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Revisiting de novo drug design: Receptor based pharmacophore screening

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The authors declare no competing financial interests

Key Words: 3D-QSAR, Analog based drug design, Consensus scoring, De novo drug design, Fragment based drug design, Docking, Pharmacophore, Receptor based pharmacophore, Structure based drug design, Scoring and Virtual screening, SAR by NMR.
Abstract
De novo drug design methods such as receptor or protein based pharmacophore modeling present a unique opportunity to generate novel ligands by employing the potential binding sites even when no explicit ligand information is known for a particular target. Recent developments in molecular modeling programs have enhanced the ability of early programs such as LUDI or Pocket that not only identify the key interactions or hot spots at the suspected binding site, but also and convert these hot spots into three-dimensional search queries and virtual screening of the property filtered synthetic libraries. Together with molecular docking studies and consensus scoring schemes they would enrich the lead identification processes. In this review, we discuss the ligand and receptor based de novo drug design approaches with selected examples.

Graphical Abstract
Structure or Receptor based pharmacophore modeling on active site residues identify the key features requisite for ligand binding. Together with virtual screening, consensus scoring, impact the success of lead identification.

Introduction
Over the years, structure-based drug discovery has become an important tool in drug design and discovery process serving as an alternative to experimental high throughput screening. With the advent of higher computational resources, simultaneous increase in the number of public and commercial synthetic small molecule libraries [1] and a concurrent increase in the deposition of high resolution crystal structures for a number of protein targets have brought structure based drug design (SBDD) methods to the forefront of drug discovery process [2].

Drug discovery methods are broadly categorized into 1) ligand-based and 2) structure-based methods. Ligand-based methods mainly emphasize on comparative analysis of the structural or topological similarity, common or diverse pharmacophore descriptors of known ligands. As this method does not rely on the structural information of protein target, the knowledge of experimentally characterized active compounds is of paramount importance to the success of ligand-based methods [3]. On the contrary, the structure-based methods do not entirely rely on experimentally active compounds but rather identifies new molecules that are complementary to protein active site. In particular, structure-based methods have enhanced the prospects of identifying novel scaffolds by inclining the search towards the active site or binding pocket itself. Molecular docking, which mainly utilizes computers to predict the binding mode and the affinity of a given compound towards a target receptor, forms the basic framework in receptor-based virtual screening procedures [4] and in lead discovery strategies [5, 6].

Most of the molecular docking algorithms consider the target protein as rigid single low-energy conformation and explores the conformational flexibility of a ligand. The use of single receptor conformation in docking experiments is often debated as the flexible nature of protein conformation could lead to altered binding modes. Further the conformational changes induced by ligand binding could increase the computational errors in identification of binding modes and prediction of binding affinities [7]. This can significantly hamper the chances of finding new ligands [6, 8]. Recent works attempted to consider the protein flexibility when docking the ligand into a rigid receptor using reduced van der Waals radii [9] (to allow overlap of protein and ligand atoms) with the receptor side-chain conformations sampled for each receptor-ligand binding pose followed by an energy minimization and a second round of docking [10-12]. Alternatively, an experimental (crystal and solution NMR structures) or molecular dynamics ensemble of multiple receptor conformations [13] was employed to select the subset of receptors conformations that can account for the protein flexibility.
Structure or receptor based search for better binding fragments was carried in a more focused way by ensuring the selection of features that are complementary to binding site of the protein. This is achieved by appropriate positioning of functional groups or fragments such as methyl, carbonyl, amino, benzene or heterocyclic groups onto the active site residues. Better binding groups are identified by scoring and ranking based on their interaction energies. The best ranking fragments are then linked in silico to obtain a new molecule. However, these early methods suffered from problems of combinatorial explosion and synthetic feasibility. To overcome the synthetic tractability of molecules, template (ligand) based or scaffold hopping approaches by retrograde fragmentation of the template with known cleavage reactions are adopted, which helps to rebuild the ligand by employing the similar reactions and thereby evolving the virtual synthetic scheme for the in silico generated ligands. Alternatively, the synthetic tractability can be overcome by biasing the search towards synthesized fragments or whole molecules. Molecular docking offers the advantage of docking of small molecule library into the active site and predicting the protein-ligand interaction energies. Virtual high-throughput screening (vHTS) were popular and enabled the users to quickly identify most probable binders from non-binders and thereby reducing the data set of molecules from millions to thousands and followed by a standard docking protocol that further filters the better binders. However, vHTS do possess inherent pitfalls in terms of scoring and ranking the high affinity compounds.

**Scoring and Ranking**

Warren *et al.*, in their critique on performance of 10 docking programs and 37 scoring functions on eight proteins belonging to seven protein types evaluated that though most docking programs could simulate the crystal conformations for at least one of the protein type, but none of the scoring functions could successfully differentiate the crystal conformation from the docked conformations. Similarly, none of the docking programs or scoring functions works well for all the targets, even though they were able to identify active ligands from a decoy dataset and are good at predicting binding affinity. Numerous studies done for improving docking and scoring in particular pose prediction have shown that by combining results from multiple scoring functions, evolving consensus yields better results than the use of single scoring function [14-19]. Consensus scoring schemes have shown to increase the chances of identifying true hits and provide enrichments better than single scoring functions [15, 17, 19, 20].

**Fragment-based de novo design**
Receptor based de novo design programs (Table 1) such as HSITE [21], LUDI [22] employ rule based methods to map interaction sites from the three dimensional structure of the protein. Hydrogen bond donors, acceptors and lipophilic features are mapped based on the ideal geometry of hydrogen bond and non-bonded contacts deduced from statistical analysis of Cambridge Structural Database (CSD) [23]. Likewise grid based methods such as GRID [24] generate a grid of points onto the binding site by placing an appropriate probe to evaluate the hydrogen bonding ability or lipophilic properties; their interaction energies are estimated - providing interaction map of the binding site. De novo approach such as Multiple Copy Simultaneous Search (MCSS) [25] docks fragments and determines the energetically favorable positions and orientations of a functional group or fragment, by randomly placing multiple copies of functional groups at the binding site and simultaneous minimization of all the fragments or functional groups. An empirical scoring function is utilized to output the best set of pre-docked fragments forming the source of ideas for synthesis and optimization for assembling the fragments into a complete ligand. These ideas led to the genesis of whole molecule docking approaches, which later became more popular and formed the basic step in most of the structure based drug design methods.

Assembly, linking and growing are the three main concepts that are involved in de novo drug design at the binding site of the receptor. Once the fragments are assembled on to the binding site, the sampling of the assembled fragments is achieved either by linking the fragments or growing the fragment into vacant crevices of the binding site or by lattice-based sampling, random structure mutation, molecular dynamics simulations of fragments, and graph-based sampling. The linking approach starts with the successful placement of building blocks at the key interaction sites of the receptor either by the user or by the de novo program itself. Depending on the positioned or pre-docked building block or fragment, the linkers are automatically selected from linker library. Each of the fragments is connected by a suitable linker to yield a molecule that can map to all key interaction sites. In order to avoid the combinatorial explosion, most of the programs such as LUDI, PRO_LIGAND [26], SPROUT [27] employed a small linker library and predefined linking site rules. The fragments are linked to each other by a suitable linker to yield a completely new ligand that can occupy all the interaction sites at hydrophobic pocket (Fig. 1).

**Fragment based screening - SAR by NMR**

Successful pioneering studies from Abbott and Astex employing the NMR and X-ray crystallography as a screening platform have revolutionized the concept of fragment based drug design [28-30]. Fragment based screening approach have now becoming increasingly popular both in academia as well as in pharma industry.
Many of the fragment based studies targeting protein such as Hsp90, β-secretase, MMP-3 and Bcl-2 have now reached clinical trial stage. More recently PLX4032 / Zelboraf targeting B-RAF V600E kinase became the first FDA approved drug developed (Fig. 2) from fragment based approach [31-33]. Fragment based approach involves i) assembly of low molecular mass fragment molecules conforming to the rule of three [34], ii) Screening of fragment library by biophysical methods – NMR or SPR (surface plasmon resonance) or X-ray crystallography and iii) growing the size of the validated fragments either by linking the fragments (which dock at distinct sites of the active site) or grow the ligand into adjoining pockets or merging/ hopping the fragments to yield a new molecules.

Fesik group from Abbott showcased the utilization of low affinity fragments in lead design by employing SAR by NMR [28, 29, 35]. In these works, two low affinity fragments that bind to the distinct sites were structurally characterized by using two dimensional isotope edited NMR spectroscopy and medicinal chemistry efforts successfully linked the two fragments generating a high affinity ligand. The authors in their attempts to identify a high affinity MMP-3 selective inhibitor have employed two weak affinity inhibitors – a zinc chelating acetohydraoxamate (Kₐ 11 mM) and a biaryl compound fragments which bind close to the substrate binding pocket. The two fragments were then linked based on their three dimensional structure information leading to identification of a series of potent and selective inhibitors of MMP-3 (ABT-518) (Fig. 2). Similarly, NMR based fragment screening identified fragments such as biaryl carboxylates (Kₐ 300-1000 μM) that bind to the peptide binding groove of Bcl-2 in presence of tetra-hydonaphthols like compounds (Kₐ 2-6 mM). Elucidation of three dimensional structure information and extensive medicinal chemistry efforts have yielded ABT-737 which potently inhibited the tumor cells in animal models. Experimental methods such as NMR, X-ray crystallography and other sensitive biophysical assays such as thermal shift, SPR are now widely employed to validate the binding modes or nature of fragment binding. In a very recent example of fragment based screening, Takeshi Hondo and colleagues have identified novel inhibitors of D- amino acid oxidase (DAAO). DAAO functions as a co-agonist of N-methyl-D-aspartate receptor (NMDAR) and was shown to play a vital role in regulating schizophrenia [36]. Using a fragment 4-hydroxypyrindazin-3(2H)-one (8, IC₅₀ 1.15 μM), which docks perpendicular to the flavin ring of FAD (flavin adenine dinucleotide) with stacking interaction between the flavin-quinolone and Tyr 224 and the hydrogen bonding of carbonyl and 3-hydroxyl group of 4-hydroxypyrindazin-3(2H)-one with Arg283, Tyr228 and G313 residues further stabilizes the fragment binding. The optimized ligand with 2-phenethyl linker (12, IC₅₀ 3.9 nM) was more potent than the parent fragment and it
not only maintained the similar binding mode but was also involved in additional hydrophobic interactions with Tyr224, Leu51, Leu215 residues (Fig. 3).

**Receptor based Pharmacophore Screening**

Receptor based pharmacophore screening involves the development of a pharmacophore model based on active site residues that play a critical role on the activity of the protein. Receptor or structure based pharmacophore modeling involves three steps i) construction, ii) subtraction phase and optimization similar to that of ligand based pharmacophore development. In construction phase, the program, LUDI, identifies the key interaction or vector sites that are possible the specified active residues based on the acceptor, donor, hydrophobic features and generates numerous vector sites at the active site. In subtraction phase, the interaction vector sites are hierarchically clustered by feature types (using RMS distance or number of features) and key vectors sites anchoring the important residues are only kept and finally to optimize the model, an exclusion volume(s) representing the protein active site atoms was added as shape constraint or volume of active site to prevent clashes with ligand atoms. Carlson *et al.*, employed a dynamic receptor pharmacophore modeling to account for the inherent flexibility of active site of HIV-1 integrase receptor. The flexibility of apo-protein is sampled via molecular dynamics (MD) simulations and the overlay of multiple snapshots of the apo-protein delineated the most conserved binding region defining the dynamic pharmacophore model. Screening the available chemical directory (ACD) database with such dynamic receptor based pharmacophore model led to identification of novel inhibitors [37]. Cerius² structure based focusing (SBF) program generates 3D pharmacophore queries grounded on the binding site residues of the receptors. Presence of known ligand data was often useful to verify the selectivity of these queries and employ a selective query to screen the commercial database. Kirchhoff *et al.*, in their studies on estrogen receptor have shown that the selection of features up to six features together with the exclusion volumes could play a key role in reducing the percentage of less active hits and improve the hit rate [38]. Pocket 2.0 program attempted to automate the receptor based pharmacophore modeling by using a grid based model to identify the interaction sites at the active site region. Empirical rules are employed to reduce the numerous hydrophobic and hydrogen bonding features (that are available with the active site) and together with an abstraction of features that are close to the ligand binding atoms are used to refine the receptor based pharmacophore model development [39]. Alternatively, Sherman *et al.*, have developed a hybrid approach which utilizes the energy terms computed from Glide SP docking / scoring to rank the important features for pharmacophore model development and database screening. Thus developed energy optimized structure based
pharmacophore was shown to outperform the contact based or 2D- similarity methods upon screening the decoy set of ligands [40]. In our recent study, we have successfully employed the de novo method of receptor based pharmacophore screening to identify novel ligands targeting *Plasmodium* FKBP35. In this study, we mainly aimed to target residues C106, S109 that are well conserved in *Plasmodium* species but are varied with human FKBP family members. The receptor based pharmacophore screening on *Plasmodium* FKBP35 (Fig. 4A) has indeed identified novel purine like scaffold (D44) that binds favorably towards the Cys106 and Ser109 and the thio-acetamide moiety compensates the lack of canonical ‘pipercolyl’ moiety which is shown to be perquisite for FKBP binding. D44 not only inhibit the enzymatic activity (IC_{50} 132 nM) of FKBP35 but also inhibited the growth of *Plasmodium falciparum* (IC_{50} 234.5 nM) in ex vivo culture. Further, elucidation of X-ray crystal structure of PfFKBD35 with D44 clearly shows that the purine like ring was positioned towards the vicinity of C106, S109 and interacts with important catalytic residues such as Y101, D56, I75 that are important for ligand binding (Fig. 4B) [41]. Therefore, receptor based pharmacophore screening not only biases the search towards biologically relevant scaffolds but also obviates the synthesesizability issues by considering the synthesized libraries as input during database screening.

**Ligand or ‘template’ based de novo design**

Ligand based de novo design methods do not require the receptor information, but solely rely on the presence of at least one known active ligand serving as a template to generate novel structures with no similarity. The molecules generated from either receptor or ligand based de novo drug design often face synthetic accessibility issues. This problem has mainly limited the success of de novo programs, as only a small percentage of molecules are tractable for synthesis. Many of the recently developed de novo programs addressed this problem by employing rules to guide the assembly by avoiding the implausible fragments or building blocks. Most of the ligand based methods are template driven and need the reference fragment structure to initiate the design to generate a novel molecule [42]. Application of retro synthetic rules as illustrated in RECAP [43] program leads to identification of unique database of building blocks that can be deduced from drug like molecules with a definite set of 11 cleavage reactions. The same virtual reaction scheme can be employed to assemble the candidate compounds. Similarly, SYNOPSIS [44] also employed a database of building blocks that are guided by 70 different virtual organic synthesis schemes enabling the user to propose the synthetic route for the generated structure. Design of genuine structures (DOGS) successfully generated alternative diverse scaffolds by preserving the pharmacophore features of the ‘template’ molecule as detailed in earlier review [45].
program makes use of its 83 chemical reactions on available building blocks and compounds are virtually synthesized by iterative cycles of synthetic reactions. In each of the iterative construction steps, it maintains the key pharmacophore features and structural similarities of the template (1, Fig. 5) and guides the growth of the ligand. DOGS program has successfully designed a de novo compound 4 (Fig. 5) which inhibits the hPlk1 kinase activity and proliferation of HeLa cancer cell lines. Furthermore, the compound 4 lacked inhibitory activity on 48 active kinases including Aurora Kinase A [46].

Fragment Shuffle 47 program utilizes a set of known ligands for a particular target and their 3D bio-active conformation is rigorously sampled by recombination using fragment hopping. The recombination is carried out by i) aligning the known ligands and calculation of their atom scores, ii) a three step fragmentation of ligands and computation of the fragment scores and iii) incremental construction of derived fragments with best scores by recursive tree search and the candidate compounds that emerge from recombination are outputted to the pool of novel actives. As the knowledge of bioactive conformation was used to constrain the search for novel ligands, the generated structures were implied to inherit the bio-active conformation.

It is now widely recognized that for a successful drug design, it is imperative to satisfy multiple objectives (rather than single objective) ranging from binding to primary receptor, synthetic tractability, similarity / diversity, and favorable ADMET constraints to improve the outcome of drug discovery efforts [48-51]. Nicolaou et al., have combined multiple variables such as i) evolutionary techniques and graph theory to handle the efficient structure sampling, ii) RECAP fragmentation rules to generate fragments and virtual reassembly, iii) Chilliscore / Tanimoto similarity score to evaluate the fitness to receptor / ligand and iv) drug like properties calculated via the chemical graphs of generated molecules via multiple objective evolutionary graph algorithm [50].This study clearly showed that satisfying multiple objectives via MEGA produces structurally diverse molecules with the supplied constraints and could impact the drug discovery efforts by reducing the attrition rate.

QSPR - QSAR / QSTR

As discussed previously, ligand-based methods are of much utility for diseases with no target or protein information known and mainly consists of either developing a 3D QSAR or pharmacophore model based on descriptors of active and inactive molecules by identifying the common / diverse pharmacophore features that are important for activity among the actives and inactive dataset. Further the inclusion of shape feature of most active ligand to simulate hypothetical or pseudo receptor shape of protein active site and is critical to recall hits
that could bind with the protein in a manner similar to known actives. 2D or 3D quantitative structure activity or property relationship studies identify the hidden knowledge from ligands properties that are critical for pharmacological activity or toxicity. A known data set of molecules with diverse structural features and varied activity enables to train a model. A well trained model can identify the underlying hidden patterns by relating the structural descriptors to activity and thereby predict the activity of the molecule. These methods are important as they not only identify the statistically significant descriptors that define the activity but also the toxicity properties. QSAR/QSTR models can predict the both the pharmacological activity as well as pharmacokinetic properties of similar or diverse scaffolds with good precision. QSAR studies have also lead to the formulation of drug likeliness rules in the form Lipinski’s rule of Five [52], Veber rules [53] and rule of three [34] for fragment libraries. QSAR studies on solubility [54], absorption [55], intestinal permeability [56], hepatotoxicity, serum protein binding [57], hERG channel binding [58] (cardio-toxicity) have streamlined combinatorial library design and enabled the researchers to not only to filter out molecules with unfavourable drug like properties but also exclude ligands that may have the probability of carcinogenicities and mutagenicity [59-61]. Some of these QSAR products such as TOPKAT are patented and included in commercial software packages such as Discovery Studio.

Of all the drug design approaches, “QSAR” studies are the most popular studies with ~9856 articles in PubMed Central Database followed by studies involving a combination of pharmacophore modeling and docking studies (1728 articles). These trends suggest the fusion of both analog and structure based methods are employed to enrich the decision making in the identification of best hits in drug design and discovery efforts. Impact of pseudo receptor models on drug design is beyond the scope of this review and was well reviewed previously [62]. Works of Hawkins et al., show that inclusion of ligand shape in virtual screening approaches could be superior or on par with the efficient docking programs such as GOLD or GLIDE [63].

**Challenges**

In most cases, SBDD methods are very successful in enriching hit rates by eliminating bad hits [16]. Fragment based approaches, receptor based pharmacophore modeling together with the virtual screening enrich the hit rates and are successful in finding de novo novel chemical entities. The failures of SBDD methods are mainly attributed to the inherent lapses during protein preparation [64], scoring and ranking algorithms [15-20, 65-70]. Warren et al., in their study summarized that most of the docking and scoring procedures do well at predicting binding mode, reproducing ligand poses within 2 Å, but none could rank the ligands by affinity [20].
Furthermore, it is also still not possible to identify “activity cliffs” indicating that nanomolar inhibitors cannot be consistently ranked over μM inhibitors. Though the recent fusion procedures have improved the performance of structure-based methods retrospectively, it is still difficult to predict a priori what data fusion or which approach would give better results for a given system. The receptor-based virtual screening method that could account the structural flexibility of both the ligand and receptor are still computationally expensive for screening large library of compounds. Employing an ensemble of de novo generated multiple receptor or experimental conformations to simulate protein flexibility in virtual screening studies could provide novel binding modes but yet there might be no clear relationship between docking and ranking and may tend to increase the false positive rate than improving the hit rate [71]. Most docking and scoring algorithms are still not adequate to handle the above mentioned complexities. With advances in computing power and further improvements in computer assisted drug design methodology could reliably impact industrial drug discovery process [20, 66, 72].

Acknowledgements

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Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>ACD</td>
<td>Available Chemical Directory</td>
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<tr>
<td>ADMET</td>
<td>Absorption, Distribution, Metabolism, Excretion, Toxicity</td>
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<tr>
<td>CSD</td>
<td>Cambridge Structural Database</td>
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<tr>
<td>DAAO</td>
<td>D- Amino Acid Oxidase</td>
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<tr>
<td>DOGS</td>
<td>Design of Genuine Structures</td>
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<tr>
<td>FAD</td>
<td>Falvin Adenine Dinucleotide</td>
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<tr>
<td>FKBP</td>
<td>FK506 Binding Protein</td>
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<tr>
<td>MCSS</td>
<td>Multiple Copy Simultaneous Search</td>
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<td>MD</td>
<td>Molecular Dynamics</td>
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<tr>
<td>MEGA</td>
<td>Multiple Objective Evolutionary Graph</td>
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<tr>
<td>MMP</td>
<td>Matrix Metallo Proteinase</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>QSPR</td>
<td>Quantitative Structure Property Relationship</td>
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</table>
QSTR Quantitative Structure Toxicity Relationship
SAR Structure Activity Relationship
SBDD Structure-Based Drug Design
SBF Structure Based Focusing
SPR Surface Plasmon Resonance
vHTS Virtual high-throughput screening

References


### Table 1. Recent programs used in structure and ligand-based methods

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<td>2008</td>
<td>Fragment</td>
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<td>Ligand</td>
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<td>Ligand</td>
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<td>Receptor/Ligand</td>
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<td>2013</td>
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<td>2013</td>
<td>Receptor/Ligand</td>
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**Figure Legends**

**Figure 1.** Fragment based de novo drug design of FKBP12 inhibitors. A) Surface view depicting the important binding site residues and the assembly of adamantyl fragment which interacts with I56 residue and a pyridine fragment at the vicinity of H87 and D37 residues. B) The two assembled fragments are tethered together by a sulphur atom and this molecule potently inhibited the enzymatic activity of FKBP12 (K<sub>i</sub> 9.2 µM). C) Ligand grown by linking the two fragments with a carbonyl moiety and further with a hydroxyl group grown on 3-position of benzene to improve the interaction with D37 residue and thus grown ligand inhibits the FKBP12 activity with a K<sub>i</sub> of 16.7 µM [85].

**Figure 2.** Successful examples of fragment-based lead generation on drug targets such as Hsp90, BACE, Bcl-2, MMPs and kinases.

**Figure 3.** Fragment-based ligand design of D-Amino acid oxidase (DAAO) inhibitors. A) Cartoon model of DAAO in complex with benzoic acid (PDB: 2DU8) showing the key hydrogen bonding interactions with R283, Y228 and stacking hydrophobic interactions of benzoic acid with the falvin ring and Y228 residue. B) Binding mode of (compound 8) in complex with DAAO (PDB: 3W4I) showing similar hydrogen bonding interactions with R283, Y224, flavin and hydrophobic interactions (IC<sub>50</sub> of 1.1 µM). C) Shown is the optimized compound 12 maintaining a similar binding mode. The grown phenethyl group was engaged in additional hydrophobic interactions with L51, L225, Y228 residues and had a enhanced IC<sub>50</sub> of 3.9 nM. D) 2D structural depiction of fragments leading to potent inhibitor Compound 12.

**Figure 4.** Receptor-based pharmacophore model on Plasmodium FKBP35. A) Overlay of FKBP35 structure based pharmacophore model mainly comprising of two acceptors features anchoring the I75 and Y101 residues, a donor feature targeting the D56 residue, two hydrophobic features in the vicinity of hydrophobic residues such W78, F55,C106 residues. B) Surface view of crystallographic complex of
top scoring ligand (D44) with Plasmodium falciparum FKBD35 obtained from receptor based screening and this ligand potently inhibits the peptidyl-prolyl cis/trans isomerase activity (IC\textsubscript{50} 132 nM) as well as growth of Plasmodium falciparum 3D7 strain [41].

Figure 5. De novo design of compound 4 using previously identified human Plk1 inhibitor (1) as template by program Design of genuine structures (DOGS). The de novo ligand 4 shows close similarity to known antidepressant fluoxetine and further evaluation of compound 4 confirmed that it binds to the inactive form of human Plk1 selectively and prevents the HeLa cell proliferation [46].

Figure 1

**Figure 2**
Figure 4

Figure 5

\[
\begin{align*}
1 & \quad \text{Cl} \\
2 & \quad \text{DOGS} \\
3 & \\
\text{Fluoxetine} & \\
4 & 
\end{align*}
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