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Research Article

PATTERN BASED DETECTION OF POTENTIALLY DRUGGABLE BINDING SITES BY LIGAND SCREENING

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Abstract: This article describes an innovative way of finding the potentially druggable sites on a target protein, which can be used for orthosteric and allosteric lead detection in a single virtual screening setup. Druggability estimation for an alternate binding site other than the canonical ligand-binding pocket of an enzyme is rewarding for several inherent benefits. Allostery is a direct and efficient way of regulating biomacromolecule function. The allosteric modulators can fine-tune protein mechanics. Besides, allosteric sites are evolutionarily less conserved/more diverse even in very similarly related proteins, thus, provides high degree of specificity in targeting a particular protein. Therefore, targeting of allosteric sites is gaining attention as an emerging strategy in rational drug design. However, the experimental approaches provide a limited degree of characterization of new allosteric sites. Computational approaches are useful to analyze and select potential allosteric sites for drug discovery. Here, the use of molecular docking, which has become an integral part of the drug discovery process, has been discussed to predict the druggability of novel allosteric sites as well as the active site on target proteins by ligand screening. Genetic algorithm was used for docking and the whole protein was placed in the search space. For each ligand in the library of small molecules, the genetic algorithm was run for multiple times to populate all the druggable sites in the target protein, which was then translated into two dimensional density maps or "patterns". High density clusters were observed for lead like molecules in these pattern diagrams. Each cluster in such a pattern diagram indicated a plausible binding site and the density gave its druggability score in terms of weighted probabilities. The patterns were filtered to find the leads for each of the druggable sites on the target protein. Such a novel pattern based analysis of the clusters provides a way to probe new druggable sites on a target protein in a much simpler setup. This structure based analysis method might help researchers to develop allosteric modulators and to identify novel target sites on drug resistant proteins.

Keywords: virtual screening; docking; ensemble analysis; clustering; density distribution; genetic algorithm

Note - Coloured Figures and Supplementary Information available on Journal Website in "Archives" Section

Introduction

Allosteric modulators are ligands of proteins that bind in a binding site other than the site of the natural substrate. The site to which the ligand binds is termed the allosteric site. Allosteric regulations are a natural example of control loops, such as feedback from downstream products or feed forward from upstream substrates in a catalytic cascade (Mayes and Bender, 2003). Allosteric regulation is also particularly important in a cell's ability to adjust enzyme activity by providing short-term, readily reversible regulation of metabolite flow in response to specific physiologic signals without altering gene expression (Rodwell and

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Kennelly, 2003). Moreover, the allosteric modulators are interesting drugs. Allosteric modulators can enable modulation of targets that are difficult to target orthosterically (Christopoulos, 2002). There are a number of advantages in using allosteric modulators as preferred therapeutic agents over classic orthosteric ligands. For example, allosteric binding sites have not faced the same evolutionary pressure as orthosteric sites to accommodate an endogenous ligand, so are more diverse. Therefore, greater selectivity may be obtained by targeting allosteric sites (Christopoulos, 2002). This is particularly useful for targets such as GPCRs (Burford *et al*., 2011; Ivetac and Andrew McCammon, 2010) and kinases (De Smet *et al*., 2014; Hantschel *et al*., 2012; Zhang *et al*., 2010) where selective orthosteric therapy has been difficult due to the sequence conservation of the orthosteric site across receptor subtypes. In this respect, allosteric modulators are considered as emerging strategy in rational drug development. Recent advances in targeting protein kinases include the discovery of type III inhibitors that bind a site proximal to the ATP binding pocket as well as the truly allosteric type IV inhibitors that target protein kinases distal to the substrate binding pocket (Foda and Seeliger, 2014; Lamba and Ghosh, 2012; Liu and Gray, 2006). These new classes of inhibitors are often selective and usually display high affinities. Discovery and development of non-nucleoside reverse-transcriptase inhibitors (NNRTIs) are another example of allosteric drug development (Schauer *et al*., 2014; Seckler *et al*., 2011). NNRTIs are antiretroviral drugs used in the treatment of human immunodeficiency virus (HIV) infection. NNRTIs are generally allosteric inhibitors of reverse transcriptase, an enzyme that controls the replication of the genetic material of HIV and is one of the most popular targets in the field of antiretroviral drug development. Figure 1B shows the interaction of the allosteric NNRTI drug nevirapine with the HIV-1 reverse transcriptase (Das *et al*., 2014). Allosteric inhibitors act by binding noncompetitively or uncompetitively to the target protein; however, unlike the agonists or antagonists, allosteric modulators can also amplify signals (Schwartz and Holst, 2007) rather than activate/inhibit them.

Druggability, on the other hand, refers to the ability of a protein target to bind small molecules with high affinity (Edfeldt *et al*., 2011). Sometimes, perhaps more appropriately, it is called the 'ligandability'. And the probabilities of such binding are used to predict the druggability scores. The target proteins having more than one binding sites, therefore, may have different druggability scores for the orthosteric and different allosteric sites. Orally bioavailable drug-like small molecules tend to have properties within certain parameters defined by the Lipinski's rule of five: molecular weight less than 500, logP less than 5, maximum of 10 hydrogen bond acceptors and 5 donors (Lipinski *et al*., 1997). In order to bind such compounds, a protein should have a binding site with complementary properties e.g. a buried not so polar cleft with appropriate size to accommodate a drug-like ligand, up to 10 hydrogen bond donor and 5 acceptors. Druggability score, thus, depends on the pharmacophoric competence between the binding site and the ligand. Structure-based prediction methods rely on identifying cavities in protein crystal structures and assessing the properties of these cavities to predict whether they may bind drug-like molecules (An *et al*., 2005; Hajduk *et al*., 2005; Volkamer *et al*., 2012). Rules for properties that indicate a druggable cavity are learnt from analysis of co-crystal complexes with drug-like ligands e.g., volume, surface area, polar surface area, surface exposure etc. These rules are then applied to new targets to predict/score druggability. There are several algorithms available to predict the druggability, which includes PocketFinder, Druggability Indices, DoGSiteScorer and DrugEBIlity (An *et al*., 2005; Hajduk *et al*., 2005; Volkamer *et al*., 2012). However, the druggability can also be described in terms of the types of ligand. A protein or a binding site may be more druggable for a particular type of ligands and less druggable for another type. Similarity, allosteric and orthosteric sites on a protein often show differential preference for ligands. Mixing of different types of ligands in the dataset, in turn, produces noise in the large scale screening against a druggable protein. Therefore, in order to reduce entropy and streamline the lead discovery process, it is necessary to develop a method to filter orthosteric and allosteric ligands in real time in a virtual screening setup.

In this article we discussed a docking based method that combines the binding site detection and ligand with the virtual screening. This method also enables us to segregate orthosteric and allosteric ligands from a mixed dataset for lead discovery in drug design. Our approach also

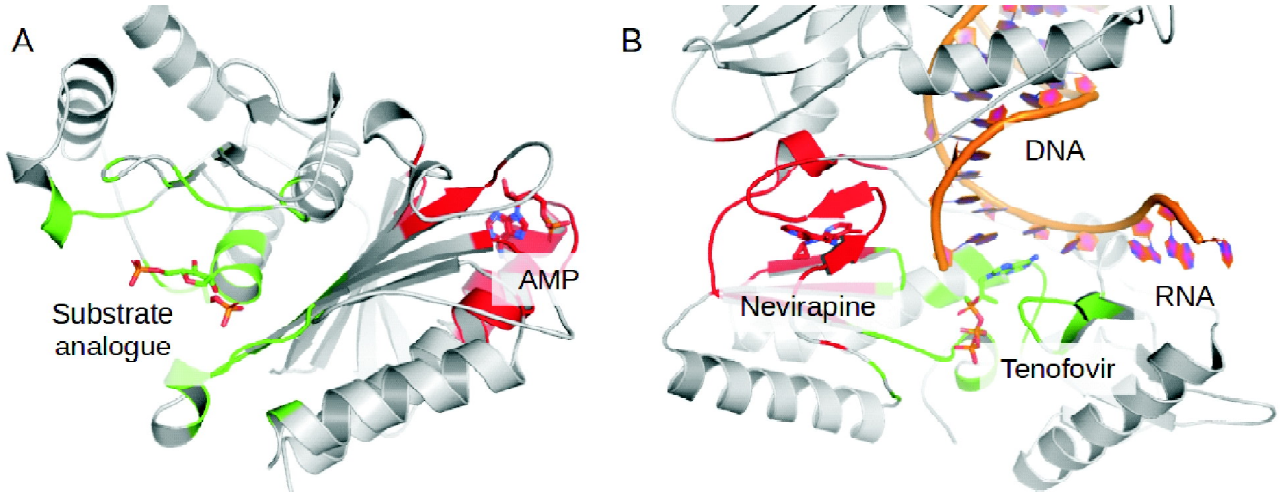

Figure 1: **Allosteric modulation of enzyme. (A) Fructose 1,6-bisphosphate (PDB: 1FPG), a rate limiting enzyme in gluconeogenesis, bound to a substrate analogue (2,5-anhydro-d-glucitol-1,6-bisphosphate) and the indigenous allosteric inhibitor AMP. Orthosteric sites are colored green and the allosteric sites are colored red. Ligands are shown in stick model. (B) Nevirapine and tenofovir, allosteric and orthosteric inhibitors of HIV-1 reverse transcriptase (PDB: 1T05, 4Q0B)**

provides a smart way to depict and compare the molecular docking results. We took macrophage migration inhibitory factor (MIF) as a model target protein and two of its reported inhibitors (one orthosteric and the other allosteric) for the illustration. Genetic algorithm was employed for docking of ligands on the target protein. Blind docking approach was taken and the whole protein was placed in the search space to probe all possible binding sites present in the target. Relying on the stochastic property of docking we generated enough docked conformers to populate all the druggable sites in the target protein. State of the bound conformers in the ensemble was then transcribed into two dimensional density maps (patterns). Cluster densities were probed for lead like molecules for the druggable sites on the target. This novel pattern based analysis of the clusters provides a way to probe new druggable sites on a target protein in a much simpler setup. More diverse the ligand library is, more probable the allosteric lead detection becomes. This structure based analysis method would be useful to develop allosteric modulators and to identify novel target sites on drug resistant proteins.

Materials and Methods

This docking based method to find the binding sites and their druggability indices for a test ligand requires an ensemble of docked conformers to be analyzed. In order to generate a statistical ensemble of bound conformers for a test ligand, AutoDock 4.2 of The Scripps Research Institute was used as the base program in this method (Morris *et al.*, 2009). The structures of the proteins were obtained from the Protein Data Bank (Berman *et al.*, 2000)and the ligand molecules were either obtained from PubChem (Kim *et al.*, 2016) or drawn on Avogadro (Hanwell *et al.*, 2012) followed by geometry optimization in molecular mechanics force filed (UFF) using the steepest descent algorithm. The center of mass for all the ligands in the library were set to (0,0,0) and the target protein was placed some distance away (around 40 Å) along a Cartesian axis in order to avoid any overlaps. Moreover, the protein was oriented in such as way so that it breaks any symmetry in this overall arrangement. The ligand and the target was place in such a way in order to get a better resolution in the output. Proteins and the ligands were preprocessed in AutoDockTools (Morris *et al.*, 2009) for docking, where the non-polar hydrogen atoms were merged, Gaisteiger charges were added and the atom types were defined. Any water, duplicate atoms or undesirable chemical entities were also removed from the protein structure files. Rotatable bonds in the ligands were defined for the flexible docking. Search spaces were defined by encompassing the whole protein, which is essential in order to explore all the possible binding sites on the target. Lamarckian genetic algorithm with Solis & Wets' local search methods was used as the search algorithm (Morris *et al.*, 1998). Genetic algorithm runs were set to 100 or 1000 with 25 million energy

evaluations each time. Spatial deviation of the conformers and their binding energies were then distributed on a smooth density histogram using Wolfram Mathematica. Intensity of the densities on these histograms corresponds to the druggabiligy of a site by the ligands. Comparison of densities across a population of ligands contributes to computation of the global druggability score of the site or the protein as a whole. A detailed methodology along with the commands and the scripts is given in the Supporting Information file.

The scheme of the protocol is shown in the Figure 2. According to this protocol a ligand is picked up from a library of small molecules and then docked onto the target protein. The docking runs in a loop until a specified number of iterations is reached. Each docking calculation in this loop is independent of the previous ones. After a certain number of docked conformations are generated, their binding energy, spatial orientation and position are logged. Ranges for the distributions are then defined and the system states are distributed over the energy and RMSD axes on two dimensional smoothed density histograms. Density scaling is performed to compare the distribution patterns for different ligands. The smoothed density histograms are then converted into machine readable patterns (Figure 3). Each of these patterns is associated with a particular ligand in the dataset. The patterns describe their binding site preference on the target and affinity for that site. These patterns are then compared with the pattern generated by a successful self-docking experiment of an orthosteric ligand, which serves as a hallmark for the detection of orthosteric leads. For an unknown protein, active sites may first be predicted by an active site prediction tool (Goyal *et al.*, 2007). In case of enzymes, such as kinases, active site can be easily identified from the structure and the pattern generated by docking ATP/ATP analogues to that site serves as control pattern. Now, if the pattern generated by a new ligand in the library matches with the control pattern, it is classified as an orthosteric lead. If a pattern shows good density but mismatches with the control, the ligand is classified as an allosteric lead and the density indicates the presence of a potentially druggable allosteric binding site in the target. The different types of ligands are then scored according to the binding energy. Maximum densities observed for a particular site on the target, in turn, indicates the druggability of the target protein/sites toward small molecule ligands.

Results and Discussion

In very large scale analysis of drug like molecules, virtual screening often suffers from noise (wrong predictions); one possible cause for which, is mixing of orthosteric and allosteric ligands. Here we described the development of a docking based method under virtual screening setup to explore all the druggable binding sites on a target protein for a set of test ligands in order to segregate the orthosteric and allosteric hits. This method relies on the stochastic nature of docking and pharmacophoric properties of the binding sites as discussed above.

Figure 4 shows the interaction of ISO-1 (4,5-dihydro-3-(4-hydroxyphenyl)-5-isoxazoleacetic acid methyl ester) with human macrophage migration inhibitory factor (MIF). ISO-1 is a known inhibitor of human MIF. It binds to the active site of the enzyme and competitively inhibits the tautomerase activity (Lubetsky *et al.*, 2002). Details of ISO-1 and MIF binding interaction are known from the crystal structure of the complex (PDB: 1LJT). Human MIF is a target protein for the development of anti-inflammatory drugs. MIF is a homotrimeric enzyme that catalyzes the conversion of Keto-phenylpyruvate to enol-phenylpyruvate and L-dopachrome to 5,6-dihydroxyindole-2-carboxylate. The homotrimeric enzyme shows a three-fold symmetry and there are three active sites are located near the N-terminal regions in between two adjacent subunits of the enzyme. The binding of ISO-1 with human MIF was analyzed using the protocol described in Figure 2 and compared with the known crystal structure (PDB: 1LJT). The ligand was separated from the complex and placed in the origin (0,0,0); the receptor was moved to around 40 angstroms away from the ligand. 100 docked conformers were generated from independent docking simulations and are shown superimposed in Figure 4A. It was found that ISO-1 docked exclusively into the active sties of the enzyme. Then, we generated the probability pattern from this docking experiment and it is shown in the Figure 4B. Three high probability densities can be observed in this two dimensional diagram, each of which corresponds to the binding to one of the three active sites. Probability densities are also shown in a 3D histogram representation in Figure 4C. When the absolute positions of the ligand and the target are kept fixed, each of such densities along the RMSD

axis of the density diagrams represents a separate binding site. In this particular example, all the three densities indicate the orthosteric sites due to the multimeric nature of the protein. Binding to allosteric sites also produces distinct densities. Therefore, using machine learning techniques allosteric and orthosteric ligands can be filtered by analyzing their docked patterns.

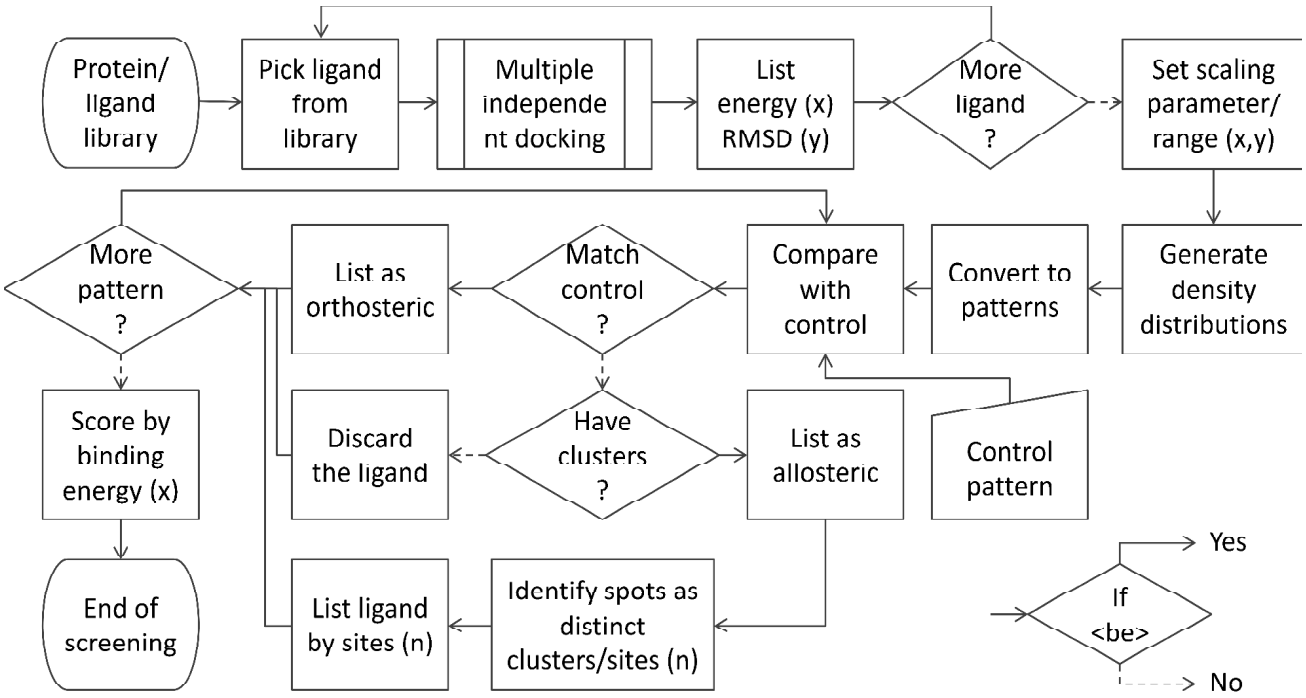

Figure 2: **The algorithm for the detection and segregation of orthosteric and different allosteric leads in a virtual screening setup. <be>, Boolean expression**

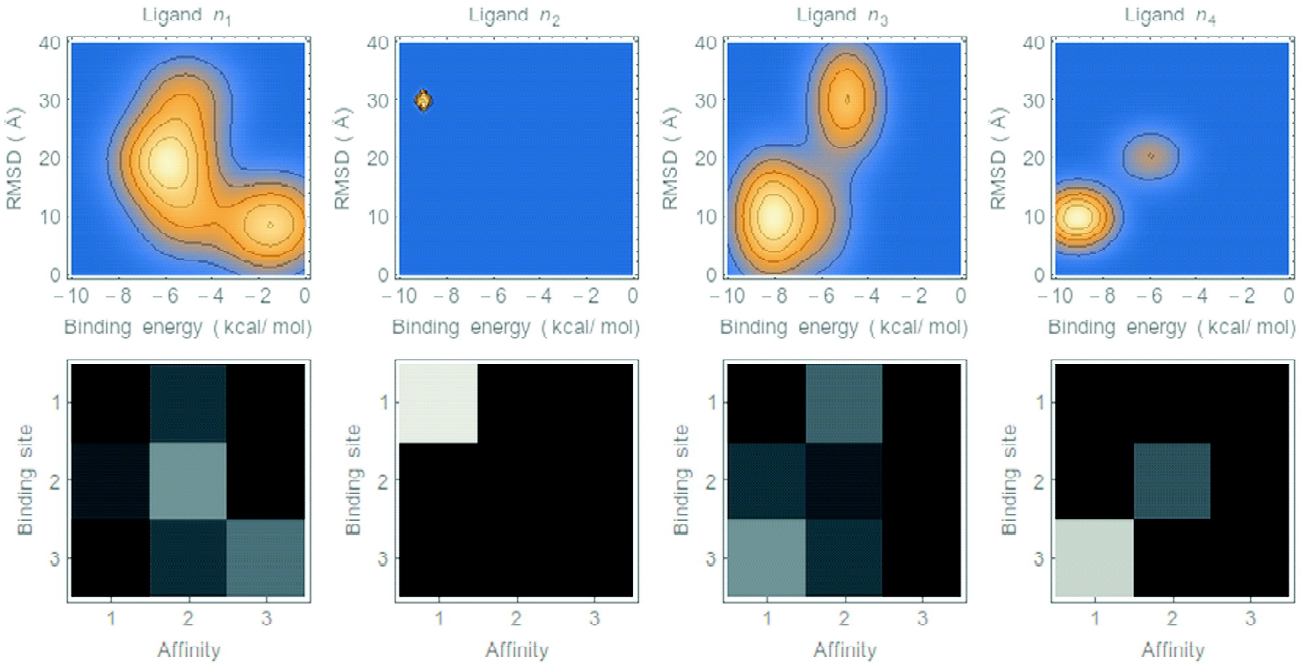

Figure 3: **Machine readable pattern generation from the density distributions. Upper panel shows the representative distributions. Lower panel shows the patterns generated from the distributions. Each of the distributions is associated with a particular ligand in the dataset describing their binding site preference on the target and affinity for that site**

4-((3-(4-Hydroxy-3-methoxyphenyl)-4,5-dihydroisoxazol-5-yl) methoxy) benzaldehyde (CP4b) is another reported inhibitor of human MIF (Alam *et al*., 2011). The docking pattern of this molecule on MIF was also analyzed and compared to that of ISO-1 (Figure 4D to 4F). All the docked conformers are superimposed and shown in Figure 4D and the Figure 4E shows the density patterns. It was observed that, unlike ISO-1 binding, the binding of CP4b was not exclusive to the active sites (Figure 4D). Three densities were observed in the density diagram (Figure 4E) corresponding to the binding into the three active sites and these were comparable energetically to that of ISO-1. However, the probabilities of binding of CP4b into the active sites of MIF were very low (Figure 4F). Other densities corresponding to the binding into other sites of MIF were observed (Figure 4E and 4F). Although CP4b is an active compound against human MIF, from the analysis of docked patterns it appears that the CP4b binding lacks specificity of interaction. Therefore, this pattern analysis technique also provides us a way to study the specificity of interactions. More specific the interaction is, denser the cluster becomes. The spread of the cluster inversely correlates with the specificity of interaction, as well. Highly specific interactions produce localized and solitary high density spots, whereas, non-specific interactions produce very low density spots spanning large regions on the density map.

Figure 5 shows the virtual screening of six ligands (epoxyazadiradione, azadiradione, azadirachtin A, azadirachtin B, nimbin and salannin) against a target protein (human MIF). These compounds are natural products isolated from *Azadirachta indica*. One of the compounds, epoxyazadiradione, is reported to be a potent inhibitor of human MIF (Alam *et al*., 2012). Binding of these compounds was analyzed following the protocol given in Figure 2. It was observed that these molecules produce densities at around 29 Å along the RMSD axis on the 2D pattern. This density does not correspond to the active site of the protein but an allosteric site located on the N-terminal side along the C_3 symmetry axis of the protein.

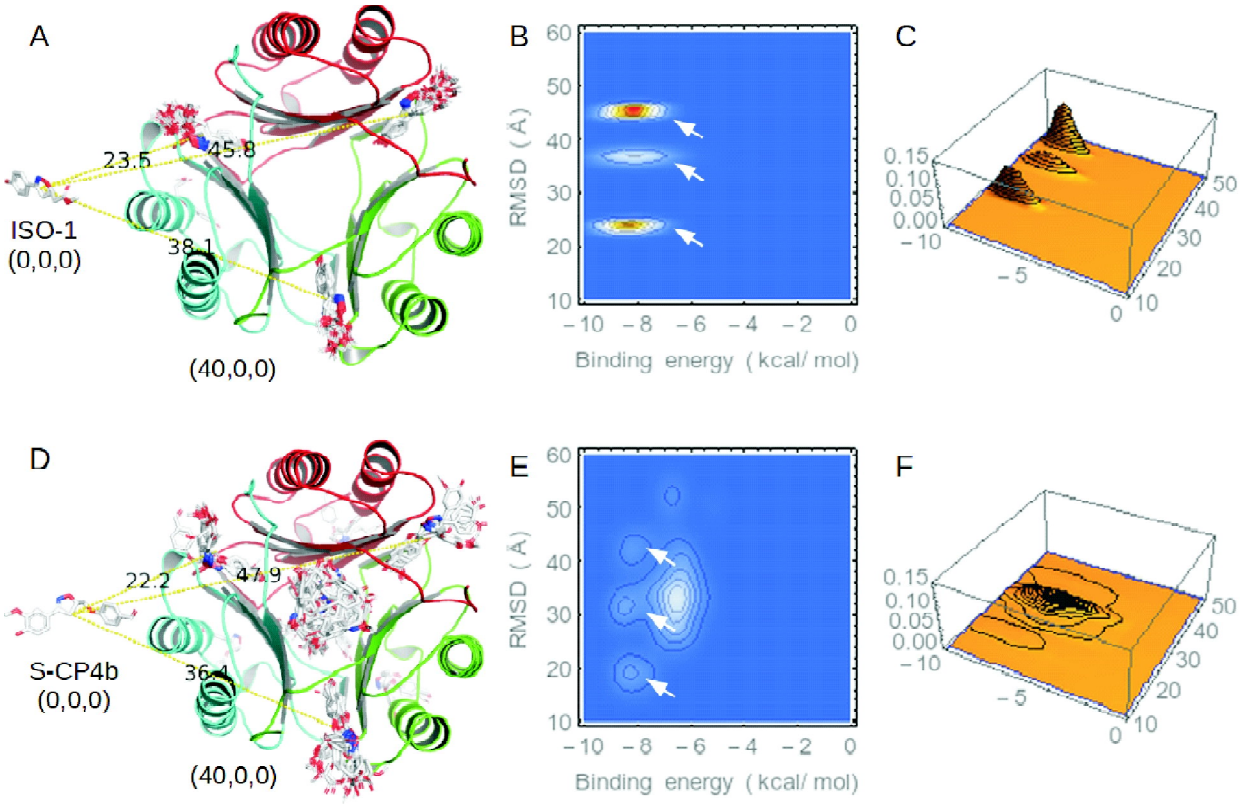

Figure 4: **Orthosteric ligand binding into the active site. (A) ISO1 binds to the active sites located in between the adjacent subunits of human MIF. (B) Densities of the docked confomers of ISO-1 on the energy-RMSD surface. (C) 3D histogram of the probability densities. (D) Binding of CP4b with human MIF. (E) Densities of the docked conformers of CP4b. Densities in the active sites are marked with arrows. (F) 3D histogram of the probability densities for CP4b binding with MIF**

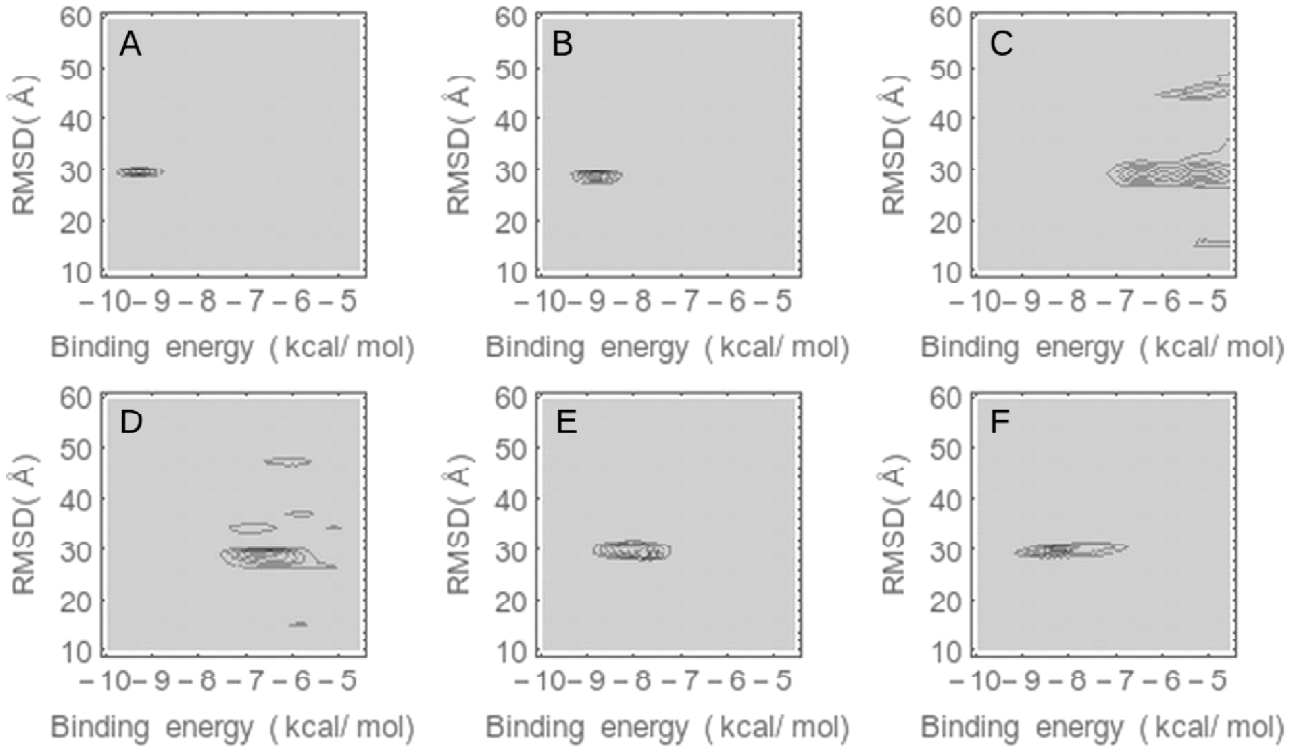

Figure 5: **Screening of six ligands against human MIF. (A-E) Epoxyazadiradione, azadiradione, azadirachtin A, azadirachtin B, nimbin and salannin binding to human MIF, respectively**

Epoxyazadiradione produced the most condensed spot suggesting it to be a highly specific allosteric inhibitor of MIF. Azadiradione also produced a condensed spot; however, it was energetically less favorable. All the other compounds were less specific or energetically less favorable. However, all these molecules showed affinity toward an allosteric site on the target. The origin of such affinity is in their structural similarity. It illustrates the potential of this approach in identifying and sorting of allosteric ligands from a mixed dataset and reduce the noise in the virtual screening.

It was observed that not all the sites are druggable by all the ligands. Binding of ISO-1 was exclusive to the active site, whereas, epoxyazadiradione targeted exclusively the allosteric site. Therefore, in order to probe all the possible binding sites on a target protein a heterogeneous set of ligands is required to be docked, which eventually gets sorted during the analysis. In this algorithm, RMSD was used as the major parameter to distribute the system states; other parameters such as the center of mass/ geometry etc. may also be used, instead. One of the limitations of this study is that, it relies solely on the structural parameters; chemical parameters of the ligands or the biding sites are not directly considered. The method separates the different types of ligands and the binding modes on two dimensions as in a 2D gel electrophoresis, but unlike electrophoresis, here one can go beyond the two dimensions. Several other properties of the ligands such as the change in the solvent accessible surface area etc. can also be added in other dimensions.

Conclusions

In the analysis of complex scientific problems, visualization has become an important tool. Here, we derived an elegant and visually sensitive way that could depict intricate details of molecular docking and linked allosteric phenomena with it. A new algorithm has been described to probe the potentially druggable sites (both allosteric and orthosteric) on a target protein and to reduce noise in virtual screening by segregating the different types of ligands in a mixed dataset. A projection method to spread the surface of a protein into a topological map has been demonstrated, on which the orthosteric and the different allosteric sites appear as probability densities for ligand binding. Utilizing this method, allosteric sites on the target protein can be probed and the druggability of such sites can be determined.

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Abbreviations

AMP, adenosine monophosphate; ATP, adenosine triphosphate; CP4b, 4-((3-(4-Hydroxy-3-methoxyphenyl)-4,5-dihydroisoxazol-5-yl) methoxy) benzaldehyde; GPCR, G-protein coupled receptor; HIV, human immunodeficiency virus; ISO-1, 4,5-dihydro-3-(4-hydroxyphenyl)-5-isoxazoleacetic acid methyl ester; MIF, macrophage migration inhibitory factor; NNRTI, non-nucleotide reverse transcriptase inhibitor; RMSD, root mean square deviation; UFF, universal force field

Conflict of interest

The authors do not have any conflict of interest with the contents of this manuscript.

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