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Review

In silico design of low molecular weight protein–protein interaction inhibitors: Overall concept and recent advances

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ABSTRACT

Protein–protein interactions (PPIs) are carrying out diverse functions in living systems and are playing a major role in the health and disease states. Low molecular weight (LMW) "drug-like" inhibitors of PPIs would be very valuable not only to enhance our understanding over physiological processes but also for drug discovery endeavors. However, PPIs were deemed intractable by LMW chemicals during many years. But today, with the new experimental and in silico technologies that have been developed, about 50 PPIs have already been inhibited by LMW molecules. Here, we first focus on general concepts about protein–protein interactions, present a consensual view about ligandable pockets at the protein interfaces and the possibilities of using fast and cost effective structure-based virtual screening methods to identify PPI hits. We then discuss the design of compound collections dedicated to PPIs. Recent financial analyses of the field suggest that LMW PPI modulators could be gaining momentum over biologics in the coming years supporting further research in this area.

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1. Introduction

About 20–30 years ago, it was generally considered that protein–protein interactions (PPIs) could not be inhibited by low molecular weight (LMW) "drug-like" compounds. This was in part due to the fact that protein–protein interfaces were perceived as flat, large and apparently lacking tractable cavities that could accommodate small chemical compounds and also because, in general, protein interfaces are not known to bind LMW molecules as compared to enzymes or GPCRs (Arkin and Wells, 2004). The lack of clear binding pockets at the interfaces and the lack of endogenous ligands or substrates to start the design process together with the difficulty of developing experimental assays further accentuated this opinion. This view was also supported as, in general, very low hit rates (or no hits) were obtained after high throughput screening (HTS) experiments (Arkin and Wells, 2004; Macarron et al., 2011). As such, modulators of PPIs were essentially proteins (e.g., antibodies) and peptides (or modified peptides), yet these

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molecules are still difficult (or impossible) to administrate orally (the preferred mode of administration for both the clinicians and the patients), may not reach intra-cellular targets and are usually very expensive to develop (the costs still do not go down as expected some years ago) (Kinch, 2014a, 2014b). Starting around the years 2000 and up to now, although this traditional view is still dominating text books and many recent reviews in drug discovery, numerous investigations suggest a new emerging view: PPIs can be modulated by biologics AND by LMW "drug-like" compounds (see for instance several reviews reported in 2014 (Arkin et al., 2014; Cierpicki and Grembecka, 2015; Falchi et al., 2014; Jin et al., 2014; Johnsson, 2014; Lage, 2014; Milroy et al., 2014; Nero et al., 2014; Petrey and Honig, 2014; Rognan, 2015; Villoutreix et al., 2014; Watkins and Arora, in press; Zhang et al., 2014a)). With regard to LMW inhibitors (the focus of this review), it is known that they can act via one or several mechanisms: for example orthosteric inhibitions (here understood as a small molecule binding at the interface) or allosteric inhibitions (the small molecule binds some distance away from the interface and induces structural and/or dynamic changes over the target) (Jin et al., 2014; Szilagyi et al., 2013; Wells and McClendon, 2007). We decided to comment here binding pockets (for orthosteric LMW compounds) at the protein interfaces and in silico methods that can help to predict these special regions of the molecular surface. We then discuss recent reports describing the design of "smart" compound libraries dedicated to the direct inhibition of protein–protein interactions (i.e., we do not discuss peptides, small chemical fragments and allosteric molecules, readers can find valuable information in several recent studies like for instance (Baaden and Marrink, 2013; Chen and Tou, 2013; Craik et al., 2013; Fayne, 2013; Kaspar and Reichert, 2013; Khan et al., 2013; London et al., 2013; Ma and Nussinov, 2014; Morley et al., 2013; Nussinov and Tsai, 2015; Petrey and Honig, 2014; Pevzner et al., 2014; Rognan, 2015; Schon et al., 2011; Szilagyi et al., 2013; Thevenet et al., 2015; van Westen et al., 2014; Wang et al., 2012)). In addition, while LMW inhibitors of PPIs are valuable for therapeutic interventions, stabilizers can obviously be of importance. Stabilizers are outside the scope of the current review but interested readers can for instance find information in two recent reports (Giordanetto et al., 2014; Zhang et al., 2014b).

2. Protein–protein interfaces and ligandable pockets

General principles about protein–protein interactions at the atomic levels (e.g., for transient complexes) have been proposed some 20 years ago (Bogan and Thorn, 1998; Janin et al., 2008; Janin and Chothia, 1990; Jones and Thornton, 1996; Nooren and Thornton, 2003) and have been recently revisited and/or reviewed (see for instance (Andreani and Guerois, 2014; Chen et al., 2013b; Cukuroglu et al., 2014; Higueruelo et al., 2013a; Jubb et al., in press, 2012; Kastritis and Bonvin, 2013; Levy, 2010; Makley and Gestwicki, 2013; Smith and Gestwicki, 2012; Sudha et al., 2014; Surade and Blundell, 2012; Winter et al., 2012)). Analysis of several hundreds of transient PPIs gave a general trend about interactions and showed that the minimum protein surface that must be buried to form a functional complex is in the order of 900 Å^2 (about 500 Å^2 provided by each partner) with about 12 residues involved on each partner (Janin et al., 2008). A large majority of atoms in transient protein–protein interfaces are usually still accessible to the solvent. Relative to the accessible protein surface, the interfaces of such protein complexes are generally depleted in Glu, Asp and Lys and enriched in Met, Tyr and Trp (Janin et al., 2008). From these initial observations about protein–protein interactions, several additional structural, biochemical and computational investigations were performed and suggested, as seen below, that it should be possible to use LMW molecules to modulate such biological systems.

Protein interfaces can be divided into a core region and a rim region (Janin et al., 2008). The rim is made of residues in which none of the atoms are fully buried and has an amino acid composition close to the protein accessible surface, the rim regions by definition, are located around the core region. The core comprises buried atoms and about 55% of all interface residues. This core region is enriched in aromatic residues and to a lesser extent, in aliphatic residues but Arg residues can be present in both the core and the rim regions. Another region was also recently described, the so-called support zone that seems similar in composition to the protein interior (Levy, 2010). A related way to model protein recognition is based on the concept of hotspots. Hotspots in this context were first proposed after site directed mutagenesis (alanine scanning) experiments (see for instance (Clackson and Wells, 1995)). Analysis of these experiments suggested that the binding energy was not equally distributed among all amino acids present at the interfaces, some residues were directly responsible for the stabilization of the complex and conferred most of the binding energy. Indeed, in the investigation of the human growth hormone (HGH)/HGH receptor system by Clackson and Wells, 31 interface residues were mutated on the receptor, but only 11 mutants showed a significant loss of affinity for the hormone. Hotspot residues in the context of protein–protein interactions are typically defined as those amino acids contributing to about 2 kcal/mol to the total binding energy of the complex (Clackson and Wells, 1995). Hotspots tend to occur in clusters and are generally located on both protein partners, these regions can be in contact with each other in the complex and form a network of interactions that is often called hot region (Keskin et al., 2008). As mentioned above, hotspot regions can be identified experimentally using alanine scanning but a number of computational approaches can also be used, with as input, the amino acid sequence alone or the 3D structures (experimental or homology models) of each individual partner (e.g., by docking, see for instance a protein docking computation guided by site directed mutagenesis data which predicted an overall contact area between the two protein partners that is partially confirmed by X-ray crystallography (Autin et al., 2006; Pomowski et al., 2014)) or the macromolecular complex (Fernandez-Recio, 2011; Sudha et al., 2014; Thangudu et al., 2012; Villoutreix et al., 2014, 2013). Hotspot residues (among the most conserved amino acids) are generally located around the center of the interface, and are protected from bulk solvent by energetically less important residues forming a hydrophobic O-ring (Bogan and Thorn, 1998). This view is indeed very similar to the core–rim-support model reported above and support further the concept of direct LMW modulators. Tryptophan (21%), arginine (13.3%) and tyrosine (12.3%) are often hotspot residues (i.e., thus hotspot regions would tend to be hydrophobic and aromatic) whereas leucine, serine, threonine and valine tend to be disfavored (Bogan and Thorn, 1998; Fernandez-Recio, 2011; Moreira et al., 2013). The surface area of a region containing some hotspot residues is around 600 Å^2, a size that is compatible with a small molecule (NB: traditional protein–small ligand interaction ~300–1000 Å^2 and the solvent accessible surface of many small molecule drugs usually ranges from 150 to 500 Å^2), and much smaller than a typical protein–protein interface (e.g., 1000 to 2000 to well over 3000 Å^2) (Janin et al., 2008). Also, it is important to note that the term hotspot can have a different meaning in drug design and be considered as a site on a therapeutic target that has high propensity for (small) ligand binding. In such case, investigation of these regions can be performed experimentally with for instance investigation of fragment binding using NMR or X-ray approaches (Hajduk et al., 2005a, 2005b). Another important observation suggesting that small compounds binding at

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PPI interfaces could modulate an interaction in spite of the large difference in size between the small molecules and the interface is that protein–ligand binding hotspots seem to correlate with protein–protein hotspot residues (Jubb et al., 2012; Zerbe et al., 2012) (i.e., the same amino acids could be involved in both types of interactions).

An additional critical observation that should assist the rational design of PPI inhibitors (iPPIs) comes from molecular dynamics studies. Two major concepts were explored these last ten years, the possibility of pocket formations at the PPI interfaces along the simulation run and the fact that many hotspot residues important for protein–protein interactions could be relatively rigid. It has indeed been shown that hotspot residues important for protein–-protein interactions tend to be more rigid as compared to the surrounding interface residues (Rajamani et al., 2004). Transient binding pockets have been observed at the protein–protein interfaces (Eyrisch and Helms, 2007) and indeed, these small binding pockets can be formed with little energetic cost at some PPI interfaces as compared to other regions of the protein surfaces (Johnson and Karanicolas, 2013). By combining these observations and several other structural investigations of protein–protein interfaces (Cukuroglu et al., 2014; Kozakov et al., 2011; Sudha et al., 2014), we propose to conceptualize PPI and small molecules binding at the interface as follow (some PPIs involve a disordered partner and as such the present view may only apply to the other well folded partner): hotspot residues are pre-organized in the unbound protein form and their conformation in the unbound form resembles the one they have in the bound state (Cukuroglu et al., 2014; Kozakov et al., 2011; Sudha et al., 2014) (see below the example of interleukin-2). Several of these hotspot residues are centered in and around peculiar pockets that often involve 3 to 5 (in general superficial) small volume subpockets (Fuller et al., 2009). These hotspot residues are important for protein–protein interaction but also have high propensity to bind small chemical compounds (Jubb et al., 2012; Zerbe et al., 2012). Then, around these somewhat rigid and concave areas containing hotspot residues, flexibility is possible (e.g., side chains and some backbone atoms) and generally required, the upcoming ligand is then expected to select a low energy conformation of the receptor or induce fit takes place (or both are possible according to some studies) (Arkin et al., 2014; Wlodarski and Zagrovic, 2009). This should be possible as small binding pockets could form with little energetic cost on the druggable PPI interfaces (Bohnuud et al., 2014; Johnson and Karanicolas, 2013).

To design a LMW inhibitor targeting a protein interface, experimental HTS, experimental fragment-based methods and other related biophysical approaches have been shown relatively successful but the output obviously varies depending on the target and the compound collection used (Arkin et al., 2014; Illendula et al., 2015). However, the cost can be very high as compared to in silico methods (Clark, 2008; Kapetanovic, 2008) (e.g., a HTS campaign of 1 million compounds is estimated to cost anywhere from $500,000 to $1,000,000, requires many months of analysis with hit rates typically ranging from 0.01 to 1% and being generally lower for PPIs as historically synthetic efforts have been focused on a limited number of target classes and definitively not on PPI inhibitors (Davies et al., 2006)). Furthermore, some targets tend to resistant or very challenging to investigate with those methods (e.g., membrane receptors, and in some cases PPIs because, for instance, a low affinity fragment, generally lacking tridimensional features, may have difficulty in inhibiting a protein–protein interaction) (Chen et al., 2013a; Dias et al., 2014; Macarron et al., 2011; Mathieu et al., 2014; Ngounou Wetie et al., 2014; Sun et al., 2011; Villoutreix et al., 2014; Wells and McClendon, 2007). Although not specifically on PPIs, the important failures observed after running 70 HTS campaigns over a period of seven years on antibacterial targets with compound collections containing from 260,000 to 530,000 molecules (Payne et al., 2007) suggest that experimental work could certainly benefit from in silico studies and in that specific case the investigation of targets' ligandability (Hopkins et al., 2007). In addition, other in silico tools generally well established in the field of drug discovery (yet one notes healthy and unhealthy resistance to such technologies (Muchmore et al., 2010)) could have been used such virtual screening approaches. For example, we could find PPI inhibitors by testing a list of 200–1000 compounds starting from compound collections of over 300,000 molecules using structure-based virtual screening strategies. The work was carried out for the vascular endothelial growth factor (VEGF)–VEGF receptor (VEGFR), one of the flattest protein–protein interfaces known (Gautier et al., 2011) (hit rate ~9%), the interaction between VEGF and neuropilin (Starzec et al., 2014) (hit rate ~3% for the first round of computations), the interaction between the anticoagulant protein C and its substrates, blood coagulation factor V and factor VIII (Sperandio et al., 2014; Villoutreix and Sperandio, 2010) (only low affinity compounds could be find in part due to the very challenging targeted binding pocket), and one protein–protein exosite on the tyrosine kinase SYK (Mazuc et al., 2008; Villoutreix et al., 2011) (hit rate ~8.5%). Yet, it is important to note that it can be challenging to identify hits for some PPIs using structure-based virtual screening because the docking and/or scoring accuracy can drop as compared to regular targets presenting clearly defined binding cavities (i.e., docking a small ligand in small superficial cavities is difficult and in general scoring functions have not be tuned for ligands that remain largely solvent exposed after docking (Gowthaman et al., 2013; Kruger et al., 2012)). It is thus clear that all these technologies, experimental and in silico, have strengths and weaknesses (Davies et al., 2006; Dragiev et al., 2011; Kar and Roy, 2013; Macarron et al., 2011; Nero et al., 2014; Parker et al., 2006; Scior et al., 2012; Varin et al., 2012; Villoutreix et al., 2014), yet a good complementarity between all these approaches has often been noted (Bajorath, 2002; Bienstock, 2012; Davies et al., 2006; Ferreira et al., 2010; Gul and Hadian, 2014; Makley and Gestwicki, 2013; Wanner et al., 2011). A fast and cost effective rational design strategy can be envisioned for the design of PPI inhibitors if one has for instance only the 3D structure of one protein partner in the apo (free) form (or a comparative model (Cavasotto and Phatak, 2009; Fan et al., 2009; Skolnick et al., 2013)) and the possibility to screen only a couple of hundreds of compounds. Since small cavities likely to bind a small chemical ligand can be predicted by computational approaches (see for instance (Johnson and Karanicolas, 2013; Kozakov et al., 2011; Li et al., 2014; Perot et al., 2010) and below) and that hotspot residues likely to be at a protein–protein interface can be proposed using in silico approaches (see for instances in silico tools to predict hotspots (Fernandez-Recio, 2011; Villoutreix et al., 2014)), then hit compounds could be found using structure-based virtual screening carried out on several conformations of the receptor followed by in vitro screening of a short list of molecules selected in silico (Cherkasov et al., 2014; Clark, 2008; Cross and Cruciani, 2010; Cukuroglu et al., 2014; Ekins et al., 2007a, 2007b; Falchi et al., 2014; Fernandez-Recio, 2011; Gautier et al., 2011; Gowthaman et al., 2013; Grosdidier and Fernandez-Recio, 2012; Heikamp and Bajorath, 2013; Jain, 2004; Kastritis et al., 2014; Kruger et al., 2012; McInnes, 2007; Phatak et al., 2009; Rester, 2008; Ripphausen et al., 2012; Schneider, 2010; Scior et al., 2012; Shoichet, 2004; Starzec et al., 2014; Stumpfe et al., 2012; Sudha et al., 2014; Taboureau et al., 2012; Totrov and Abagyan, 2008; Villoutreix et al., 2014; Zarzycka et al., 2015; Zhu et al., 2013).

An important step for structure-based screening approach involves the identification of binding pockets and the ranking of

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these pockets. It is indeed well known that LMW drug molecules bind to some types of protein cavities, the cavities that are said to be druggable or ligandable or bindable (Edfeldt et al., 2011; Sheridan et al., 2010; Surade and Blundell, 2012). On regular targets, these cavities tend to be characterized by several properties such as pocket volume (~500 Å^3 and above but the value obviously varies depending on the method used, often LMW drugs bind to one of the top 3–5 largest cavity identified on the molecular surface of a conventional protein target) and by a single cavity with a pocket depth ranging from about 7 to 11 Å, among others (Brown and Hajduk, 2006; Hajduk et al., 2005a, 2005b; Kufareva et al., 2012b; Lahti et al., 2012). By contrast and also because PPIs have not evolved to bind a LMW chemical compound, interfaces tend to lack this type of LMW ligand-binding cavity. However, it has been found that protein–protein interfaces that bind small molecules in general possess a special type of binding surface containing 3–5 relatively superficial subpockets (each of about 50 Å^3) (Fuller et al., 2009) (Fig. 1). This observation is critical as it suggests that the molecules present in compound collections (i.e., the chemistry and 3D structure of these molecules) are not really optimal to block PPIs since indeed, molecules present in screening libraries are most of the time legacy compounds, that is, molecules developed to hit conventional protein targets (e.g., ion channels, GPCRs and enzymes) (Arkin et al., 2014; Fry et al., 2013; Kuenemann et al., 2014; Mullard, 2012; Neugebauer et al., 2007; Reynes et al., 2010). As such, small molecules found in most commercial screening collections are well suited to bind to relatively deep cavities possessing a relatively large volume as generally found in enzymes or GPCRs while protein–protein interaction inhibitors would need in general to have a 3D structure and shape that allow the distribution of functional groups in several low volume subpockets (Arkin et al., 2014). The shape of these molecules is thus expected to be very different from the ones seen in molecules hitting regular targets (Fry et al., 2013; Kuenemann et al., 2014).

Binding pockets can be investigated in silico if the 3D structure of the target is available but it is important to note that most of these prediction methods were not developed for PPIs. Binding pocket detection algorithms (numerous tools are listed at www.vls3d.com) are essentially subdivided into two major classes, geometry-based (i.e., search of cavities) and energy-based (i.e., probe the energetic of a binding surface) tools (Fig. 2a) (Chen et al., 2011; Dessailly et al., 2013; Fauman et al., 2011; Koes and Camacho, 2012; Kufareva et al., 2012a; Leis et al., 2010; Perot et al., 2010; Trosset and Vodovar, 2013; Villoutreix et al., 2013, 2007; Wirth et al., 2013; Xie and Hwang, 2015; Zheng et al., 2012). In addition, some tools report not only a list of binding pockets or a list of regions that could interact favorably with small chemical compounds but also provide a druggability score (Perot et al., 2010; Wirth et al., 2013; Zheng et al., 2012). In general, the algorithms work on a static protein structure but some take into account amino acid side chain flexibility on the fly or the different receptor conformations have to be generated prior to the use of these binding pocket prediction methods are used. Because of the peculiar binding pockets (Fuller et al., 2009) present at protein interfaces, it would seem that purely geometric methods (i.e., these methods are usually calibrated to find large and deep cavities and not binding sites that involve small and generally superficial subpockets) are not the best approach to use while energy-based approaches would appear more suited for PPIs (Li et al., 2014) (Fig. 2b, see below). Examples of energy-based approaches involve probe mapping algorithms (e.g., Ruppert et al., 1997) and docking of fragments and of small compounds (e.g., Huang and Jacobson, 2010) or the use of other simulation tools such as molecular dynamics (Alvarez-Garcia and Barril, 2014). To date, two fragment/compound docking approaches have been specifically developed for PPI-pocket prediction (including also allosteric sites in some cases), FTMAP (Kozakov et al., 2011) (computational mapping with 16 different chemical probes) and FindBindSite (Li et al., 2014) (docking of several thousands of fragments). In fact, it has been shown that when a main hotspot region at a protein–protein interface has a concave topology, with one or two additional hotspots close enough to be reached from the first main hotspot site by a drug-sized molecule, then the region is likely to be druggable (Kozakov et al., 2011; Zerbe et al., 2012). Of importance also with regard to the ligandability of protein–protein interface, as mentioned above, is the observation that protein–ligand binding hotspots in PPIs seem to correlate with protein–protein hotspots (Jubb et al., 2012; Zerbe et al., 2012). It is interesting to note that inhibitors of protein–carbohydrate interactions could have binding pockets that share many similarities with the binding pockets for LMW compounds found at the protein–protein interfaces (Liu and Finzel, 2014).

Proteins are flexible and this is of course of major importance when using structure-based design approaches. Protein–protein interfaces tend to dynamically adapt to upcoming ligands (small or large molecules), and transient cavities not visible in experimental protein structures can appear on the molecular surface during (or

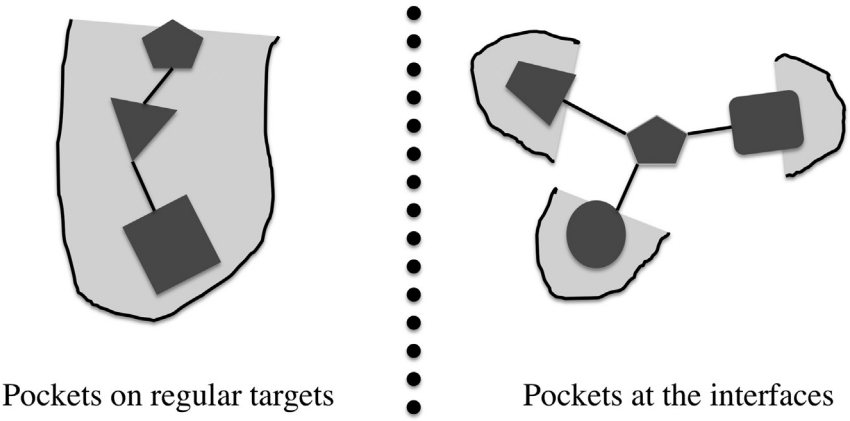

Fig. 1. Binding pockets for LMW molecules. Binding pockets have been investigated during many years for the so-called regular therapeutic targets: enzymes, GPCRs and ion channels. Often, these pockets form a single and deep cavity (~7–11 Å) with a volume of about 500 Å^3. Binding pockets for LMW compounds identified at protein–protein interfaces are in general very different, they display three to five subpockets, each of about 50 Å^3 and tend to be superficial (Fuller et al., 2009). This observation suggests that chemical compounds (shown here in dark gray) for regular targets may not be very efficient for binding to pockets present at the protein–protein interfaces (see text for discussion).

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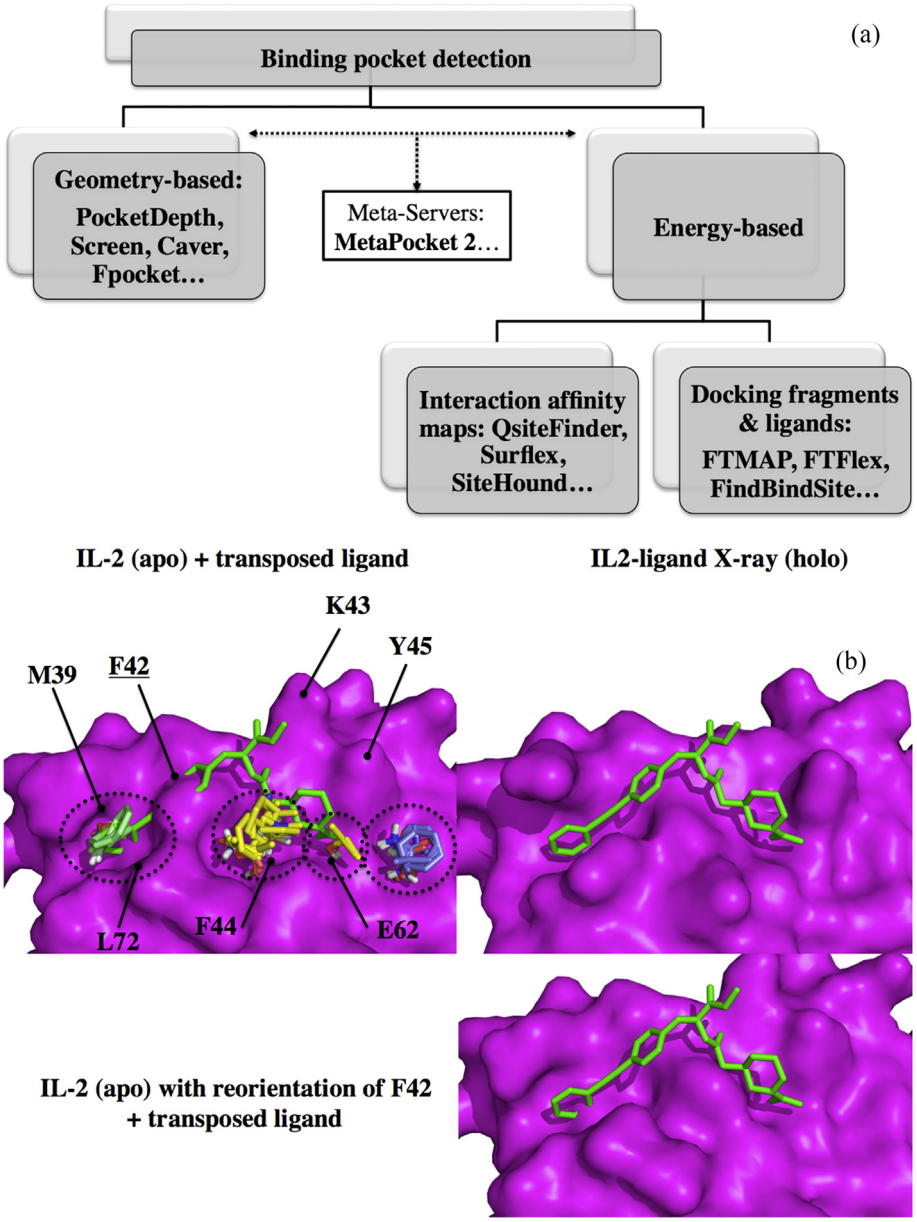

Fig. 2. In silico pocket predictions. a: In silico methods to predict and score binding pockets. We maintain a small database of free tools that assist drug discovery at www.vls3d.com (Villoutreix et al., 2013; Villoutreix et al., 2007). There, about 100 methods can be found to investigate binding pockets. Some tools available online are presented here. The two types of methods are often used, methods that search for cavities, generally called geometry-based approaches (Chovancova et al., 2012; Kalidas and Chandra, 2008; Le Guilloux et al., 2009; Nayal and Honig, 2006) and methods that probe the molecular surface of the protein to find regions that could interact favorably with small chemical groups and fragments, these methods are said to be energy-based (Grove et al., 2013; Hernandez et al., 2009; Kozakov et al., 2011; Laurie and Jackson, 2005; Li et al., 2014; Ruppert et al., 1997). Some meta-servers combine these two main concepts (Zhang et al., 2011). b: Pocket predictions on a protein–protein interface. We have tested energy-based approaches on several biological systems and illustrate the output of FTMap for interleukin-2 (IL-2) (Arkin and Wells, 2004). IL-2 interacts with its receptor and several small molecules have been identified for this system that binds at the interface on IL-2 and inhibit the interaction with the receptor. The crystal structure of the free IL-2 protein (apo, PDB code 1M47) is shown (left) and the structure of the protein in complex with a small ligand is also presented (right) (PDB code 1M48). Another rotamer could be found for F42 in a rotamer library, this move was applied to the apo form (bottom figure) and the ligand of the holo form could be transposed without major steric clashes. The same ligand was also transposed on the apo form (left figure) and it is seen that it could not fit essentially because of the side chain of F42. Hotspot residues are indicated (left figure), they are important for the binding of the receptor and of the small ligand. The key residues are E62, Y45 and F42 for both IL-2R binding and for the interaction with the small ligand. All hotspot residues have essentially the same orientation in the apo and holo form but F42 (this residue is underlined to highlight this point). The FTMap pocket prediction tool was used on the apo form and several subpockets were identified (regions surrounded by dashed lines with the small chemical probes/fragments displayed). See text for additional comments.

prior to) the binding event (Eyrisch and Helms, 2007). In such cases, while the flexibility at the interface can be challenging to study, it is known that various molecular simulation tools and protocols can be used to complement or replace in some cases X-ray or NMR studies (Craig et al., 2011; Eyrisch and Helms, 2007; Loving et al., 2014; Metz et al., 2012; Pitt et al., 2014; Rueda et al., 2010; Schmidtke et al., 2011; Sotriffer, 2011; Sperandio et al., 2010a; Ulucan et al., 2012).

Taken together, our increased understanding of protein–protein interfaces at the atomic level already assist the structure-based design of PPI modulators and in silico methods under development will certainly boost even more the field in the coming years.

To illustrate several concepts reported above on the rigidity of hotspot residues (often pre-organized in space) present at the interface, the fact that residues important for protein–protein interactions can also be important for small-ligand binding (the two

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types of concept about hotspots) and the fact that binding pockets can be predicted using as starting point the 3D structure of a free protein form, we tested several pocket prediction methods and analyzed several biological systems. In the following, we illustrate the main observations resulting from our analysis on interleukin-2 (IL-2) (Arkin and Wells, 2004). It is of course possible that some PPIs will not function along the line proposed here, yet our observations are in agreement with previously reported work (see for instance the recent review of Arkin et al. (2014)). IL-2 interacts with its receptor (IL-2R) and several small molecules have been identified for this system that bind at the protein–protein interface and inhibit the interaction with the receptor (Thanos et al., 2006). In Fig. 2b, the crystal structure of the free IL-2 protein (apo) is shown and the structure of the protein in complex with a small chemical ligand is also presented while the apo structure with another rotamer selected manually from a rotamer library and after inspection of steric clashes is reported below the apo and holo structures. The ligand has been transposed on the apo form and it is seen that it could not fit on that structure essentially because the side chain of F42 impedes binding. Indeed, selecting another (low energy) rotamer for F42 removed most of the steric clashes. Several hotspot residues for the interaction with IL-2R are shown, M39, F42, K43, F44, Y45, E62, and L72. These residues are not only important to bind the receptor but play also a role in the binding of the small ligand (Thanos et al., 2006). These residues are essentially in the same orientation in the apo and holo form but for the side chain of F42 that needs to move. The three residues that contribute most significantly to the binding of IL-2R and of the small ligand are conserved and involve E62, Y45 and F42. As noted above, these residues have essentially the same orientation in the apo and holo form but for F42. This illustrates the results reported in Rajamani et al. (2004) and shows that some regions that contain hotspots are pre-organized in the unbound state prior to the interaction with the ligand (i.e., several concave regions containing or surrounded by hotspot residues have essentially the same conformation in the bound and unbound states). Next to these “rigid regions”, there are flexible areas that allow for fine-tuning of the interaction and that often involve side chain movements and minor backbone re-adjustments. These movements open some subpockets often not visible in the apo form or on a single 3D conformation (Eyrisch and Helms, 2007). These types of movements can generally be reproduced with available simulation methods such as NMA or molecular dynamics or by simple side chain re-orientations (Eyrisch and Helms, 2007; Garzon et al., 2007; Grove et al., 2013; Rueda et al., 2010; Sperandio et al., 2010a). This analysis also shows that hotspot residues for protein binding and for ligand binding can be identical (Kozakov et al., 2011; Zerbe et al., 2012). Because these residues are close in space, this suggests that a small chemical compound binding there would indeed block the interaction with IL-2R despite being significantly smaller than the entire interface. Structural analysis of the IL-2 apo form highlights several subpockets in an overall relatively flat surface. If one wants to screen IL-2 using structure-based virtual screening, an important first step will be the identification of a binding zone. Using geometric methods (that usually search for relatively large cavities) and the apo form of IL-2, we obtained either a long list of binding pockets covering basically the entire surface of the protein with PocketDepth (Kalidas and Chandra, 2008) (among the top 10 pockets, one was correctly pointing the area between M39 and L72) or a short list of four top pockets with Fpocket (Le Guilloux et al., 2009) that were away from the region involved in the binding of the small ligand. These methods were not calibrated to investigate PPI pockets or small and superficial pockets. They may work in some cases but here failed when using a single apo IL-2 structure as input (i.e., to use these approaches with PPIs it is more appropriate to generate several conformations of the protein and to analyze some selected structures). Because protein–protein interaction inhibitors, as mentioned above, bind to pockets that are subdivided into several small subpockets and because hotspot residues involved in protein binding and in small molecule binding should be in general pre-oriented in space, one expects that energy-based approaches (that probe regions of the surface that could bind tightly small chemicals) applied on a single protein structure would be more efficient to find PPI pockets than geometric methods. Indeed, the FTMap tool (Kozakov et al., 2011) was used on the apo form and several subpockets were identified (regions surrounded by dashed lines with the small chemical probes/fragments displayed) that fully cover the binding site of the small ligand. A manual rotation of the F42 side chain was done using PyMol and was enough to open most of the binding pocket. At this stage, a structure-based screening procedure could be applied suggesting that at least, on this target, existing in silico approaches could be used.

3. Compound collections for PPIs and ADMET properties

As commented above, the pocket properties at protein–protein interfaces tend to be different from the ones observed on traditional targets and as such it is not surprising that hit rates obtained after experimental (Macarron et al., 2011) screening and in silico screening are not high for PPIs since screening collections contain essentially legacy compounds that were synthesized for traditional targets. Thus, the chemical structures that would be needed to efficiently hit PPIs appear to be under-represented suggesting that new collections dedicated to PPIs should be designed (Macarron et al., 2011). Depending on the goals (chemical biology or drug discovery), different types of in silico filters could be used (e.g., structural alert filters, physical-chemistry filters, etc. that can be combined with diversity filtering protocols) to prepare high quality compound collections (Dahlin and Walters, 2014; Macarron et al., 2011). This point, as seen below, represents an important challenge to the field, as LMW PPI inhibitors tend to have physicochemical properties that stand outside the boundaries usually well accepted in drug discovery. The earliest efforts to develop small protein–protein modulators were based on the mimicry of linear structural elements of the interacting partners and thus at compounds able to mimic beta-turns, alpha helices and beta strands (Cummings and Hamilton, 2010; Lao et al., 2014; Wilson, 2009). Such approaches are still valuable today (Azzarito et al., 2013; Fry et al., 2013; Lao et al., 2014; Watkins and Arora, in press) (yet possibly 50% of the protein interactions do not involve a linear epitope). Indeed, the greatest successes for HTS experiments have been for PPIs in which one helix of one protein binds into the groove of the interacting partner (e.g., the Bcl family, yet the interacting helix might be partially disordered when not bound to its partner) (Nero et al., 2014). This observation illustrates the potential of compounds mimicking such secondary structure elements (NB: there are different subcategories of secondary structures present at the interfaces, see for instance (Bullock et al., 2011)). Yet designing rationally such compounds is not simple. In general, the 3D structure of the complex is needed to design these compounds but the secondary structure element involved in the interaction that is visible in the crystal structure represents the end product of the binding process while in reality, in many cases, this secondary structure element may not be formed in the isolated protein but only appears in the presence of the partner (e.g., intrinsically disorder protein). In some cases, observation of the final 3D structure of the complex can be misleading for designing secondary structure mimics because some amino acids may appear of importance for the protein–ligand interaction while they are

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indeed important for the folding or nucleation of the secondary structure element (Yang et al., 2004).

Another possible way to prepare a collection dedicated to PPIs, as more and more LMW inhibitors are described in the literature and patent applications, would be to find molecular descriptors and properties of the small molecules that would be specific of this large essentially untouched target class (i.e., a general trend in the chemistry and shape of the small molecules that would make them well suited for binding at the protein interfaces). In order to explore these new regions of the chemical space, machine-learning methods could be applied. Indeed, three chemoinformatics studies making use of various training sets have been reported that describe statistical filters that should assist the rational design of compound collections dedicated to the inhibition of PPIs. A first decision tree was reported in 2007 underlining the importance in PPI inhibitors of a shape descriptor (Neugebauer et al., 2007). A second decision tree model was reported by Reynes et al. (2010) and led to the development of the PPI-HitProfiler filter which is available as a standalone script or online via the ADME-Tox filtering tool FAF-Drugs2 (Lagorce et al., 2011). The third study reported a statistical filter that was designed using support vector machine to discriminate PPI inhibitors from non-PPI inhibitors (Hamon et al., 2013) ($2P2I_{HUNTER}$).

Additional information about privileged scaffolds or substructures particularly well-suited to bind at the PPI interface or about physicochemical thresholds will come by investigating databases of PPI modulators. At present, there are three databases dedicated to PPI modulators that will greatly assist the process, the 2P2Idb (manually curated) (Basse et al., 2013), TIMBAL (Higueruelo et al., 2013b), and iPPI-DB (manually curated) (Labbe et al., 2013) (Fig. 3). Using these databases or smaller datasets, it was found that PPI inhibitors indeed differ from other regular target inhibitors, they for instance tend to be more lipophilic, larger, with more complex three-dimensional structure and more aromatic (and in turn these differences allow the use of machine learning approaches to develop statistical filters) (Higueruelo et al., 2013b, 2009; Morelli et al., 2011; Neugebauer et al., 2007; Pagliaro et al., 2004; Sperandio et al., 2010b; Villoutreix et al., 2008, 2012; Wells and McClendon, 2007). More specifically and to illustrate this point, known PPI inhibitors have in general a higher molecular weight (average MW of 540 Da for protein–protein inhibitors in the iPPI-DB versus 341 Da for regular drugs), higher log P (a mean value of ~4.6 in iPPI-DB was found while it is around 3.5 for enzyme inhibitors) and a more complex three-dimensional structure than typical drugs, underlining further the need of rationally designing the screening collections. Obviously, these properties do not apply to all PPI modulators as many (~60%) of the about 2000 PPI inhibitors analyzed (Labbe et al., 2013) comply with the rule of five (see below) (Bologa et al., 2006; Higueruelo et al., 2009; Labbe et al., 2013; Morelli et al., 2011; Sperandio et al., 2010b; Villoutreix et al., 2012).

Definitively, as we gain more knowledge about PPI modulators, it might be possible to develop smaller and less lipophilic molecules (for the time being many PPI inhibitors were found using generic compound collections not rationally designed for PPIs). This will be of major importance in drug discovery projects (and in some cases for chemical biology) because it is known that molecules displaying such properties tend to be difficult to administrate orally (the vast majority of patients prefer to take oral drugs as compare to invasive delivery methods (Verbrugghe et al., 2013)) or difficult to optimize or because they tend to interact with anti-targets. At this stage of the discussion, it is important to underline that they are many fiery debates about filtering or not molecules in compound libraries or for hit selection based on physicochemical analysis or related rules and we would like to summarize below several viewpoints.

The rule-of-five guideline makes use of computed physicochemical properties (a log P less than 5, a MW under 500, no more than five hydrogen bond donors and no more than 10 acceptors) to delineate a region of the chemical space where compounds are more likely to be orally absorbed (Lipinski et al., 2001) (about 85% of the FDA approved drugs obey this rule, considering two violations of the rule). By examining the rule-of-five and the physicochemical properties of PPI inhibitors, one could first think that designing oral PPI inhibitors is going to be very difficult. Yet, it is important to note that there are many exceptions to the rule of five and that a number of orally bioavailable drugs exist far beyond these physicochemical boundaries (Doak et al., 2014; Harvey et al., 2015). Many other rules have been developed following the rule-of-five concept, to predict not only oral absorption but also properties such as toxicity (Bohnert and Gan, 2013; Dahlin and Walters, 2014; Doak et al., 2014; Gleeson, 2008; Hann and Keseru, 2012; Leeson and St-Gallay, 2011; Ritchie and Macdonald, 2009; Taboureau et al., 2012; Ward and Beswick, 2014). For example, the 3/75 rule (related to *in vivo* toxicity in animal models) states that compounds with high lipophilicity (computed log $P > 3$) and low topological polar surface area ($TPSA < 75$) can have an increased risk of toxicity (about 6 times more likely to be toxic in short-term animal studies possibly due to increased interactions with off- and anti-targets) (Hughes et al., 2008). When analyzing oral drugs, we noticed that many approved drugs would not pass the rule and that the situation was worst for PPI inhibitors (Labbe et al., 2013). However, the 3/75 guideline and some others like molecular complexity defined as the fraction of sp3 carbons or Fsp3 suggested to correlate with success in drug development (Lovering et al., 2009), have been seriously challenged in a recent study by Muthas et al. (2013). In our opinion, the problem is not the rules but the misuse and over-interpretation of these guidelines. Definitively it would seem better to start a drug discovery project with a molecule having a MW under 400 and a low log P but on the other hand a too strict implementation of such rules will obviously result in lost opportunities as for instance, larger compounds could reduce their effective size and lipophilicity through hydrophobic collapse or by forming internal hydrogen bonds, thereby enhancing membrane permeability and possibly impacting the overall bioavailability (Alex et al., 2011; Bruncko et al., 2010; Ettorre et al., 2011; Faller et al., 2011; Zhao, 2011). At the same time, it is important to remember that the ("oral") chemical space is almost infinite (e.g., the recently reported virtual collection that contains over 166 billion compounds (Polishchuk et al., 2013; Reymond, in press; Ruddigkeit et al., 2012)) and that already in the rule-of-five and related LMW chemical space, there is a potential for diversity, complexity and novelty.

Taken together, we believe that it should definitively be possible to rationally design PPI inhibitors with balanced ADME-Tox properties, adequate potency and relevant selectivity by considering a soft implementation of the above-mentioned rules. For example, a recent study attempts to identify specific descriptors of PPI inhibitors that would not correlate with physicochemical and structural descriptors that are known to impede drug developments (Kuenemann et al., 2014). In that analysis, 84 inhibitors of PPI (from 2P2IDB (Basse et al., 2013) and the Protein Data Bank) and 1282 inhibitors of conventional targets (e.g., enzymes) called non-iPPIs (taken from PDBbind (Wang et al., 2004)) were collected and computationally compared in order to discover descriptors specific of protein–protein interaction inhibitors. Because the heavier and more hydrophobic nature of iPPIs is known to be a potential liability for drug development, the authors have imposed that none of the identified descriptors could correlate with the hydrophobicity or the size of the compound. The selection protocol (Fig. 4) has highlighted four 3D descriptors. The selected descriptors illustrate

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Fig. 3. The iPPI-DB. We have developed a database containing over 1500 inhibitors of protein–protein interactions (www.ippidb.cdithem.fr) (Labbe et al., 2013). This collection can be searched using various parameters, in the present example the user is looking for the IL2–IL2R system. The identified small molecules are returned with numerous annotations, ranging from physicochemical properties to the assay used to discover the compounds. By clicking on one molecule, here compound 20, many additional data are displayed, like the position of the molecule in a PCA plot computed for all the compounds of the database. The next 2015 release of the iPPI-DB will contain more molecules and additional functionalities to search the compounds as well as additional annotations.

either the layout of the hydrophilic or hydrophobic interacting regions (Volsurf descriptors (Cruciani et al., 2000): EDmin3, IW4 and CW2) or the shape (globularity) of the compounds (Fig. 5). The most important descriptor identified by this analysis was EDmin3 (local interaction minimum or EDmin3 is the third lowest local minimum of the interaction energy in kilocalories per mole of a probe). For PPI inhibitors, it illustrates how efficiently iPPIs manage to bind hydrophobic patches often present at the PPI interfaces (i.e., regions that usually contain hotspot residues). It also points out that it is not so much the number of hydrophobic groups in the compounds that will favor hydrophobic binding but rather their 3D layout within the compound. All of these descriptors were then confirmed as specific on larger dataset (selected from iPPI-DB (Labbe et al., 2013), a subset of BindingDB (Liu et al., 2007), and eDrugs3D (Pihan et al., 2012)). Interestingly, the very limited correlations of these properties with hydrophobicity and the size of the compounds open new avenues for the rational design of more "drug-like" iPPIs with better binding efficiencies.

Beside the above-mentioned physicochemical properties that are important to consider when designing a compound collection or selecting a hit, other parameters such as the presence of structural alerts (toxicophores) (Benigni and Bossa, 2011) must be born in mind, for drug discovery projects and often for chemical biology. In addition, it has been noticed that artifact compounds (e.g., PAINS, pan assay interference compounds and/or promiscuous molecules) are reported at a growing rate (Baell and Walters, 2014; Baell and Holloway, 2010; Devine et al., 2015; Schorpp et al., 2014; Taboureau et al., 2012; Whitty, 2011) and it thus seems important to search for the presence of such structures in a collection using in silico approaches (e.g., this can be done with the new version of the online tool FAF-Drugs (manuscript in preparation and Lagorce et al. (2011))). But here again, just like for the rules that monitor some physicochemical properties, it is important to remember that there are exceptions to these guidelines and many limitations in our present knowledge with possible exaggerations in the observed correlations (Mendgen et al., 2012; Mok et al., 2013; Muthas et al., 2013). The critical point here is that guidelines should not be considered as commandments as unfortunately seen more and more in many blogs and research papers.

4. Outlook

In this review we have highlighted several critical aspects about the peculiarity of binding pockets for small ligands found at the protein–protein interfaces with a special emphasis on hotspot residues and the fact that many of them seem to have the same conformation in the apo and holo form (pre-orientation of many side chains in space prior to the binding event). These residues tend also to be involved in small molecule binding suggesting that designing compounds that interact with these hotspot residues is a valuable approach to inhibit protein–protein interactions. We also noted that in silico binding pocket prediction tools could be applied even on the apo form when the 3D structure of the target is available. This also suggests that although virtual screening approaches will have to be tuned to PPIs (improve both docking and scoring), existing methods can already be used with a reasonable

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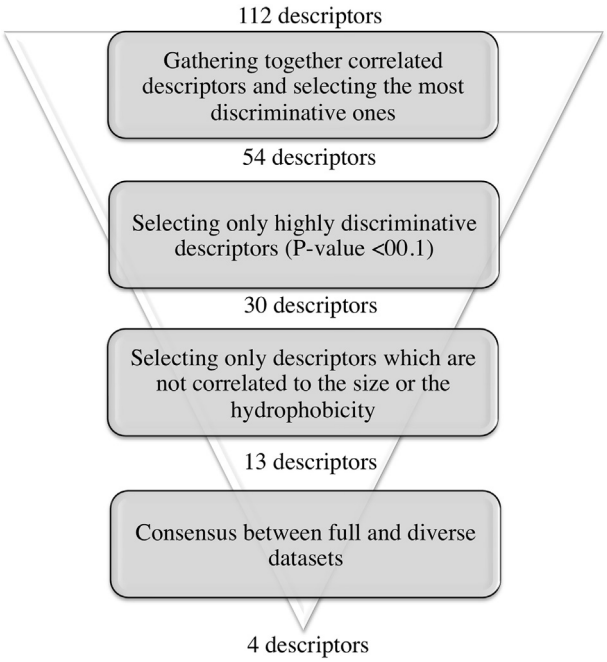

Fig. 4. Prediction of protein–protein interaction inhibitors with improved physical-chemistry properties. The protocol for the selection of descriptors specific of protein–protein interaction inhibitors. First step: group together all correlated descriptors (with a Pearson's correlation coefficient < −0.9 or a Pearson's correlation coefficient > 0.9) and pick the most discriminative descriptors from each group according to the comparison test. Second step: choose only highly discriminative descriptors (P-value < 0.01). Third step: pick only descriptors not correlated (−0.6 < Pearson's correlation coefficient < 0.6) with hydrophobicity investigated using log P and TPSA computations or the size (here considered as MW and the number of non hydrogen atoms). Fourth step: select only descriptors obtained for the full data set and a chemically diverse version of the same data set.

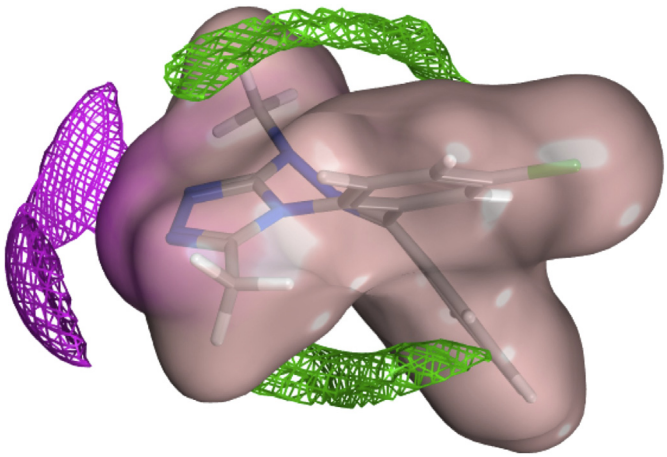

Fig. 5. Properties for ideal protein–protein interaction inhibitors. The bioactive conformation of 08K co-crystallized with Bromodomain-containing protein 4 (PDB code 3U5L) represents an "ideal iPPIs" according to the investigation protocol shown in Fig. 4. The ideal iPPIs tend be more globular (glob), with a stronger capacity to bind hydrophobic patches (EDmin3), a lower proportion of exposed polar groups (CW2) and a concentration of these polar groups to one extremity of the compound (IW4). Transparent molecular surface and molecular sticks colored by lipophilicity (polar regions in magenta) represent the compound. The values for the four descriptors identified are: a high IW4 descriptor of 5.21 (represented by the magenta molecular field calculated using MOE version 2012.10 at the levels of energy = −4 kcal/mol using a water probe), a high glob (descriptor) = 0.23 (represented by the molecular surface), a low EDmin3 descriptor of −3.32 kcal/mol (represented by the green molecular field calculated using MOE version 2012.10 at the levels of energy = −2.4 kcal/mol using a dry, lipophilic, probe), and a low CW2 descriptor of 1.83 (represented by the proportion of pink surface over the full molecular surface).

chance of success. We and others have indeed identified LMW compounds that inhibit protein–protein interactions by combining various experimental and in silico approaches (see some recent studies or reviews (Arkin et al., 2014; Buchwald, 2010; Falchi et al., 2014; Gautier et al., 2011; Gowthaman et al., 2013; Jin et al., 2014; Kruger et al., 2012; Lage, 2014; Mazuc et al., 2008; Milroy et al., 2014; Nero et al., 2014; Rognan, 2015; Sperandio et al., 2014; Starzec et al., 2014; Villoutreix et al., 2014, 2011; Villoutreix and Sperandio, 2010; Voet et al., 2013; Watkins and Arora, in press; Zhang et al., 2014a)). A rapid survey of PubMed indicates that these last 2 years, about 50 studies report the identification protein–protein interaction inhibitors using such a combined silico–vitro approach, suggesting that after optimization, new clinical candidates could appear in the coming years. A financial report analyzing drug candidates in clinical trials indicates that recently developed LMW PPI modulators may have a higher probability of success in moving from one clinical phase to the next as compared to other new chemical entities (Meier et al., 2013). Overall, the future of PPI seems bright and the many ongoing methodological and conceptual developments worldwide (from PPI network with implementation of structural information, new pocket detection engines, better handling of interface plasticity, optimization of scoring functions, new biophysical methods to exploration of the chemical space) should both continue to enhance our understanding about the importance of PPIs in the health and disease states and assist the conception of novel drugs.

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