prediction of quercetin's chemical structure similarity with Dasatanib and thus its possible use as a tyrosine kinase inhibitor has been independently validated [356]. Chen *et al.* combined networks obtained from experimental assay information in PubChem, protein-protein interaction, drug-target interaction and biological pathways to map polypharmacology within PubChem database [357].

Structure-based methods are based on the premise that proteins with similar binding sites will attract similar compounds. Examples include the similarity ensemble approach developed by Keiser *et al.* [346], three-dimensional shape based approach for identifying diverse targets [358], structural similarities of molecular scaffolds to map drug promiscuity [359]. Vulpetti *et al.* developed a shape-based binding site similarity method to elucidate polypharmacology in the kinase family [360]. Weill et al. described the binding site of

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GPCRs by encoding both, ligand descriptors and protein pharmacophoric properties, in a low dimensional fingerprint for chemogenomic screening applications [172]. Text mining approaches [361] and semantic framework based methods [362] have been applied to map and predict polypharmacology using widely available biochemical information contained in publicly available resources. Although current structure-based drug design hardly considers multi-targeting issue, efficient polyphamacology prediction may be helpful in the future to facilitate drug repurposing and to discover more potent drug with less off-target toxicity.

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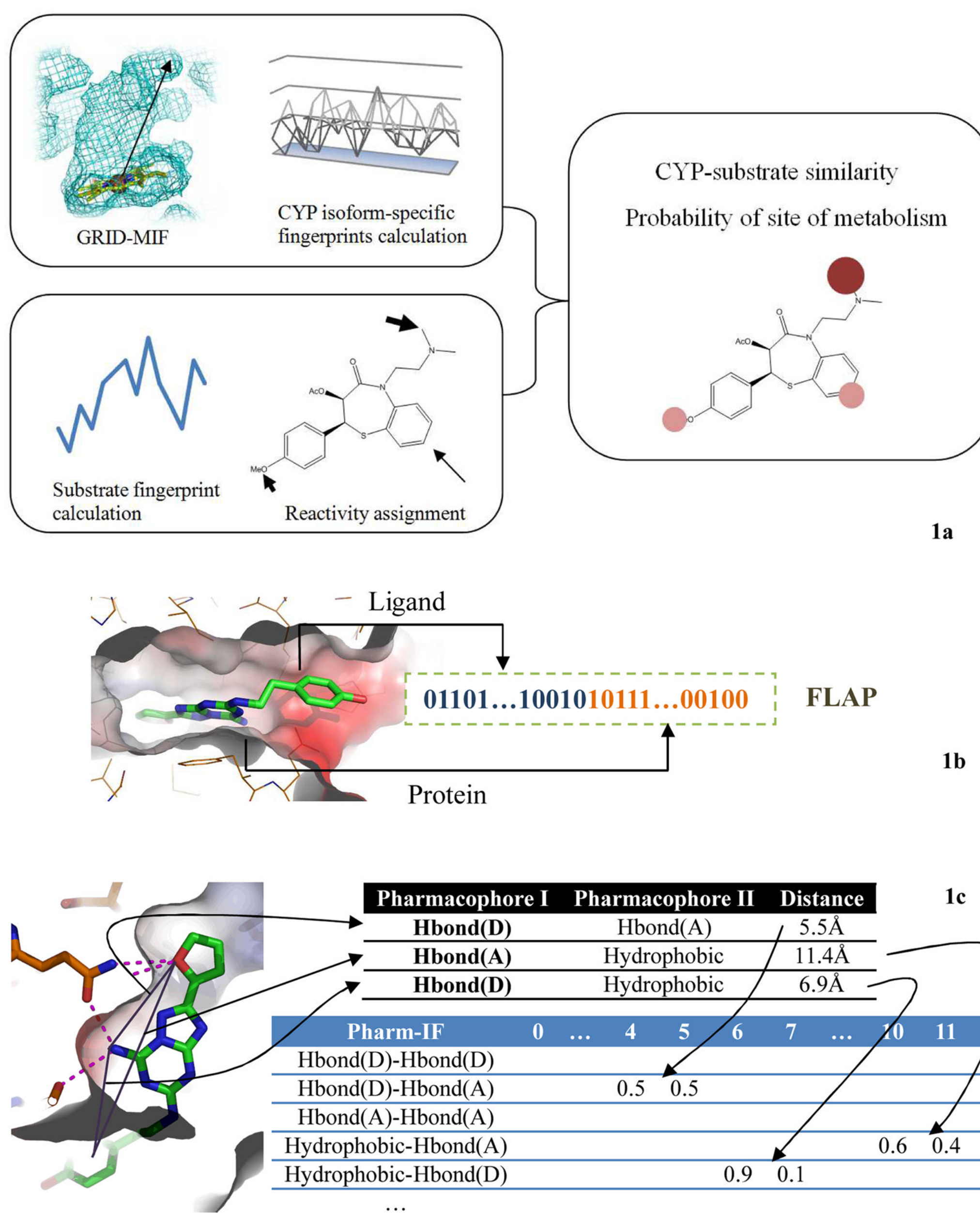

Fig. 1. Structure-based pharmacophore approaches in drug development

1a. Workflow of prediction of the metabolism sites used by MetaSite. GRID-MIF of CYP is generated by chemotype probing technique. Then, similarity between GRID-MIF of CYPs and fingerprints of substrate is calculated to determine the substrate-specific CYPs. The site of metabolism is predicted as the degree of correlation based on the hypothesis that the distance between the reactive center on CYP and GRID-MIF points should correlate with the distance between the reactive center of the substrate and the positions of different chemotypes in the substrate. **1b**. FLAP generation. Descriptors and fingerprints for both ligand and residues in the binding pockets are binned into FLAP fingerprints. Chemgenomic space is mined by machine learning method. **1c**. Pharm-IF fingerprint generation. Only ligand atoms that participate in protein-ligand interaction are involved in fingerprints calculation. Pharm-IF fingerprints are derived from the distances of different

pharmacophoric atoms. Target-specific Pharm-IF can be used to build pharmacophore models by machine learning method or similarity search.

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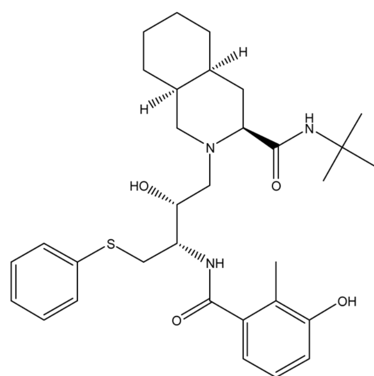

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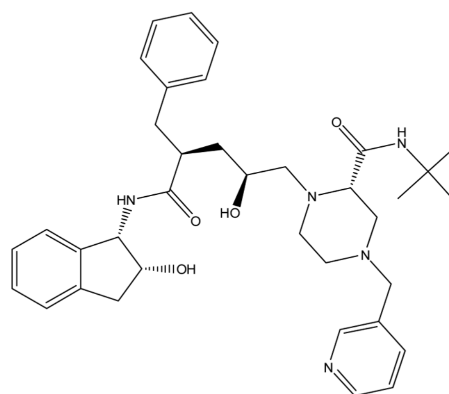

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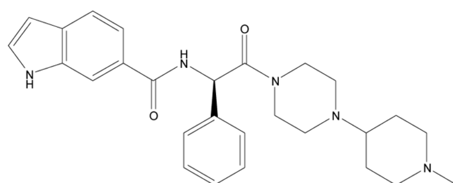

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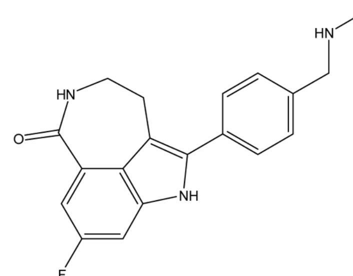

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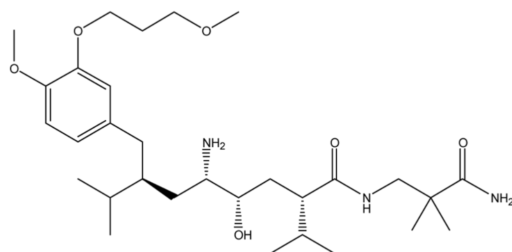

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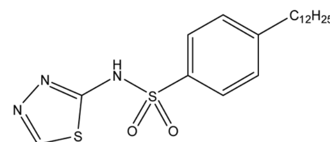

PHT-427

Fig. 2.

Example compounds that target protein-ligand interaction resulting from *in silico* structure-based design

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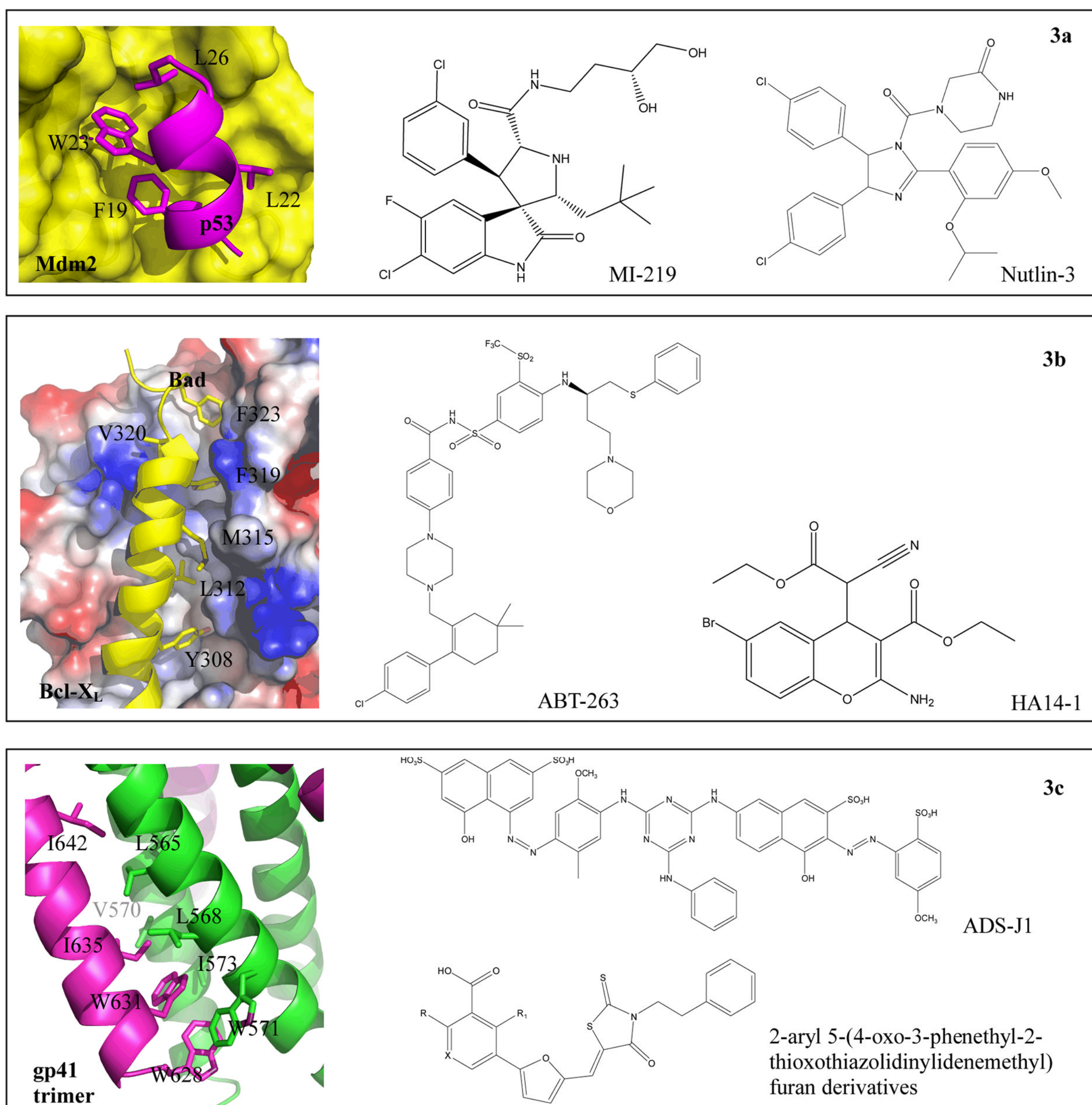

Fig. 3. Successful protein-protein interaction targets for therapeutic developments

3a. Four hot-spot residues of p53 (magenta)-MDM2 (yellow) complex and two inhibitors (MI-219 and Nutlin-3). Complex structure was got from (PDB ID: 1YCR). **3b.** Interactions between Bcl-X_L and Bad (PDB ID: 1G5J) and structures of two inhibitors (ABT-263 and HA14-1). Peptide of Bad are colored in yellow, and the electrostatic potential surface of Bcl-X_L is displayed. **3c.** Hydrophobic groove of C-terminal heptad repeats from HIV-1 gp41 trimer (PDB ID: 1AIK). Two representatives of inhibitors (ADS-J1 and 2-aryl 5-(4-oxo-3-phenethyl-2-thioxothiazolidinylidenemethyl) furan derivatives) are in right side. All critical residues are displayed in sticks.

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Table 1

Major methods for flexible active site modeling

Methods	Level of flexibility	Algorithm optimization	Reference
oft potentials	Side-chain	Toleration of minor steric clashes	[79–82]
Rotamer libraries random sampling	Side-chain, backbone	Discrete rotamer libraries, Monte Carlo minimization	[80, 81, 83–85, 99]
MD ensemble sampling	Side-chain, backbone, domain	Normal mode (low mode), temperature replica exchange	[88–90, 93]
4D docking	Side-chain, backbone	Flexibility as the 4th discrete dimension of docking grids	[96]
QSiCR	Side-chain, backbone	Knowledge-based machine learning	[97, 98]

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Table 2

List of popular docking software and associated specifications

Program	OSC	lFlex	pFlex	Parallel	Algorithm	SF	SF class	Owner
GOLD			SP, RTML, MRC	CG	GA	GOLD_Fitness, Chemscore,	Empirical	CCDC
						ASP	Knowledge-based	
eHiTS			SP	CG, FG	Fragment	eHiTS SF	Knowledge-based	SimBioSys
Surflex			SP	CG, FG	Protomol	Surflex SF	Empirical	Tripos
Glide			SP, side-chain repacking[a]	CG	Energy funnel, MC	GlideScore	Empirical	Schrödinger
						Emodel	Empirical+Force field-based	
LigandFit			SP	CG, FG	MC	LigScore1/2	Empirical	Accelry
FlexX			MRC[b]	CG	Fragment	FlexX score	Empirical	BioSolveIT
ICM			SP, MRC	CG	MC	ICM SF	Empirical	Molsoft
						ICM-PMF	Knowledge-based	
Fred				CG	RB	Chemgauss3, Chemscore, OEChemscore	Empirical	OpenEye
						Zapbind[c]	Force field-based	
AutoDock			RTML	FG	GA, SA	AD4	Empirical	Scripps
DOCK			Full-atom flexibility during AMBER score calculation	FG	Fragment	DOCK3.5 score, GB/SA score, PB/SA score[c], AMBER score	Force field-based	UCSF
RosettaLigand			Side-chain repacking	FG	MC	Rosetta energy function	Knowledge-based	University of Washington

OSC=open source code; lFlex=ligand flexibility; pFlex=protein flexibility; SP=soft potentials; RTML=rotamer libraries; MRC=multiple receptor conformers; CG=coarse-grained parallelization; FG=fine-grained parallelization; SF=scoring function; GA=genetic algorithm; RB=rigid-body docking; SA=simulated annealling; Fragment=fragment-based algorithm; MC=Monte Carlo energy minimization; GB/SA=Generalized Boltzmann Surface Area; PB/SA=Poisson-Boltzmann Surface Area;

[a]Prime (Schrödinger) is needed for induced fit docking.

[b]FlexE (Tripos) is needed for MRC sampling.

[c]Zap package (OpenEye) is needed for PB/SA calculation.

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Table 3

List of structure-based library design method

Library design method	Target: Disease	Reference
Optimized hit and scaffold based library design	NS5B Polymerase: Hepatitis C	[147]
Docking-based combinatorial library design	Soluble epoxide hydrolase: Cardiovascular disease	[148]
Anchor-based library tailoring approach	Receptor tyrosine kinase EphB4: Cancer	[150]
Combination of substructure-search and pharmacophore-based library design	Falcipain-2: Malaria	[151]

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Table 4

Recent development in drug development starting from homology modeling

Target	Indication	Inhibitor	Reference
Renin	Hypertension	Aliskiren (Tekturna®)	[235]
EtCRK2	Avian coccidiosis	BES062021, BES143551, BES241415, BES252034	[238]
Dynamin receptor	Epileptic seizures	Pthaladyn scaffold	[237]
hIKK-2	Chronic inflammatory diseases	ZINC03871389	[239]
Cdc25A	Cancer	1-phenyl-2-(5-phenyl-4H-[1,2,4]triazol-3-ylsulfanyl)ethanone scaffold	[240]
α-glucosidase	Diabetes	13 inhibitors (< IC_{50} 50μM)	[241]
PH domain leucine-rich protein phosphatase	Diabetes, heart disease	NCS45586, NCS117079	[242]
NF-κB inducing kinase	Rheumatoid arthritis	4H-isoquinoline-1,3-dione scaffold	[243]
Matriptase-2	Iron homeostasis, breast/ prostate cancer	Three N-protected dipeptide amides conjugated to 4-amidinobenzylamide	[244]
Chemokine receptor -2 (CCR2)	Anti-inflammation	G365-0350	[245]

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Table 5

Strategies and mechanisms of protein-protein interactions inhibitors

Molecule	Mechanism of action	Identification method	Stage in clinical trials
Nutlin-1/-2/-3	MDM2 binding	High-throughput screening	Phase I
MI-219	MDM2 binding	Structure-based *de novo* design	Phase I
benzodiazepine-based inhibitors	MDM2 binding	High-throughput screening	Preclinical
ABT-737	Bcl-2, Bcl-X_L and Bcl-w binding	Structure-activity relationship by NMR	Phase I
ABT-263 (Navitoclax)	Bcl-2, Bcl-X_L and Bcl-w binding	Lead optimization from ABT-737	Phase I/II
HA14-1	Bcl-2 binding	Virtual screening by DOCK	Preclinical
J042	Bcl-2 binding	Receptor-based pharmacophore modeling	Preclinical
Embelin	XIAP binding	Virtual screening by DOCK	Preclinical
Azabicyclooctane derivatives	XIAP binding	Computational *de novo* optimization from peptide using CAVEAT program	Preclinical
Enfuvirtide	gp41 protein folding	Peptide mimetics	Approved
ADS-J1/ADS-J2	gp41 protein folding	Virtual screening by DOCK	Preclinical
NB-64, NB-2 and derivatives	gp41 protein folding	Sandwich ELISA screening combined with molecular docking	Preclinical
NSC23766	Rac-1 binding	Virtual screening by UNITY	Preclinical
ZINC08010136	Rac-1 binding	Pharmacophore filtering, virtual screening by AutoDock and MOE	Preclinical
Ro26-4550	IL-2 and IL-2Rα binding	Peptidomimetic of IL-2	Preclinical
SP-4206	IL-2Rα binding	Fragment-based design from Ro26-4550	Preclinical