Modulating Protein-Protein Interactions: From Structural Determinants of Binding to Druggability Prediction to Application

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Abstract: During the last decades, a large amount of evidence has been gathered on the importance of protein-protein interactions in tuning and regulating most important biological processes. Since many of these pathways are deeply involved in diseases, extensive research efforts have been undertaken towards the modulation of protein-protein interactions. At the early stage of this challenge most of the attention was drawn to the drawbacks of such a therapeutic approach. Encouragingly, however, several recent studies provided a proof of concept that protein-protein interactions are actually valuable targets and that they do have a promising therapeutic potential.

This review is divided into three sections. In the first section we summarize the general features of protein-protein interfaces, focusing on the characteristics that make them different from classical protein-ligand binding sites, as well as on problematic aspects that hamper the application of classical drug discovery approaches. In the second section, we present how some of the characteristics of protein-protein interactions can be exploited fruitfully in drug design, hence focusing on the druggability of protein-protein interfaces. Methods successfully applied to protein-protein interactions will be introduced, giving special attention to the computational ones. In the third section, three case studies are presented. First, we describe protein-protein interaction modulators targeting HDM2 and the computational methods applied to identify them. Next, we present the retrospective application of the discussed approaches on the well-examined target IL-2. We conclude with a prospective application to the NHR2 protein, a target just recently validated experimentally with the aid of computational methods.

Keywords: Alanine scanning, binding pocket detection, druggability, DrugScore, high throughput screening, hot spots, IL-2, MM-GBSA, NHR2, virtual screening.

INTRODUCTION

Recently, multiprotein complexes have become attractive targets for drug discovery [1, 2] due to the essential role of non-covalent association of proteins in the communication of cell components [3]. This is highlighted by the importance of these systems in signaling [4-7] and the regulation of, e.g., cellular growth [1] and apoptosis [8, 9]. It does not come as a surprise then that protein-protein interactions (PPIs) are involved in many diseases, such as cancer, neurodegenerative diseases [10], and viral and bacterial infections [11]. For this reason, interfering with PPIs has a great therapeutic potential, providing attractive opportunities for pharmacological intervention [3, 11-14]. However, modulating PPIs is a daunting task. First, in contrast to "classical" targets such as enzymes or receptors, much less experience has been gained so far due to the novelty of many protein-protein targets. Second, the intrinsic complexity of PPIs requires innovative methodological approaches. Encouragingly, extensive investigations have proved the general feasibility of interfering with PPIs as a valuable approach for treating a number of diseases [3, 11-15]. Here, the most important goal is to identify small molecules protein-protein interaction modulators (PPIMs) that efficiently and selectively affect processes involving protein-protein binding. These successes have benefited from remarkable steps towards an understanding of PPI properties, the determinants of binding to protein-protein interfaces, as well as the implications of modulating PPIs for biological systems. This knowledge originates from an interdisciplinary approach, including the fields of structural biology, biochemistry, genomics, medicinal chemistry, and computational chemistry.

In this review, first, we present those characteristics of PPIs that are important for molecular recognition with a special emphasis on how to identify the determinants of binding of PPIMs, which is important from a drug discovery point of view. Second, we focus on the question of how to estimate the druggability of protein-protein interfaces. Finally, we describe case studies that elucidate the application of the approaches discussed in the first two parts.

PROTEIN-PROTEIN INTERACTIONS: FUNCTIONAL AND STRUCTURAL ASPECTS

"Classical" targets versus PPIs. In the case of protein-ligand binding, an enzyme or receptor (hereafter together referred to as "receptor") interacts with a small molecule or a peptide within a relatively small and well-defined binding site located in a cavity on the receptor surface. When there are no conformational changes on the binding site of the receptor, this situation can be described by the simplistic "lock-and-key" model already suggested by Emil Fischer [16]. According to this model, high affinity and specificity are achieved through shape and chemical complementarity, leading to a compact and tight fit between the binding partners [17]. When trying to interfere with such a system, the most direct and obvious approach is to develop small molecules resembling the natural ligand, i.e., bearing chemical groups that can be accommodated by and form interactions with the binding site of the receptor. Especially for enzyme targets, it is possible to identify protein families that share the same biological function [14]. Usually, members of the same protein family have common interaction mechanisms and binding pocket architectures. This allows exploiting information gained on one enzyme when trying to identify small-molecule ligands for other targets of the same family [18-22]. As discussed in the following, PPI targets are intrinsically different from "classical" targets, such as enzymes and G protein-coupled receptors. This makes it difficult to target protein-protein interfaces by approaches established for classical targets.

Surface size and shape of PPIs. Structural characteristics provide the biggest challenge when aiming at modulating PPIs. First, on a global level, protein-protein interfaces are generally much larger than binding site regions of classical targets. In fact, ligand-receptor contact areas are typically about 300 to 1000 $Å^2$ in

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size [23-25], while protein-protein contact areas can range from ~1500 to 3000 Å^2 or even be larger [26, 27]. Second, protein-protein interfaces are often shallow and lack deep grooves or indentations (Fig. **1a**) that are usually present in classical targets (Fig. **1b**). Third, interactions between protein binding partners often occur through several, not necessarily sequentially connected spots, thus leading to a discontinuous epitope. All of the above make identification of a spatially defined region within the interface that is responsible for binding a difficult task. Encouragingly, counterexamples have been presented that benefited from a deep knowledge of the respective protein-protein interface [11]. Finally, proteins are usually promiscuous molecules [28, 29] that are able to bind more than one binding partner, possibly even at the same site. While this allows proteins to take part in intricate interaction networks, it increases the level of difficulty for finding a small molecule that modulates a specific protein-protein interaction only.

Specificity and complementarity. Cells are crowded environments and, hence, potentially all molecules populating the same cellular compartment can contact each other [30, 31]. Accordingly, it is especially important for proteins that essential interactions maintain a high degree of specificity and occur only when needed, limiting the myriad of possible contacts [30, 32]. Thus, identifying the determinants of binding at protein-protein interfaces is an important goal in molecular biology with high relevance also in related fields, notably in pharmacology, genomics, and biological chemistry. Although no common strategy can be devised to achieve binding affinity and specificity in PPIs, one can nevertheless identify some mechanisms that occur preferentially in PPIs. First, proteins are marginally stable molecules [33] forming an ensemble of conformational states, each of which could potentially interact with a binding partner [31]. These conformational changes can result in the formation of cavities in the interfaces that could not be detected by visual inspection of the static representation of a crystal structure [34, 35]. That way, proteins can exhibit grooves that allow for molecular recognition and binding [36]. Therefore, it is worth investigating conformational ensembles in solution by analyzing the dynamics of the protein of interest in detail. Several tools can assist in this task, among them NMR and scattering techniques [37, 38] for determining protein structures in solution and molecular dynamics (MD) simulations for exploring the dynamic behavior of the system by computational means [34, 39-45]. The importance of accounting for receptor flexibility to identify adequate receptor conformations complementary to a PPIM is demonstrated in a study by Isvoran *et al.* [46] combining both experimental and computational approaches. Here, docking into multiple crystallographic and NMR receptor structures in connection with complex relaxation and rescoring identified binding poses of a terphenyl PPIM with calmodulin and human centrin 2 that are considerably closer to the native one than those from docking into individual, non-relaxed, and non-complementary structures.

Nussinov *et al.* pointed out that in protein-protein interfaces *unfilled pockets* and *complemented pockets* can be distinguished [47]. Unfilled pockets are present both before and after protein-protein association. They are not crucial for complex formation, but are important for the overall flexibility. In contrast, complemented pockets are detectable at the interface before binding, but disappear after association. These pockets are then filled by the binding partner, being responsible for tight and highly complementary binding of the proteins involved. The same authors also demonstrated that pre-existing pockets do not undergo significant rearrangement after binding. This means that complemented pockets offer a favorable setting for binding interactions. Interestingly, they also found that there is a weak correlation between the conservation of residues and their frequency of occurrence in complemented pockets [47]. Such residues are often also hot spots because of their enlarged contact area and the exclusion from solvent [47, 48]. Conversely, this implies that it should be possible to identify hot spots and, hence, complemented pockets through the identification of conserved amino acids [49].

Hot spots. A fundamental characteristic of protein-protein interfaces is their energetic non-homogeneity [50]. Evidence from alanine scanning experiments shows that the binding energy is not equally distributed among all amino acids participating in the interaction [51-54]. Within the large surfaces involved in the interaction, generally some patches suffice for complex formation, the so-called *hot regions* [55]. These often have a conserved residue composition for binding similar proteins but can also differ in composition for promiscuous binding by the same interface [56]. Furthermore, only some of the residues belonging to these regions account for most of the binding energy. These amino acids are called *hot spots* if, by definition, a mutation to alanine leads to a change in the binding free energy of ≥ 2 kcal mol^{-1} [57]. Hot spot amino acids on one face of the complex are usually located in correspondence to hot spots on the other face, forming interactions that lead to complex stabilization [29]. Within the hot regions, there is a very tight geometric and energetic complementarity between the binding partners. Thus, bulky side chains on one protein are accommodated in indentations on the other protein, hydrophobic groups on one protein form close contacts with hydrophobic groups on the other protein, and polar residues establish hydrogen bonds or salt bridges between the two proteins. Rajamani *et al.* showed that *anchor residues*, which are highly buried, preordered in the unbound state, (structurally) conserved, and often energetic hot spots of PPIs, are present in many protein-protein interfaces and can possibly be exploited as starting points for PPIM development [58]. Similarly, Yogurtcu *et al.* found that hot spots are more rigid than the surrounding interface in MD simulations [59]. Hot spots within one hot region work together in a cooperative fashion, thereby stabilizing the complex [31, 55, 60]. In contrast, energetic contributions from different patches are additive [61-63], suggesting that hot regions are independent from each other. As a consequence, protein-protein interfaces appear to have a hierarchical and modular architecture being formed by separate patches, within which each hot spot amino acid strongly depends on the other close-by amino acids for an efficient interaction [31]. Interestingly, hot spots are among the most conserved residues [47, 48, 64, 65]. This relation has also been proposed to be a way to distinguish between binding interfaces and otherwise exposed protein surfaces [48]. This hypothesis is strengthened by the observation that no residue conservation was found within solvent exposed surfaces [48]. Overall, this highlights the importance of hot spots for protein-protein complex formation and explains why evolutionary changes rarely lead to a significant modification in hot spot composition [64].

Although the leading role in driving the interaction between protein binding partners relies on hot spots, the surroundings amino acids are also important. According to the *O-ring theory* [51], surrounding residues have the function to protect hot spots from solvent molecules, favoring hydrophilic or even hydrophobic interactions that would be otherwise disturbed by the presence of water. A high degree of complementarity between the binding partners is sometimes also achieved through water mediated interactions [66-68]. Such structural water molecules are particularly important in regulating hydrogen bond networks within the interface: I) by bridging interactions between the binding partners or II) by favoring the formation of a dry core in the interface that maximizes the interactions between hot spots surrounded by a rim of amino acids and water molecules [24].

Interaction types and amino acid composition. Given that protein-protein interfaces have considerable areas of hydrophobic residues, resembling cores of globular proteins [69], it has been suggested that the hydrophobic effect is the driving force leading to protein-protein association [70-72]. However, a careful analysis shows a situation similar to protein folding: the hydrophobic effect is a leading force but the proteins do not necessarily adopt a con-

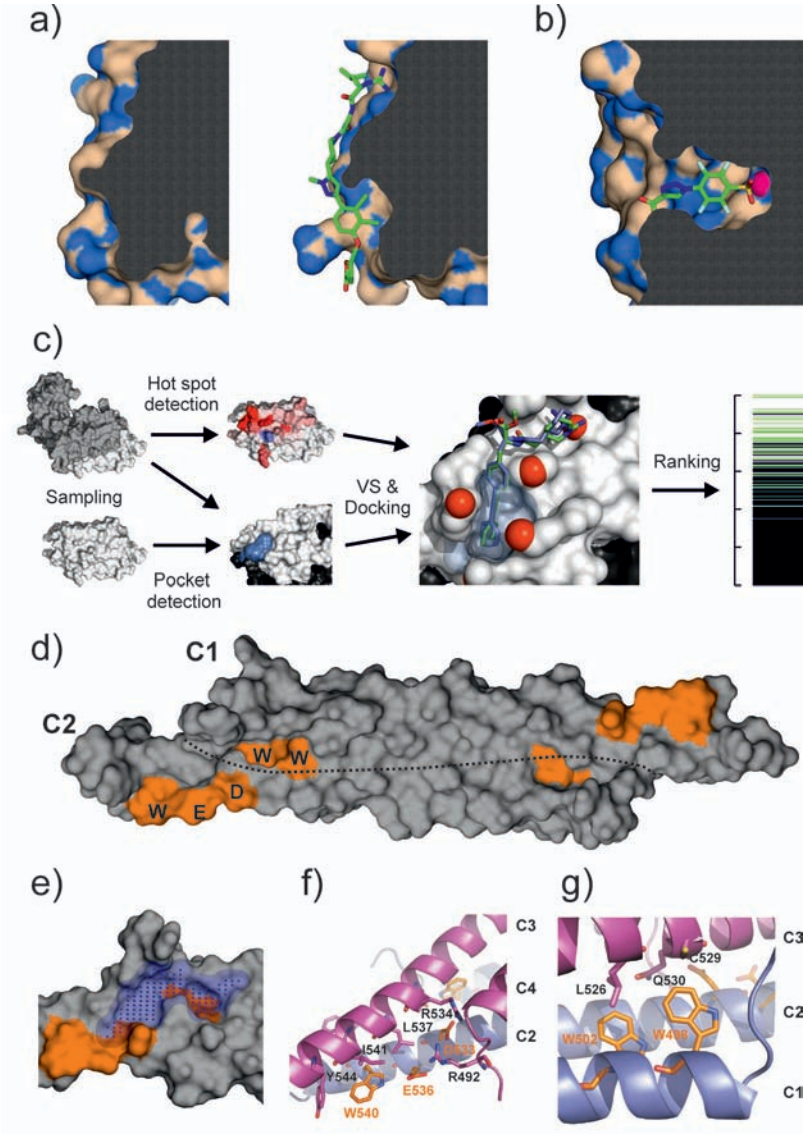


Fig. (1). Druggable binding sites at protein-protein interfaces. (**a**) Protein-protein interfaces usually lack deep pockets in the unbound state, as depicted for unbound IL-2 (left panel, PDB code: 1m47). Still, surface flexibility can allow for the formation of druggable pockets, as depicted for a PPIM-bound conformation of IL-2 (right panel, PDB code: 1py2). (**b**) In contrast classical targets have narrow and deep binding pockets, as depicted for carbonic anhydrase in complex with an inhibitor (PDB code: 3p25). The surface coloring in (**a**) and (**b**) highlights polar (blue) and non-polar (beige) atoms; Zn^{2+} highlighted in purple. (**c**) Strategy for PPIM identification on protein-protein interfaces. Surface coloring of hot spots (red) on a linear color scale as calculated by per-residue free energy decomposition; pocket volume depicted as blue surface. (**d**) Hot spots (W498, W502, D533, E536, and W540) in the dimer/dimer interface of NHR2 (PDB code: 1wq6) calculated by per-residue MM-PBSA free energy decomposition. The dotted line represents the border between the two antiparallel α-helices C1 and C2. (**e**) Potentially druggable pocket in the dimer/dimer interface of NHR2. The blue dots mark the location of the largest indentation in the binding epitope. Intermolecular contacts involving (**f**) hot spots W540, D533, and E536 on helix C2 as well as (**g**) hot spots W502 and W498 on helix C1.

formation with optimally buried non-polar surface area [73-76]. This hints at further mechanisms being involved. In fact, even though hydrophobicity is important in this context, the role of electrostatic interactions cannot be neglected [77-80]. In fact, the hydrophobicity of protein-protein interfaces is usually intermediate between the one found for a protein core and a solvent exposed protein surface. The amino acid composition in the hot spots, which has been shown to be non-random [81], reflects this situation. In fact, it has been observed that hot spots are enriched in tryptophan, tyrosine, arginine, and, to a lesser extent, isoleucine [50], whereas leucine, serine, threonine, and valine were found slightly depleted [48, 51]. One could argue that large side chains just contribute more, but functional considerations prevail. In particular, tyrosine and tryptophan allow establishing stacking and hydrophobic interactions owing to their aromatic, non-polar side chains, but at the same time offer the possibility to create hydrogen bonds due to the phenolic OH group and the indolic nitrogen. On the contrary, arginine, being a polar amino acid bearing a charged guanidinium group, is mostly involved in hydrogen bonds and salt bridges across the interface although the electron delocalization of the guanidinium π-system also confers a pseudo-aromatic character [51]. This dual side chain behavior exemplifies the two-faced chemical nature of protein-protein interfaces. As a word of caution, even though the mentioned residues are the most frequent ones in PPIs, this knowledge should neither lead to neglecting the importance of other amino acids for binding nor to uncritically considering these residues hot spots just because of their occurrence in an interface.

DRUGGABILITY OF PROTEIN-PROTEIN INTERACTIONS

PPIs are far from being widely exploited targets in drug development. Even though there are some examples of marketed small-molecule drugs acting on PPIs [3, 82-86] and some further molecules are in advanced clinical trials (Fig. **2**) [1, 87-92], PPIs are usually considered high risk targets by pharmaceutical companies [2, 93]. This is for two reasons: First, initial attempts to identify PPIMs by high-throughput screening (HTS) were mostly unsuccessful, particularly when using chemical libraries designed for traditional targets [11]. Second, the wideness of protein-protein interfaces, the lack of defined binding pockets, and the stability of PPIs led to PPIs being considered difficult to target if not undruggable [93, 94]. Also due to this overgeneralization, there is still a large gap between the knowledge gathered on these systems [1, 3, 12-14] and its actual use in the development of therapeutics. Yet, some prominent counterexamples, such as the well-studied systems p53/MDM2 [11, 14] and Bcl-x_L/Bac [11], have contributed significantly to expose the myth depicting PPIs as undruggable systems [29, 94].

A big challenge associated with PPIs is the high degree of diversity in terms of the molecular recognition properties encountered. Each interface is unique, bearing its own particular characteristics and, thus, requiring a specifically tailored approach. In fact, binding sites at protein-protein interfaces are often not well conserved, which is different from enzymes that bind the same type of substrate and, therefore, share many common features in the binding regions if they belong to the same family [14]. Nevertheless, as the amount of structural data of bound PPIMs increases approaches that exploit PPIM binding information from homologues [95] will become increasingly applicable. Furthermore, there are differences between protein-protein interfaces and the non-interacting surface of a protein that allow the sequence- and structure-based prediction of residues in the interface and an enrichment of hot spots, which often stand out in such analyses [53, 96-100].

For establishing the suitability of a protein-protein interface as a target for drug discovery, first, one needs to define what is meant by "druggability" in this context. In the straightforward definition of Egner and Hillig, druggability can be considered as the likelihood of finding a selective, low molecular weight molecule that binds with high affinity to the target [101]. But what are the characteristics of a PPI that allow targeting the interface? Due to the inherent complexity of the issue, it seems impossible to answer this question unambiguously. Aside from the particular characteristics of protein-protein interfaces, as presented earlier, it is important to consider that druggability is not an absolute property of a target molecule such as chemical class, molecular weight, or logP, but always refers to a specific application. Accordingly, authors have provided different concepts for assessing druggability both qualitatively and quantitatively [14, 23, 101-106]. Utilizing computational techniques to assess a target's druggability is appealing. An important reason for this is that it should permit to cut down research costs relating to experimental investigations that otherwise must be carried out in a more extensive fashion. However, despite large research efforts, initial progress is only emerging in this field [107].

Even though a unified approach to unarguably establish the druggability for a certain PPI is not available yet, there are some general considerations valid for all PPIs, which can be used for a preliminary assessment. An interesting approach to select protein-protein interfaces suitable for drug discovery is the decision tree proposed by Chene [14]. The author showed that considerations on the physicochemical properties of an interface allow assessing whether a PPI could be a suitable target for the design of modulators. A first point concerns the natural binding state of the protein of interest, i.e., whether it falls within the *obligate* or *non-obligate* class of protein-protein complexes. In the former class, the monomers involved do not exist in the non-associated form in the cell, while in the latter class the protein binding partners can be bound or dissociated at different times or conditions. Consequently, targeting a permanent PPI should be much harder than a transient one. Other important factors to be considered are the availability of structural information, the presence of cavities, the degree of interface hydrophobicity, and the size and complementarity of the interface. In an ideal case, there is a detailed characterization of the PPI by structural studies that clarifies the determinants of binding. Next, there should be cavities on the surface with appropriate sizes to accommodate PPIMs and to allow specific targeting. In addition, the overall hydrophobic character of the interface should be intermediate, permitting to develop molecules with an adequate trade-off between optimal binding and favorable pharmacokinetic properties.

Another important factor influencing the druggability of PPIs is the presence of helices at the interface. With α-helices being the most frequently occurring type of secondary structure both in the protein core and in exposed regions [108, 109], helices located on accessible surfaces are often responsible for molecular recognition. Along these lines, a survey on the Protein Data Bank [110] by Arora *et al*. revealed that 62% of the protein-protein complexes present in the database have helical interfaces [105, 106]. Furthermore, the authors divided these interfaces into three categories according to the helical character: I) receptors containing a cleft for helix binding, where a minimum of two close amino acids contribute importantly to the interaction (as in the p53/MDM2 complex [11, 14, 111]); II) extended interfaces where strong binding is achieved through multiple contacts between two- to five-turn helices and a higher number of residues; III) proteins featuring both of the described characteristics and showing quite weak interactions [106, 108]. From a drug discovery point of view, it appears obvious that complexes belonging to the first category offer better chances for developing PPIMs than complexes falling in the second and third categories. In addition, knowledge about how amino acids are arranged within interfacial helices can guide the design of α-helix mimetics with different chemical scaffolds [108]. This may be a first step in the development of PPIMs.

Any analysis of a protein-protein interface should take these aspects into account in order to assess the druggability of the system. In addition, (computer-aided) binding pocket and hot spot detection have a great impact for characterizing the PPI and assessing the druggability of a protein-protein interface. These methods will therefore be presented in the following.

BINDING POCKET DETECTION

Identifying binding pockets in protein-protein interfaces. Identifying binding pockets is often the first step in assessing target druggability and has important implications for docking and structure-based drug design [102, 112]. In fact, when the goal is to develop a PPIM with drug-like characteristics (e.g., as compliant as possible with Lipinski's rules [113]) it is necessary to figure out where such a molecule can efficiently bind to the target interface. In an ideal situation, knowledge on experimentally validated binding sites is available from the literatures but this is not always the case. Additionally, proteins are usually part of complex interaction networks [114] such that multiple binding interfaces can be present [112], which may even be interlinked allosterically. Therefore, the choice of the correct target site is affecting the entire drug discovery pipeline, and caution should be taken in identifying and evaluating this site.

Binding is a complex event arising from several factors of which shape and physicochemical complementarity are of major importance [112]. Accordingly, binding pocket detection algorithms have been developed that can be sub-divided into two major classes [36], geometry-based [115-122] and energy-based ones [123-130]. Methods using structure and sequence comparison [131-135] or techniques taking into account the dynamics of protein structures [34, 35, 107, 136-139] have been reported less frequently. Several authors reviewed the available methods [36, 107,

112, 140], especially those applied to the identification of protein-ligand binding sites in classical targets. In this review, we focus on methods applied to detect binding pockets at protein-protein interfaces.

Pocket detection algorithms applicable to classical targets might not be readily applicable to protein-protein interfaces due to the size and flatness of the latter. Fuller *et al.* compared protein-ligand and protein-protein binding interfaces with Q-SiteFinder [93, 125]. Q-SiteFinder scans the protein surface with hydrophobic probes, clusters positions of favorable interaction energy, and ranks these clusters by their accumulated interaction energy to identify the most important binding sites. This procedure confirmed that classical targets exhibit larger pocket volumes than pockets located in protein-protein interfaces. Thus, inhibitors of classical targets tend to target a single high-volume pocket, while PPI inhibitors target multiple smaller pockets [93, 125]. The immediate consequence is that the identification of binding sites in a protein-protein interface solely based on geometrical considerations is challenging [34]. Q-SiteFinder could also successfully identify the actual PPIM binding pocket in the unbound conformation of PPIM targets [93]. Even when employing a rather stringent scheme to assess the accuracy of found pockets, penalizing overly large pockets reaching beyond the ligand, Q-SiteFinder was able to find the correct binding pocket in the top three predicted sites for 90% of the investigated proteins [125]. As an alternative, PocketFinder defines pockets enveloping grid points with attractive Lennard–Jones potential [130]. With PocketFinder, from 5,616 ligand bound pockets 95% were detected successfully with comparable results for *apo* structures; likewise, pockets involved in PPIs were successfully detected. Bourgeas *et al.* compared binding pockets/interfaces of PPIMs and the corresponding protein-protein interfaces found in the 2P2I database to those of heterodimeric protein-protein complexes without known PPIM and identified discriminating properties [141, 142]. In addition, the authors classified those protein-protein interfaces with known PPIMs into two groups, either with a single important secondary structure element or a more globular domain in the interface, by principal component analysis and clustering with respect to the discriminating physicochemical descriptors of the proteins [141]. The physicochemical properties of PPIMs, in comparison to common drugs, were found to be shifted towards higher molecular weights, hydrophobicity, and rigidity as well as towards an increased occurrence of aromatic moieties [142].

Molecular dynamics simulations for binding pocket detection. As it is often not possible to identify well-defined binding pockets at the interface present in the crystal structure of a protein-protein complex, it is important to go beyond analyzing the static structure of PPIs and take into account protein dynamics [29, 44, 45]. This yields a more detailed view of the conformational space accessible to a protein-protein interface. For example, it was shown that nanosecond MD simulation started from an unbound conformation can sample a bound conformation in many cases [34, 35, 143]. Accordingly, Eyrisch and Helms successfully applied a pocket detection protocol that makes use of MD simulation-derived conformations and the PASS (putative active sites with spheres) algorithm [122]. Starting from the crystal structure of unbound Bcl-x_L, IL-2, and MDM2, the authors were able to detect binding pockets that would have been missed when just applying any of the available algorithms to the unbound conformation because of the transient nature of these pockets [34]. Mimicking the experimental multiple solvent crystal structure (MSCS) [144] and NMR solvent mapping experiments, cosolvent MD simulations favor the opening of hydrophobic binding pockets that would be unfavorable in purely aqueous environment. Yang *et al.* recently demonstrated the feasibility of this approach for Bcl-x_L [43]. Miranker and Karplus presented the multiple copy simultaneous search (MCSS) where thousands of probe molecules are positioned on a protein interface and energy minimized to identify favorable binding sites [145] and potentially plastic interface regions [146]. MCSS has been applied to identify anchor residues in PPIs and to design pepidic Ras/Raf inhibitors [147]. Landon *et al.* applied the CS-MAP computational solvent mapping method [148] to a clustered structural ensemble of H5N1 avian influenza neuraminidase generated by MD simulation to identify novel hot spots while accounting for target flexibility [149]. Eyrisch *et al.* presented a comparative study showing that more and larger pockets open in methanol cosolvent MD simulations than when performing conformational sampling based on normal mode analysis or by (t)CONCOORD [42]. Coarse-grained simulations have been shown to be capable of sampling the bound state of proteins starting from an unbound one, too [150, 151]. Accordingly, the grid-based pocket detection algorithm PocketAnalyzer^{PCA} [152] was successfully applied to identify PPIM binding sites in ensembles of IL-2 generated by a constrained geometric simulation method; these sites provided an entry point for a subsequent virtual screening (see also below) [35].

Machine learning approaches. Machine learning-based and empirical scoring functions have been applied as binding site prediction methods for PPIs. These methods make use of the characteristic differences between binding sites in protein-protein interfaces and the remaining protein surface, e.g., with respect to sequence conservation, amino-acid occurrence, secondary structure, solvent accessibility, and side chain conformational entropy [153]. For a description of the numerous tools and web servers available we refer to more detailed reviews [53, 153]. Recently, Li *et al.* used ray casting to identify pockets and protrusions in protein surfaces, which can be used as a filter for detecting surface shape complementarity and help speeding up protein-protein docking [154]. Tan *et al.* presented a pocket detection algorithm based on *depth* [155], i.e., the distance of a residue to the solvent, which has been proposed to be superior to using the solvent accessible surface area (SASA) for the prediction of shallow binding pockets [156]. Ertekin *et al.* showed that residues near cavities exhibit high frequency vibrations (HFVs) that can be identified using an elastic network model [157]. Fragment docking has been presented as a valuable tool to identify pockets and evaluate their druggability [158].

A characterization of a protein-protein interface that aims at identifying potential binding sites for small molecules is definitely an important starting point for any drug discovery project targeting a PPI. Nevertheless, as already pointed out by Cheng *et al.*, the presence of a pocket on the protein surface is "necessary but not sufficient" for the development of PPIMs [23], and additional investigations of the interface are in order.

HOT SPOT DETECTION

Spatially clustered hot spots are crucial for the binding of small drug-like PPIMs in a large protein-protein interface [11, 50]. Thus, methods for the detection of hot spots [96] do not only provide a more detailed understanding of the energetic determinants of binding but yield information that complements the one derived from binding pocket detection. Initially, we will briefly introduce experimental methods for hot spot determination, which is followed by a more detailed discussion of computational methods.

Experimental hot spot detection. Mutagenesis of interface amino acids is the most significant method to detect and validate hot spots. Mutating selected or, seldom, all such amino acids to alanine is called *alanine scanning* and yields a finger print of the amino acids important for a PPI [54, 159]. A mutation to alanine is usually chosen because it has a small neutral side chain devoid of polar interactions and does not significantly influence the protein backbone as, e.g., glycine would do. Still, even a mutation to alanine can potentially introduce larger structural changes in the complex or influence the unbound state of a protein such that changes in relative binding free energies observed between wild-type and mutant complex do not necessarily originate from interac-

tions lost in the interface [159]. Also, if alanine partially carries over interactions of the original amino acid, e.g., in terms of backbone interactions or because the original amino acid is similar to alanine, the change of affinity upon mutation will be less than the total contribution of the original amino acid. Furthermore, alanine scanning is very laborious because it requires protein purification and analysis. This bottleneck can be alleviated by combinatorial alanine scanning using phage display [160, 161] or combinatorial solid-supported peptide libraries [162]. Alternatively, methods of fragment-based drug design, including covalent tethering [163-166], co-crystallization [167], SAR by NMR [168, 169], and SOS-NMR [170], can identify binding fragments of rather low affinity and, thereby, probe druggability [171]. Also, solvent mapping by MSCS [172] and chemical shift perturbation (CSP) NMR experiments [173, 174] are methods that suggest where organic molecules will preferably bind and so have been exploited in data-driven docking [107, 175]. All these methods can help identify a smaller, druggable, and hot spot-containing sub-region of the interface, even if there is no open binding pocket detectable in the unbound state of the receptor [176]. Information about experimentally determined hot spots are available in several databases [57, 177-181], although the coverage is low when compared to the number of PPIs considered to be interesting drug targets.

Since experimental methods for detecting hot spots are laborious, there is a high demand for computational prediction methods. Methods for performing *in silico* hot spot detection can be categorized into: *in silico* alanine scanning, non-perturbing fully atomistic approaches, machine learning approaches, and approaches using nothing but unbound protein structures.

***In silico* alanine scanning.** Among the computational alternatives for hot spot detection that require experimentally determined or modeled structures of protein-protein complexes as input, *in silico* alanine scanning [159, 182, 183] is the most straightforward analogue of the above described experimental method. Here, a relative binding (free) energy is calculated for a wild-type complex and one with alanine mutants in the interface. Usually, intermolecular energy and (de)solvation free energy terms are considered for this, sometimes also intramolecular energies and entropic contributions. *In silico* alanine scanning often uses simple physical models or empirical (scoring) functions for assessing the energy change [182]. Therefore, *in silico* alanine scanning is usually fast and computationally modest, allowing a rapid detection of binding determinants. As a downside, these methods rely on approximations that often reduce their accuracy. FoldX [184] and Robetta [185-187] are widely used implementations of this approach. The alanine mutants are generated by side chain truncation with subsequent structural relaxation of the environment or the whole complex. The energy change is determined by an energy function whose terms have been parameterized based on experimental data. Kiel *et al.* performed an alanine scanning for Ras/RalGDS and Ras/Raf-RBD complexes and found very good correlations ($R > 0.95$) between experimentally measured changes in the free energy of binding and the prediction of FoldX [188]. In a similar study on various members of the ubiquitin domain superfold family, experimentally found hot spots could be determined by FoldX elucidating the basis of binding specificity, even though using homology models as input structures [189]. Carbonell *et al.* investigated the distribution of hot spots in protein-protein complexes of the non-redundant yeast interactome by FoldX alanine scanning and found that hot spots of promiscuous binding are located in independent modules while those of specific binding are arranged predominantly in one module [190]. Ivanov *et al.* predicted alanine mutations that disrupt the rabies virus phosphoprotein dimerization by FoldX, which agreed with results from a yeast two-hybrid assay [191]. Kortemme *et al.* correctly identified 79% out of 233 experimentally validated hot spots from 19 protein-protein complexes using the Robetta alanine scanning method [187]. Jochim *et al.* applied Robetta to all helix-mediated PPIs in the PDB and analyzed the distribution of hot spots in these helices to propose new PPI targets and assess their druggability, e.g., by helix mimetics [105, 108]. Donald *et al.* predicted hot spots by Robetta that are located in the interface of the extracellular stalk region of the β_3 and the complementary α_{IIb} and α_v integrin subunits whose mutation lead to destabilization and thereby activation *in vivo*, although the energy threshold for predicting a hot spot was alleviated to ≥ 0.3 kcal mol^{-1} [192]. Recently, Liu *et al.* applied a consensus strategy exploiting FoldX, Robetta, KFC, and their Z-score approach, a measure for the significance of the contribution to binding of a residue derived from knowledge-based pairwise potentials and available surface area, to compare the determinants of binding of H1N1 hemagglutinin antigen variants to an antibody [193]. Perez *et al.* predicted hot spots of the betaine transporter BetP membrane protein by applying FoldX alanine scanning to crystal structures and a structural ensemble generated by MD simulation; the hot spots were later found to disrupt the trimer [194]. We recently developed a webservice (http://cpclab.uni-duesseldorf.de/dsppi) for hot spot prediction in PPIs by *in silico* alanine scanning that uses the knowledge-based scoring function DrugScore^{PPI}. DrugScore^{PPI} consists of pair potentials derived from atom type-specific pair distribution functions from 851 experimental protein-protein complex structures. The weights of the pair potentials have been adapted by partial least squares regression on relative binding affinities for the so far largest set of 309 alanine scanning results [195]. DrugScore^{PPI} efficiently predicted affinity changes for an external set of 22 alanine mutants of the Ras/RalGDS complex showing higher correlation to experiment ($R = 0.66$) than FoldX ($R = 0.52$), Robetta ($R = 0.43$), and CC-PBSA ($R = 0.23$). Tuncbag and Keskin *et al.* presented the HotPoint method [196], an empirical model based on knowledge-based residue pair-distribution potentials [197] and solvent occlusion to predict hot spots. Notably, many methods for hot spot prediction are applied to single experimental or modeled structures of a protein-protein complex. However, caution is needed because the hot spot detection outcome from a single complex structure may be less representative if the proteins are flexible *in vivo*. Therefore, it is preferable to perform calculations on conformational ensembles of the proteins, e.g., obtained from MD or coarse-grained simulations.

Non-perturbing fully atomistic approaches. As a complementary alternative to *in silico* alanine scanning, there are methods that calculate the contribution of individual amino acids to the free energy of binding without mutating them. In one of the first studies, Novotny *et al.* correctly predicted residues important for binding in antigen-antibody complexes by a physics-based energy approximation [198]. Similarly, the nowadays most widely used molecular mechanics-generalized Born surface area (MM-GBSA) [35, 199, 200] and the molecular mechanics-Poisson Boltzmann surface area (MM-PBSA) methods allow a per-residue decomposition of the binding free energy. A critical review of these methods also covering PPI applications has been published recently [201]. Both methods predict the total binding free energy by means of endpoint free energy calculations. The underlying energy function consists of electrostatic and van-der-Waals terms from the molecular mechanics (MM) force field, which are complemented by polar (based on the generalized Born (GB) or Poisson-Boltzmann (PB) continuum models) and non-polar (surface area-dependent (SA)) contributions to the (de)solvation free energy. Entropy changes upon binding can be determined from normal mode analysis or quasi-harmonic analysis. All of these terms can be decomposed into contributions of individual residues, which allows revealing hot spots. It is also possible to further decompose the binding free energy into pairwise contributions, which highlights important interactions between pairs of amino acids. The method is usually used for post-processing ensembles from MD simulation trajectories. If the unbound proteins and the protein-protein complex are sampled individually, this leads to the conceptually rigorous *three trajectory* approach, which takes into account energetic differences caused by

conformational changes upon complex formation but is also computationally demanding. A widely used alternative is the *single trajectory* approach in which the unbound structures are extracted from the trajectory of the complex without further relaxation. The latter approach, besides being faster, was shown to accurately reproduce experimental alanine scanning data. Indeed, the single trajectory approach often proved to be superior to the three trajectory alternative due to the cancellation of errors [35, 52]. However, it has to be mentioned that the MM-PB(GB)SA energy function has also been applied for *in silico* alanine scanning on structural ensembles from MD simulation [183, 202]. In this context, Moreira *et al.* found improved predictions when using different dielectric constants to account for the varying extent of relaxation upon mutating charged, polar, and non-polar residues to alanine [203].

CC-PBSA [204] is a conceptually related method using similar terms to calculate binding free energies but CONCOORD [205] for conformational sampling. CONCOORD generates conformational ensembles by iteratively satisfying geometrical constraints starting from a random structure. Here, weighting factors for the energy terms had to be derived by fitting to experimental data. Furthermore, the linear interaction energy approach (LIE) [206] has been applied to compare the binding energy of a large set of mutated proteases and their inhibitor proteins [207]. In the LIE approach, the binding free energy is calculated as the weighted sum of intermolecular energies and the (de)solvation energy of the ligand, and the weights are derived by fitting to experimental data.

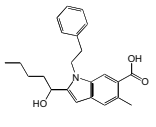
Machine learning approaches. In addition to merely training an empirical physical model on experimental data, machine learning approaches can use structural, physicochemical, or sequence descriptors for hot spot prediction without the need for a model based on first principles. Chen *et al.* compared various hot spot prediction methods to support vector machine (SVM) models trained on different sets of sequence and structure-based descriptors and found a sequence-based SVM to outperform FoldX and Robetta amongst others on an independent test set [208]. Cho *et al.* used decision-tree based feature selection to identify properties that discriminate hot spots with their SVM model MINERVA [209]. They found hydrophobicity and π-π interactions to be hallmarks of hot spots. Additionally, they found the atomic packing density weighted by the fraction of the available surface area buried upon complex formation to be highly predictive in contrast to the raw coordination number. Even in the crystal structure of the unbound proteins, the weighted atomic packing density of hot spots was found to be significantly higher than for the remaining surface residues, although calculating the surface area buried upon complex formation still required a complex structure. Kosloff *et al.* predicted hot spots of various G protein-RGS (regulator of G protein signaling) protein interactions [210] by a combination of structure-based sequence alignment, continuum electrostatics-based per-residue electrostatics, and buried surface area-dependent non-polar interaction energy [211]. In contrast to Robetta alanine scanning, they could identify hot spots with important backbone or long-range electrostatic interactions. Combining sequence conservation with hot spots energetics they distinguish *significant & conserved* from *modulatory* residues, the former ones being essential for overall binding affinity and the latter ones for binding specificity of complexes. The authors could redesign low-affinity RGS proteins to higher affinity ones by mutating modulatory residues. A comparison of experimental hot spots from the alanine scanning energy database (ASEdb) [57] and those predicted by decision trees and SVM based on buried SASA, sequence conservation, and hot spot propensity can be found in the HotSprint database [181]. Darnell *et al.* created the decision-tree based KFC model using a combination of shape specificity and descriptors of interaction types; both descriptors show a lower accuracy when used alone [212]. Here, the decision-tree machine-learning algorithm preformed slightly better than SVM and Bayesian networks or Robetta alanine scanning when applied to the (training) dataset. Nevertheless, the successor model KFC2, which comes in two variants based on different descriptors, is a SVM that was recently reported to outperform KFC and other machine learning methods [213]. Shulman-Peleg *et al.* investigated the spatial distribution of 3D alignments of physicochemical interactions (hydrogen-bond donor and/or acceptor, aromatic) in protein-protein interfaces of functionally similar PPIs and found conserved distribution patterns to predominantly contain hot spots; conversely, this allows hot spot prediction without the necessity of sequence conservation [64].

***In silico* hot spot detection without a complex structure.** The above methods require a complex structure as input. As this may often be not available, methods have been developed that aim at predicting hot spots solely based on protein sequence information or unbound protein structures. ISIS [214], a neural network based method using only sequence information, predicted hot spots with high precision [100] at the cost of a low sensitivity due to a strict threshold for the discrimination of hot spots [96]. To predict hot spots based on the unbound structure of a protein, the principle behind NMR solvent mapping and MSCS has been transferred into computational algorithms [215]. Cosolvent MD simulations, previously mentioned in the context of inducing pocket opening, provide the location of sites occupied by different cosolvent probes. The site's population with a cosolvent can be used to approximate a maximal binding affinity of residues at this site by applying the inverse Boltzmann principle [216]. Although other methods for sampling probe populations could be considered, e.g., Monte Carlo simulation, docking, or MCSS, these methods do not account for plasticity or do not generate a Boltzmann ensemble, thus aggravating the estimation of energy and entropy of binding. Nevertheless, Grosdidier *et al.* presented an approach to predict hot spots by protein-protein docking [217]. Here, the normalized interface propensity (NIP) of individual amino acids calculated from docking poses could predict hot spots with a high accuracy without the need to approximate an energy [218]. Recently, Geppert *et al.* presented iPred [219], which predicts protein-protein interfaces and hotspots based on the difference of local intramolecular atom- and residue-specific pair potentials between interface and non-interface residues. In conjunction with the geometry based pocket detection algorithm PocketPicker [120], iPred identified druggable sites in the interface of the unbound structure of interferon IFN-α to its receptor IFNAR. These sites could subsequently be addressed by pharmacophore based screening and docking to yield an *in vivo* active PPIM of the IFN-α/IFNAR interaction from a set of only six tested compounds [220]. In a retrospective study on more than 15 PPIs with known PPIM structures, Kozakov *et al.* predicted hot spots and binding sites to assess interface druggability by computational solvent mapping of 16 different probe molecules with FTMAP [221]. They found druggable sites to be structurally conserved between bound and unbound structures and that local side chain rearrangements, implemented by a rotamer search followed by energy minimization, suffice to accommodate for most adaptations in PPIM binding.

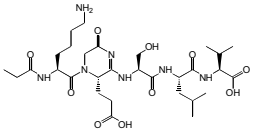
In summary, several comparative studies showed encouraging results in terms of agreement between experimental and computed results for hot spot detection [52, 195, 199, 200, 202].

CASE STUDIES

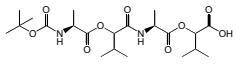
During the last two decades many studies have investigated PPIs and identified PPIMs using both experimental and computational approaches. The available experimental data has been integrated into PPI-specific databases [6, 141, 222]. Antibodies are currently the most successful class of drugs [223] inhibiting PPIs [224, 225]. As conveniently accessible high affinity PPIs, they can help reveal druggable epitopes, understand binding mechanisms [226], and may even inspire PPIM design [227] by complementing insights from non-antibody PPIs. As a drawback, antibod-



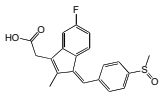
Indole derivative [235]
(PDZ/PTEN)
(K_i > 100 μM; 0.31)



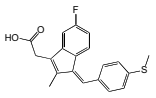
β-strand mimetic Ac-K-@E-SLV [236]
(α1-syntrophin PDZ/effector)
(K_D = 320 nM; 0.27)



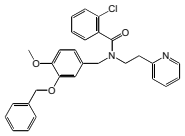
NSC668036 [237]
(PDZ/Frizzled)
(K_D = 237 μM; 0.37)



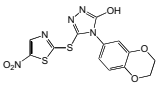
Sulindac [238]
(PDZ/Frizzled)
(K_i = 10.7 μM; 0.56)



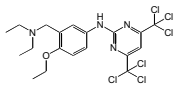
Sulindac sulfide [239]
(Ras/Raf)
(K_i = 50 μM; 0.55)



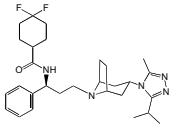
MCP1 [240, 241]
(Ras/Raf)
(IC_{50} = 17.9 μM; 0.22)



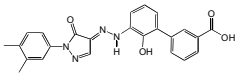
BI-78D3 [242]
(JNK/JIP)
(IC_{50} = 500 nM; 0.27)



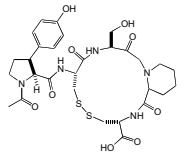
Polychloropyriminine [243]
(JNK/JIP)
(K_i = 5 μM; 0.12)



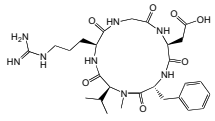
Maraviroc [244]
(CCR5/HIV-1 gp120)
(K_i < 1 nM; 0.28)



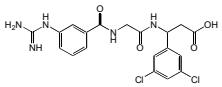
Eltrombopag [245]
(Thrombopoietin receptor agonist)
(EC_{50} = 30 - 300 nM; 0.08)



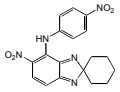
cyclic(RCDPC) mimetic [246]
(Integrin α_4 β_1/VCAM-1)
(K_i = 0.1 nM; 0.41)



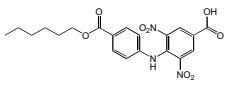
Cilengitide [84]
(Integrin $\alpha_v\beta_3$/vitronectin)
(IC_{50} = 0.6 nM; 0.63)



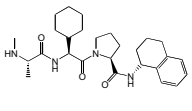
SC-68448 [247]
(Integrin $a_v\beta_3$/vitronectin)
(IC_{50} = 1 nM; 0.42)



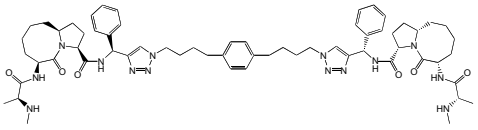
LM-11 [248]
(Arf-GDP/ARNO)
(K_i = 50 μM; 0.16)



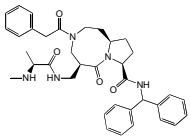
BV01 [249]
(14-3-3σ/c-Abl)
(LD_{50} = 1.7 μM; 0.10)



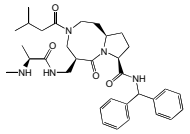
Pro-modified *N*-capped
Smac mimetic [250]
(BIR/caspase)
(EC_{50} = 13 nM; 0.67)



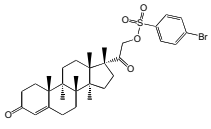
SM-164 (bivalent Smac mimetic) [251]
(BIR/caspase)
(IC_{50} = 1.4 nM; 0.19)



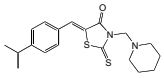
SM-337 [252]
(BIR/caspase)
(IC_{50} = 31 nM; 0.50)



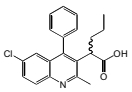
AT-406 [253]
(BIR/caspase)
(IC_{50} = 144 nM; 0.42)



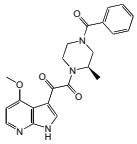
NSC88915 [254]
(CDK5/p25)
(IC_{50} = 5 μM; 0.14)



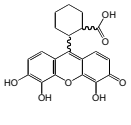
28RH-NCN-1 [255]
(c-Myc/Max)
(IC_{50} = 29 μM; 0.26)



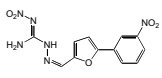
LEDGIN-6 [256]
(HIV integrase/LEDGF)
(IC_{50} = 1.37 μM; 0.21)



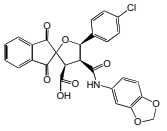
BMS-378806 [257]
(CD4/gp120)
(IC_{50} = 100 nM; 0.48)



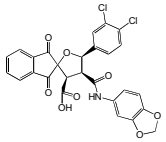
M119 [258]
($G\beta_1\gamma_2$/PLCβ2)
(IC_{50} = 3 μM; 0.42)



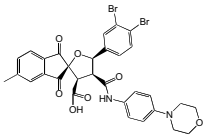
N-Nitro-hydrazinecarboximidamide [259, 260]
(ERK/ELK-1 and RSK-1)
(IC_{50} = 3 μM; 0.19)



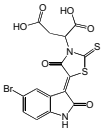
Indanone 1 [261]
(HPV/E2)
(K_i = 7.8 μM; 0.29)



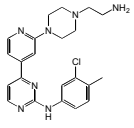
Indanone 2 [262]
(HPV/E2)
(K_i = 350 nM; 0.18)



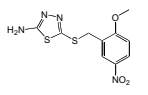
Indanone 3 [262]
(HPV/E2)
(K_i = 20 nM; 0.03)



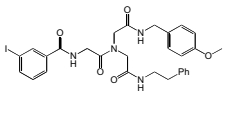
97-6 [263]
(BCL6/BBD)
(K_i = 147 μM; 0.52)



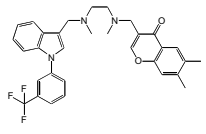
WAI [264]
(ZipA/FtsZ)
(K_i = 12 μM; 0.04)



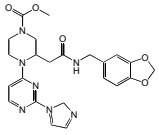
non-pY SH2 PPIM [265]
(p56Lck-SH2/ITAM)
(K_D = 4 μM; 0.24)



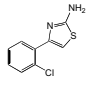
Cappsin 1 [266]
(AICAR Tfase dimerization)
(IC_{50} = 3.1 μM; 0.45)



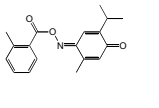
TNFα-TNFR1 inhibitor [267]
(TNFα dimer/TNFα monomer)
(IC_{50} = 22 μM; 0.15)



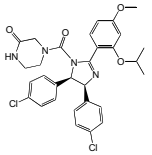
BBS-2 [268]
(allosteric iNOS dimerization inhibitor)
(IC_{50} = 28 nM; N.A.)



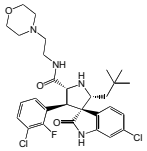
2-Aminothiazole derivative [269]
(allosteric RUNX1/CBFβ inhibitor)
(IC_{50} = 1.1 μM; 0.75)



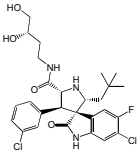
Poloxin [270]
(Plk1-PBD/phosphoproteins)
(IC_{50} = 4.8 μM; 0.07)



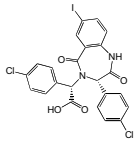
Nutlin-3 [271]
(p53/MDM2)
(K_i = 36 nM; 0.30)



MI-63 [271]
(p53/MDM2)
(K_i = 3 nM; 0.35)



MI-219 [271]
(p53/MDM2)
(K_i = 5 nM; 0.15)



Benzodiazepindione [272]
(p53/MDM2)
(K_i = 80 nM; 0.33)

WW298 [273]
(p53/MDM2)
(K_i = 109 nM; 0.24)

Benzamide helix mimetic [274]
(p53/HDM2)
(IC_{50} = 8 μM; 0.11)

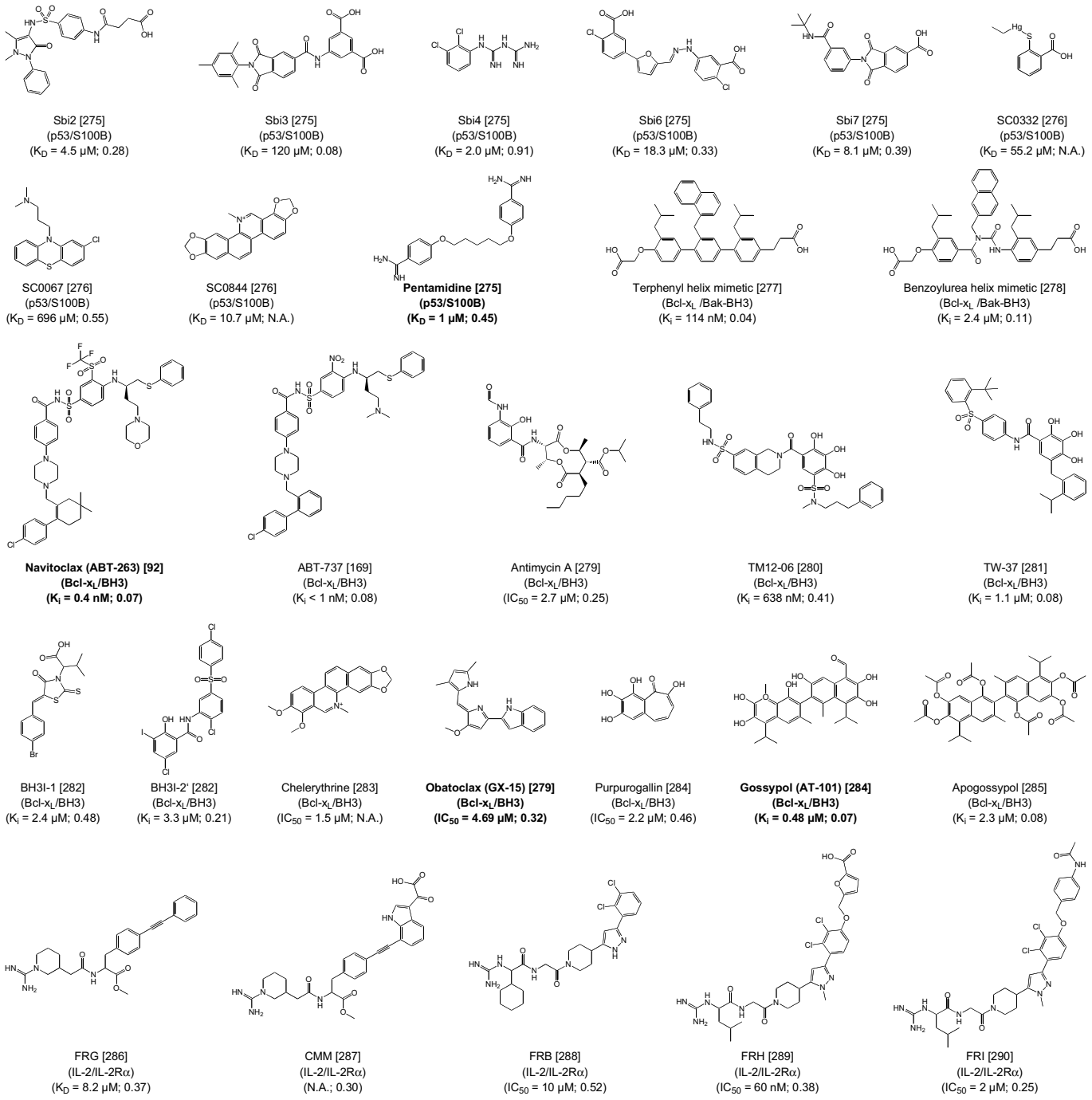


Fig. (2). A survey of protein-protein interaction modulators. Labels contain the PPIM name followed by a reference detailing the potency. Below, (PPI target/PPI competitor) and (potency; Drug-Score) in the first and second parentheses, respectively. Labels of marketed drugs or compounds that have been the subject of clinical trials are highlighted in bold. The Drug-Score was calculated by OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/druglikeness.html).

ies are not cell permeable and lack oral bioavailability [228]. We thus focus this section on small-molecule PPIMs. As related studies and discovered PPIMs have been the subject of many detailed reviews already [3, 11, 87, 107, 229-234], we solely give a survey of the so far identified PPIMs with their reported potency (Fig. **2**) without claiming completeness. In this list of PPIMs we also added a druglikeness measure, the so-called Drug-Score calculated by the OSIRIS druglikeness server. The score highlights that not all of the so far discovered PPIMs are drug-like; this also includes those being marketed or tested in clinical trials for their potential pharmacological relevance.

In addition, we describe three case studies in more detail. First, we describe PPIM design for p53/HDM2, one of the most thoroughly investigated PPI systems, as an example where a preformed binding pocket exists in the interface. Then, we describe a retro- and a prospective study from our lab concerning interleukin-2 (IL-2) and nervy homology region 2 (NHR2) proteins.

HDM2

In many tumors, p53 acts as a tumor-suppressor protein [291-293]. However, binding of the human double minute 2 (HDM2) protein (or the mouse analog MDM2), which is overexpressed in many tumors, blocks transactivation by p53 and increases p53's degradation. Thus, the p53/HDM2 interaction is an important pharmaceutical target for cancer treatment. Crystallographic structures revealed that the key interaction in the p53/MDM2 complex arises from the binding of a 15-residue α-helix of p53 into a hydrophobic cleft [111]. Furthermore, alanine-scanning revealed three hot spots on the helix [294]. Notably, this binding site suits most of the criteria in the decision tree proposed by Chene [14]. Initially, PPIMs binding to HDM2, e.g., nutlins [87, 295, 296], benzodiazepinediones [272], and others (Fig. **2**) [229, 297-299], were identified via HTS. Structure-based design and molecular modeling were then used for ligand screening and optimization [300-304] leading to *in vitro* activities down to IC_{50} = 3 nM [302]. Several computational techniques have helped designing and screening for ligands of HDM2 or MDM2. (I) MD simulation and computational alanine scanning could accurately predict the hot spots of the p53/MDM2 interaction already by the efficient post-processing of wild-type trajectories [183]. In the same study, also the change in binding affinity due to other covalent modifications, e.g., methylation of the hot spot tryptophane of p53, could be confirmed in good agreement with experimental data. Finally, the opening or widening of the binding pocket into a PPIM binding-competent conformation could be sampled by MD and detected computationally [34, 42]. (II) The molecular diversity of compound libraries (e.g., benzodiazepinediones) was maximized to optimize molecules for HTS and synthesis strategies [304, 305]. (III) Molecular docking [271, 306], also in combination with *de novo* design [299, 302, 306], was applied to predict binding modes and optimize the design of PPIMs. (IV) Virtual Screening [303, 307], QSAR [303], and receptor-based pharmacophore models using ensembles of receptor structures [308] were also applied. From this, the following general strategy emerges: If there is no binding-competent pocket in the *apo* or protein-bound structure of a PPI target, such structures can potentially be found if multiple receptor conformations from NMR ensembles or crystallography are available [46] or if an ensemble can be generated by molecular simulations, e.g., MD simulation, preferentially in solvent less polar than water [42], or constrained geometric simulation [35, 42] (Fig. **1c**). Furthermore, post-processing schemes including complex relaxation and rescoring have been demonstrated to improve the ranking and identification of native like binding poses [46]. In summary, many computational methods used in conventional computer-assisted drug design could be applied successfully to HDM2, also as a consequence of the deep binding cleft that is already preformed in the unbound HDM2 structure.

INTERLEUKIN-2

IL-2 is a key cytokine involved in the regulation of the immune system with relevance for immunological diseases, transplant medicine, and cancer [309]. Binding of the α-helical IL-2 to the trimeric IL-2 receptor is initiated by the association of IL-2 to the extracellular domain of the receptor's α subunit (IL-2Rα). The IL-2/IL-2Rα complex has been the subject of extensive studies that provided crystallographic structures and thermodynamic characterization of the protein-protein complex and five IL-2/PPIM complexes (Fig. **2**), rendering this system a perfect test case for structure-based computational methods on PPIM design. For the binding of a PPIM, a pocket in the flat but flexible interface of unbound (or receptor-bound) IL-2 has to open (Fig, **1a**-**b**). The absence of such a pocket is a major obstacle for structure-based design if based solely on the unbound or receptor-bound structure of IL-2. Additionally, it is difficult to decide which part of the large IL-2/IL-2Rα interface (~2500 $Å^2$) to address with a small molecule. Thus, it is not surprising that the first known PPIMs binding to IL-2 were not found by structure-based design but rather by high-throughput screening. Later, IL-2 PPIMs were designed using structural knowledge obtained by tethering experiments and/or fragment-based ligand design [163]. This resulted in PPIMs with affinities down to the nanomolar range [289].

As to the question of transient pockets, we were able to show that conformational sampling of the unbound IL-2 structure by a constrained geometric simulation method resulted in the opening of such pockets, whereas MD simulations in explicit solvent failed in doing so, probably due to the pockets being rather hydrophobic [35]. Not using any knowledge about known IL-2 PPIMs, we were then able to identify these pockets from the ensemble structures based on geometric criteria as provided by $PocketAnalyzer^{PCA}$ [152]. Notably, molecular docking into these pockets closely reproduced the bound state of the known IL-2 PPIMs as could these PPIMs be successfully ranked by MM-PB(GB)SA calculations and enriched in a large set of decoys. As to the question of the hot region, a narrow cluster of hot spots was predicted [35] by MM-PB(GB)SA effective binding free energy decomposition [199] starting from the IL-2/IL-2Rα complex. Performing such decomposition for IL-2/PPIM complexes showed that essentially the same hot spots are also used for PPIM binding, pointing to mimicry of the PPI by the small molecules. Notably, two recent studies applying the energy based pocket detection algorithm Q-SiteFinder [93] and the solvent mapping algorithms FTMAP [221] showed that the druggable site could also be identified in the unbound structure of IL-2. Still, the need to account for interface flexibility persists, because *apo*-docking into the unbound structure of IL-2 was unsuccessful. Being able to accurately predict transient pockets from an unbound structure, and hot spot positions and binding energetics from complex structures, strongly suggests that the strategy and methods used here (Fig. **1c**) will also be applicable in a prospective manner where nothing else than a protein-protein complex structure is known. Hence, this approach can well be the first step in a structure-based endeavor to identify PPIMs.

NHR2

NHR2 (nervy homology region 2) is the α-helical oligomerization domain of the RUNX1-ETO fusion protein present in approximately 12% of all acute myeloid leukemia (AML) [310]. The formation of NHR2 homotetramers from dimers has been shown to be essential for the leukemogenic activity of RUNX1-ETO [311]. In a prospective study, spatially clustered hot spots in the tetramer interface were predicted by MM-GBSA free energy decomposition (Fig. **1d**) and were subsequently validated by *in vitro* and *in vivo* experiments [311]. The results reveal that alanine mutants of the hot spots prevent tetramerization of NHR2 and abolish AML formation in a mouse transplant model, thereby validating NHR2 as a promising target. As for the druggability of this PPI, a shallow elongated cavity was detected next to the hot spots (Fig. **1e**). The anti-parallel orientation of helices C1 and C2 in the NHR2 dimer places D533, E536, and W540 (Fig. **1f**) in close proximity to residues W498 and W502 (Fig. **1g**), which results in a spatially compact arrangement of the hot spot residues. Furthermore, these residues are not located in the center of the interface, which is rather flat, but at its edges. These findings provided the incentive to develop a short peptide derived from the wild-type NHR2 sequence as an initial NHR2 tetramerization inhibitor (unpublished results). Based on this proof-of-principle, a virtual screening for small molecules was performed on the ZINC database [312] exploiting the knowledge about the predicted and validated hot spots. Encouragingly, some of the top-ranking small molecules from this screening exhibit *in vitro* PPIM activity in NHR2 tetramerization assays (unpublished results).

In summary, it was possible to (I) identify hot spots of the tetramerization of NHR2 that could be confirmed experimentally. These hot spots were (II) transferred to a peptide that is currently further optimized. After identifying a potent peptidic PPI modulator

(III) virtual screening for molecules exhibiting an arrangement of pharmacophoric groups as found in the peptide was carried out. These results lead us to conclude that the mutual integration of experimental and computational techniques is a promising approach to cope with the challenges of protein-protein interfaces in PPIM identification and design.

CONCLUSIONS AND PERSPECTIVES

Targeting protein-protein interfaces is currently a topic of outstanding interest in drug discovery. Since these targets offer great opportunities to interfere with PPI networks and, hence, for new therapeutics considerable effort has been undertaken for the development of PPIMs. As a result, the detailed characterization of many PPIs brought us remarkably closer towards an understanding of PPIs and their druggability [94]. While many PPIMs have been discovered by HTS, the structural insight into PPIs from experimentally determined protein-protein complexes and the experimental and computational methods for the identification of clustered hot spots and binding pockets has accelerated the rational design of PPIMs. Indeed, there are already a few examples of marketed small-molecule drugs acting on PPIs [3, 82-86, 225]. However, PPIs are different from classical targets in that binding pockets are often less pronounced, and hot spots are not in all cases arranged in a manner that they can easily be addressed by a small molecule. As pointed out by Morelli *et al.* along with the observation that there is not yet a unified approach for PPIM discovery, it appears that any such attempt has to be tailored for a specific PPI [142]. Nevertheless, the wealth of reported PPIMs shows that many PPIs are at least ligandable [94] (Fig. **2**). With respect to the druggability of PPIs, it has to be mentioned that many of the so far developed PPIMs address PPIs that are predisposed by having preformed pockets and clustered hot spots and, accordingly, are more druggable than other PPIs. Also, it has to be mentioned that many of the reported PPIMs are not drug-like in the sense of Lipinki´s rules, leaving considerable space for improvement and optimization to achieve the desired specificity and ADME properties. With the increasing number of known PPIMs, it is becoming clear that their chemical space is not identical to that of the majority of marketed drugs [313]. In fact, many of the PPIMs with pharmacological and clinical relevance do not exhibit the characteristics classically considered to be preferable for a drug-like molecule. Consequently, most currently available compound libraries, predominantly comprised of molecules with characteristics appropriate for classical targets, are not ideal for the identification of PPIMs, and methods for tailoring libraries for PPIM identification are being developed [314].

Note, however, that the trend in recently approved new molecular entities shows that the traditional criteria for drug-like properties, though desirable, are not a strict criterion for exclusion [313]. In addition, there are several examples for the optimization of non-drug-like molecules [315] and novel drug delivery approaches [316] with which some of the barriers for non-drug-like PPIMs may be overcome.

Here, we reviewed methods applicable to PPI druggability prediction and provided case studies of their successful application on PPI targets and for PPIM development. Many of these methods originate in druggability prediction of classical targets and have been adapted for application to PPIs where it is crucial to identify less pronounced potential binding sites to discern well druggable targets. Furthermore, methods for the detection of hot spots, based on the structure of the protein-protein complex, the unbound protein, the sequence, or a combination thereof, enable the identification of regions in which a small-molecule PPIM can efficiently bind. Additionally, experimental evidence shows that the flexibility of protein surfaces and protein-protein interfaces enables the opening of druggable pockets. Such pockets cannot be easily identified in the absence of a bound PPIM and, consequently, require an adequate treatment of the protein's flexibility, e.g., by molecular simulation methods.

We think that any attempt to identify or optimize PPIMs can greatly benefit from integrating computational and experimental methods of pocket and hot spot detection, screening, and rational design. However, even though the success of several such attempts has been reported, it is hard to decide which computational methods will work best for a specific PPI because many of the presented methods have only been applied to one or a few targets. Furthermore, the performance of general strategies for the prediction of pockets and hot spots is hardly comparable, for two reasons. First, the datasets used to validate many methods vary considerably, often as a consequence of the prerequisites each individual method has. Second, the definition of pockets and hot spots often varies, thus complicating a statistical comparison of the prediction performance. To overcome this situation there is a high demand for common benchmarking datasets and a comparative database with experimental data as well as predictions from the various methods for enabling a comparison amongst subsets of known targets and to extrapolate to new ones. Furthermore, adapting the content of (virtual) screening libraries in order to cover the chemical space of PPIMs [142, 314], e.g., by including large but preorganized scaffolds containing hydrophobic/aromatic groups as often found in PPIMs and privileged scaffolds such as peptidomimetics, will facilitate the identification of new PPIMs. In fact, the amount of available data on PPIs is still very low in comparison to classical targets. However, with the expected progress in experimentally determined PPI structures, targets, and affinity data thereof and of PPIMs it will eventually be possible to compare PPI targets, transfer successful strategies, and exploit the potential of modulating PPIs to its full extent.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

PPI	=	Protein-protein interaction
PPIM	=	Small-molecule protein-protein interaction modulator
RMSD	=	Root mean-square deviation
MD	=	Molecular dynamics
IL-2	=	Interleukin-2
IL-2Rα	=	α-subunit of the interleukin-2 receptor
GB	=	Generalized Born
PB	=	Poisson-Boltzmann
MM-PBSA	=	Molecular mechanics Poisson-Boltzmann surface area
MM-GBSA	=	Molecular mechanics generalized Born surface area

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