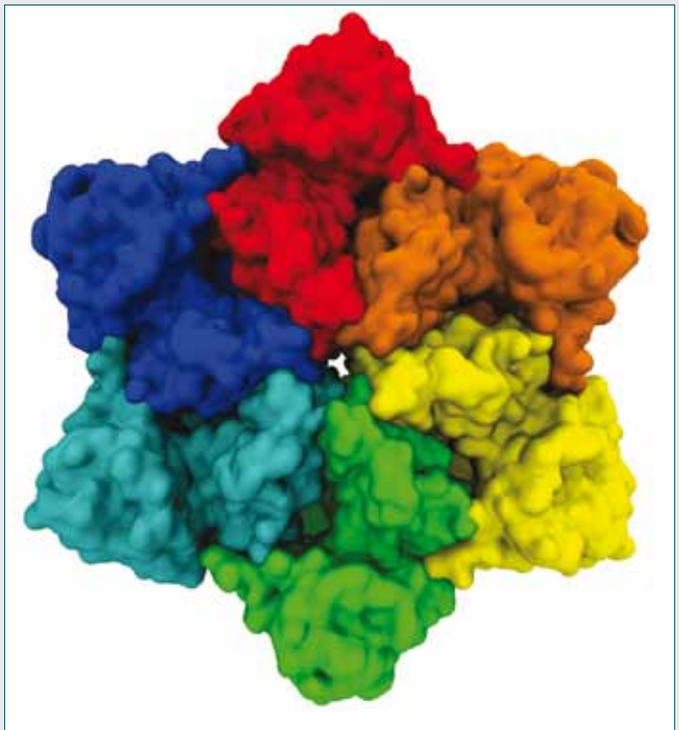

Crystallographic structure of an hexameric proteic complex

Stefano Pieraccini
Dipartimento di Chimica
Università di Milano
CNR-ISTM, Milano.
INSTM - Unità di Ricerca di Milano
stefano.pieraccini@unimi.it

PROTEIN-PROTEIN INTERACTIONS AS A DRUG TARGET: A MOLECULAR MODELING APPROACH

This review is devoted to the computer aided design of drugs targeting protein-protein interactions (PPIs). General features of PPIs are discussed in the introduction, then the problems of protein interfaces druggability and design techniques application are addressed, and finally a case of active peptides design is described.

Introduction

Proteins are fundamentals elements of the cellular machinery, being responsible for diverse functions such as enzymatic catalysis, cell signaling and transport as well as immune response and processes concerning the integrations of cells into tissues. Most often this wealth of functions is not exerted by proteins acting as isolated units, but rather by multimeric complexes of proteins interacting with one another. It appears therefore evident that the study of structural and dynamical aspects of protein-protein interactions (PPIs) has a paramount importance in the understanding of many physiological and pathological processes at the molecular level, also in the perspective of the development of novel drug molecules. Protein-protein complexes may be classified into permanent, or obligatory, and transient [1, 2]. Permanent complexes are formed by proteins that can function only when associated in the complex. Transient complexes on the other hand are continuously forming and dissociating and the monomers are in equilibrium with the associated complex. The Gibbs free energy of complex formation, also referred to as binding energy, can be evaluated from the equilibrium constant of the reaction of complex formation (K_a) or dissociation (K_d).

A dissociation constant K_d in the micromolar range for example corresponds to a ΔG of the order of 6 kcal/mol, while a K_d in the picomolar range corresponds to a ΔG of roughly 19 kcal/mol.

Proteins interact with one another through their surfaces, thus a great attention has been devoted to the analysis of the geometrical, structural and dynamical aspects of protein surfaces mediating PPIs. The aminoacidic composition of protein surfaces involved in PPIs has been investigated, analyzing the structure of protein complexes which have been resolved with X-ray diffraction techniques, and it was found to be on average enriched in hydrophobic amino acids with respect to an ordinary external protein surface [3]. This is especially evident in obligatory interactions, where protein interfaces have a composition very similar to protein interiors [4, 5], while in transient complexes this feature is less evident [3, 6]. The contact surface between two proteins forming a complex has an average area in the range 1500-3000 Å^2 [3, 7]. This surface corresponds to the protein portion that is excluded from solvent upon binding, and it is also known as buried surface. It would be tempting to infer a strict relation between the area of the buried surface and the strength of the binding between the protein partners, but in general it has been shown that the correlation between these two quantities is very week [8].

One could wonder if all residues at the protein-protein interface contribute in a roughly equal measure to the binding energy or if some of them give a dominant contribution. Experimental mutagenesis data have strongly contributed to shed light on the detailed anatomy of PPIs and to answer this key question. A typical approach is the so called alanine scanning [9], where residues located at the protein-protein interfaces are mutated into alanine and the difference in binding free energy ($\Delta\Delta G_b$) is measured between the wild type and the mutated complex. These experiments showed that only a small fraction of the residues at the interface, around 10%, shows $\Delta\Delta G_b$ values greater than 2 kcal/mol, while most of them exhibit very low $\Delta\Delta G_b$ [10, 11]. Exhaustive experimental screening of PPIs through alanine scanning is unfortunately very time consuming and expensive, so only a limited amount of data is available, which are collected in public databases such as the BID database compiled by Bogan and Thorn [10]. Residues whose mutation leads to a significant $\Delta\Delta G_b$ reduction in a complex are called hot spots. Hot spots are usually conserved more than the average residues at the interface, due to their biological function [12, 13].

Moreover, structural investigations have shown that packing along the interfaces is not homogeneous, with hot spot residues being preferentially located in more tightly packed areas [14].

A strong correlation between packing density and $\Delta\Delta G_b$ of alanine scanned residues has been found [15].

This aspect further justifies their conservation, because mutating an amino acid in a closely packed region would lead to hollows or sterical clashes depending on the relative size of the original and the mutated amino acid.

The main driving force for the formation of PPIs is hydrophobic interaction, but also salt bridges and hydrogen bonds networks make a significant contribution [16-19].

Bogan and Thorn suggested that hot spots residues are preferentially located in the interface area excluded from solvent, defined as core region, and are surrounded by an “O-ring” like structure of hydrophobic residues shielding them from water, known as rim region [20]. The relative conservation of residues in these two zones of the interfaces have been evaluated, leading to the observation that residues entropy, i.e. propensity to mutate, is higher in rim than in core regions [21].

The simple O-ring model very well explains the fact that, albeit most hot spots are large hydrophobic residues like tryptophan, tyrosine and phenylalanine, also charged residues forming buried salt bridges are often recorded. We noted above that hot spots are located within tightly packed regions, where water molecules are easily removed in the binding process, allowing a strong electrostatic contribution of the charge-charge interaction. This suggests that interface charged and polar residues can strongly interact through water exclusion mechanism, which creates a dry environment around them [22]. It has been also proposed that charged residues actually increase the rate of protein-protein association without affecting the complex stability [23].

Hot spots are not randomly distributed on the protein-protein interface, rather they tend to occur into clusters [14, 24]. Within each cluster, hot spots are tightly packed and form a network of contacts with one another building up a “hot region”.

Keskin and coworkers developed an innovative view of PPIs starting from this concept, describing the binding interface as consisting of different regions comprising amino acids strongly contributing to the complex stability [22]. This picture implies that the contribution of hot spots within the same hot region to the complex stability is cooperative, while hot spots located in different hot region contribute additively to complex stability. In other words, hot regions are independent of one another.

In this review we shall see how these general features of protein interfaces influence the possibility of exploiting PPIs as drug targets and how this has been done so far, focusing on the use of computer aided approaches relying on structural information and aiming at the design of peptides and peptidomimetics as drug candidates.

PPIs as drug targets

Many physiological and pathological processes are mediated by PPIs, which therefore are a possible target of drug molecules. In principle a PPI can be exploited as a drug target either reducing its strength, thus impairing protein complex formation, or increasing its strength to stabilize the complex.

It depends on the nature of the process mediated by the PPI and on the therapeutic aim which is the suitable strategy to adopt in each case. While PPIs stabilizing agents has recently gained a great atten-

tion [25], most active molecules developed so far for targeting PPIs act as inhibitors of the targeted interactions [26-28], and we focus on this class of molecules.

We try here to outline the main aspects that differentiate PPIs targeting with respect to the exploitation of more traditional drug targets such as enzyme binding sites [29]. First of all the typical contact surface between interacting proteins is very large compared to the size of an enzyme active site, so at first glance it cannot be easily targeted by small, drug like molecules. Second, most protein contact surfaces appear to be flat, missing deep, well defined binding pockets which could host drug molecules.

These two points seem to impair the development of PPIs targeting drugs with enough potency and selectivity, but if we carry on an in depth analysis of the problem starting from what is known about the structure of protein-protein interfaces, it appears that the main obstacles can be overtaken. We have seen in the introductory remarks that, while protein contact surfaces are large, only a small fraction of residues, the hot spots, actively contributes to the binding energy of the complex. Moreover, these residues are clustered together into independent hot regions, comprising strongly interacting residues located on both binding partners.

Considering that only few hot regions are found in the interface between two proteins, in general from one to three, it is sufficient to target one of them to greatly affect the complex stability. It is not necessary then to target the whole surface to obtain a relevant effect, but only a small subsection of it, whose size is not critically larger than a standard drug interaction site [30].

Regarding the flatness of proteins interaction surfaces, detailed structural analysis revealed a more complex picture, where surfaces are not at all fully flat, but rather studded with grooves, crevices and indentations [31], albeit less evident at first sight than enzyme active site pockets targeted by conventional drugs.

Surface pockets, depending on their size and depth, can be filled with water in the unbound protein. Upon complex formation, water molecules in these pockets may be replaced by complementary shaped protruding segments of the partner protein or remain unfilled. Complemented pockets disappear upon binding, producing tightly packed sections of the interface, while unfilled pockets give rise to more flexible, soft regions.

It has been observed that hot spots are located in tightly packed interface regions, and they are actually more likely to occur in filled rather than in unfilled pockets. Interestingly, in a study by Li *et al.* most complemented pockets have been detected both in the complex and in the apo-protein, suggesting that these hot spots rich structures are pre-organized in the separated monomers, prior to complexation [32]. Complementary pockets structures therefore confer rigidity to unbound proteins segments and minimize the entropic cost of binding, while surrounding areas conserve flexibility. It's worth noting at this point that pockets not only are present on protein surfaces, but they are likely to be located in the hot regions, exactly those regions in which we are interested in targeting. Also non complemented pockets are worth to be considered in the drug design process. Upon complex formation they are associated with less densely packed interface areas, where a drug molecule could not only mimic the interactions between the two protein forming the complex but also more easily establish new ones with the residues building up the pocket, resulting in a tighter binding [33]. Moreover, as water molecules are present in such cavities, the potency of this kind of inhibitors could be enhanced designing them in such a way that upon binding they displace some key water molecules, leading to a net entropic gain.

Protein surfaces are inherently flexible. This leads to rearrangements upon complex formation which can result in significant differences between the structure of complexed proteins and of the apo-proteins. Sometimes protein segments making up the interface can be disordered in the isolated monomer or one of the binding partners can be intrinsically unstructured [34], which make the design of PPIs inhibitors very difficult, but in most cases a limited protein surface flexibility represents an opportunity for the drug designer.

For example, small molecule inhibitors of the IL2-IL2Rα interaction bind to cavities that are not detected neither in the complex nor in the IL2 apo-protein [35], but can be observed to transiently appear and are stabilized by the binding of the drug [36, 37]. The use of long molecular dynamics simulations to monitor transient pocket formation on protein surface has been suggested as a method for locating potential target sites not evident from crystallographic data [38], which can host drug molecules able to affect PPIs by altering the protein surface dynamics and impairing the complex formation. To this purpose recent efforts have been devoted to the development of software for the accurate detection and measurement of cavities on protein structures [39, 40].

Targeting PPIs is far from a trivial task, and cannot be tackled with routine tools, due to the great variability and peculiarity of protein surface character. However, as compared with the early days, where largeness and flatness of interfaces were considered as almost insurmountable difficulties, the current *not so large, not so flat* view of PPIs targets is rather encouraging.

A decision tree has been suggested by Chéne [33] which helps in the evaluation of the druggability of a PPI. It is focused on the development of PPIs inhibitors acting at the protein-protein interface and considers four criteria. The first one is the availability of 3D structural information on the complex to be targeted, in order to make easier the drug design process. The second criterion is the presence of cavities on the interface of interest. The third is the size and polarity of the cavities. Hydrophobic pockets are preferred because they allow the design of lipophilic inhibitors, and the size should be large enough to host molecules with a surface of the order of 150-500 Å^2, but not so large to have scattered interaction sites not targetable by a molecule of reasonable size at the same time. The last one is the shape complementarity of the interacting subunits within the cavity.

In this author's opinion the most favorable case is that in which both chains are not densely packed and make few direct interactions within the cavity. This would make it possible to design a molecule fitting the cavity and establishing new contacts in addition to the interactions mimicking those present in the protein-protein complex. This last point, while valid in principle, in our opinion should not lead to an underestimation of the possibility of developing potent and selective drugs binding in complemented pockets which are statistically rich of hot spots residues.

Different approaches are known to discover molecules able to target PPIs, the most widely used are high throughput screening (HTS) [41-44] and peptide based procedures [44-47]. HTS of synthetic and natural compounds libraries, a traditional drug discovery approach, revealed to be very efficient in finding PPIs targeting hit and leads compounds, and is the only viable approach when no structural data are available about the target of interest. For a successful HTS it is basilar to use compounds libraries with a high degree of chemical complexity and diversity, in order to maximize the chance of identifying suitably potent and selective ligands. In particular, chemical diversity has shown to be more critical than the mere size of the library in determining the success of the search of hit molecules [48]. Most chemical libraries currently in use were developed to tackle traditional targets and sample a chemical space not optimal for finding PPIs inhibitors, so the development of more specific libraries is necessary to overtake this limit [49]. Natural compounds, which have recently regained interest as a huge resource for drug discovery as they exhibit a great variety of 3D scaffolds, are becoming a valuable source of molecules targeting PPIs [50].

Peptide based approaches, on the other hand, rely on structural knowledge of the protein-protein complex whose formation should be modulated. Typically, an analysis of the protein-protein contact surface leads to the location of protein subsequences that are the basis for the synthesis of peptides with potential lead character. Despite the numerous drawbacks of peptides as drugs, especially their large mass, poor pharmacokinetic and pharmacodynamics properties, limited bioavailability and chemical instability, large efforts were made to chemically improve their properties and to develop drug-like peptidomimetic molecules. Peptides shorter than 10-12 amino acids naturally enter in the cells [29] but are often unstructured, lacking the features needed for target recognition. To overcome this problem Verdine and co-workers proposed the synthesis of hydrocarbon stapled peptides [51] which show well folded stable structures. The choice of the length of the stapling chain can be also useful to fine tune the desired structure of the peptide [52]. For larger peptides, whose major problem is not the lack of correct folding, different modifications have been proposed to improve their pharmacodynamics properties. For example, D-α-peptides and retro-inverso peptides showed enhanced cell permeability as well as metabolic stability [29]. The P53-hdm2 interaction was one of the PPI to be first considered as a drug target. It is mediated by an α-helical segment of P53 which is hosted by a pocket on the surface of hmd2 [53, 54]. A peptide corresponding to this aminoacidic sequence has been found to bind to hmd2 and to inhibit the interaction between the two proteins [55].

This peptide, due to its small size, is not stable in solution, but only when bound to the target protein [56, 57]. Due to the therapeutic interest of P53-hmd2 PPI inhibition [58, 59], large efforts have been done to stabilize the pharmacophoric structure comprised in this peptide and to develop α-helix mimetics which could be potent and selective inhibitors of this interaction. An interesting study reported the design and synthesis of a β hairpin cyclic peptide reproducing the active epitope geometry of the original α helical peptide [60]. Hydrocarbon stapled peptides have been also tested [61]. On the other hand, different classes of peptidomimetic compounds have been developed to this purpose [62], and among them an interesting group of triphenylic compounds emerged as a scaffold for α-helix mimetics. These scaffolds have been widely studied from the modellistic [63] as well as the experimental viewpoint and new chemical libraries have been developed [64].

In the next section we shall discuss how molecular modeling techniques can be used to screen protein-protein interaction surfaces and to locate protein subsequences that can be used to design PPIs inhibiting peptides. The application of these techniques to the design of antimitotic tubulin targeted peptides that we have recently studied in our labs will be then discussed as an example.

Computer aided localization of hot spots

We stated that residues located at a protein-protein interface are usually considered hot spots if the change in the binding free energy of the complex ($\Delta\Delta G_b$) upon mutation into alanine is higher than 2 kcal/mol [10]. The experimental estimation of the binding free energy may be a difficult task to achieve and an experimental mutagenesis study, that could in principle test the contribution of each residue to the stability of a protein-protein complex, is very time consuming and has a high cost.

A few computational approaches allowing the estimation of the $\Delta\Delta G_b$ associated to the mutation of the interfacial residues in alanine have been developed. They are very powerful tools for the study of PPIs and are referred to as Computational Alanine Scanning (CAS) methods. Different computational methods have been developed to estimate free energy differences. Free energy perturbation [65] and thermodynamic integration [66] allow to obtain accurate estimates, but are computationally time-consuming and are not suitable for the study of the effect of a large set of mutations in a protein complex. More computationally affordable yet reliable methods have been developed to tackle this difficult task.

These methods, referred to as MM-PBSA or MM-GBSA, combine the molecular mechanical energies in gas phase, the Poisson-Boltzmann or the Generalized Born approach to evaluate solvation energy, and an empirical function to take into account the contri-

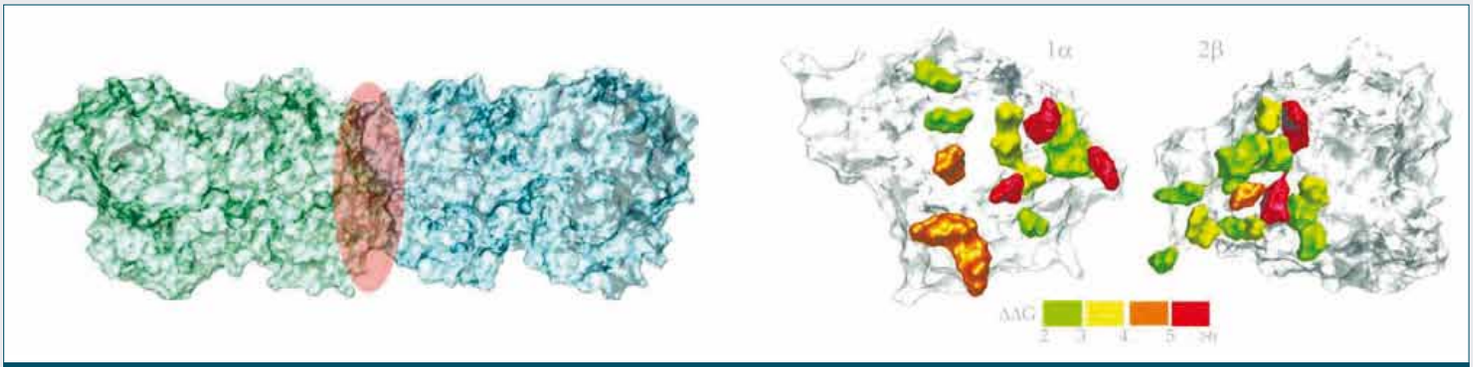

Fig. 1 - The interface between the α subunit of one dimer (green) and the β subunit of a second dimer (blue) is highlighted in red (left panel). The amino-acid residues critical for microtubule stability are distributed evenly between the α and β subunits and are complementary. Different colors, from green to red, refer to the variation in the binding energy of the complex on mutation of each of the hot spots identified

bution of protein surface exposure to solvent. They perform an *a posteriori* evaluation of binding free energy on snapshots extracted from a molecular dynamics trajectory to evaluate the total binding free energy between two proteins forming a complex. The MM-GB-SA approach uses a thermodynamic cycle to calculate the binding free energy between two protein subunits A and B in solution. Many reviews are available concerning the theory of MM-PBSA and MM-GBSA methods for evaluating free energy of binding and PPIs [67, 68], so we shall focus here on some key points of the protocols usually employed.

In principle three molecular dynamics simulations should be run to calculate binding free energy, one for the complex and one for each of the monomers in order to sample their conformations. Actually, if the conformations of the isolated monomers do not differ too much from their conformations in the complex, which is often the case, a single MD trajectory of the complex can be computed, and conformations of the monomers can be extracted from the same trajectory. This approach, when the structural modification upon binding are not extensive, is accurate and has the advantage to be less computationally expensive and to give results with a lower statistical uncertainty [69].

The calculation of the binding free energy allows to perform a CAS analysis of the protein-protein interface, in order to highlight the hot-spots. The calculation is performed by evaluating the binding free energy between the protein subunits making up the complex (ΔG_{wild}) and the binding energy upon mutation of each interfacial amino acid into an alanine ($\Delta G_{mut,X}$), so obtaining, for each residue, a $\Delta\Delta G$ value defined as:

$$\Delta\Delta G_X = \Delta G_{mut,X} - \Delta G_{wild}$$

In this way, it is possible to evaluate the contribution of each amino acid to the binding free energy, highlighting the hot spots, for which $\Delta\Delta G_X$ is higher than 2 kcal/mol [70, 71].

Also in this case in principle two MD trajectories should be computed, one for the wild type and one for the mutated complex. This would require to perform a different MD simulation for each of the point mutations to be sampled in the complex, leading to a substantial number of simulations for a large surface, which can easily contain one or two hundred residues, even if only a minor part are hot spots. It is possible to make the hypothesis that a single point mutation does not significantly affect the structure of a protein or of a protein-protein complex.

This is a strong approximation, because at least locally the protein structure may be modified by the substitution of an amino acid, but in general it is rather accurate. It is therefore possible to use the so-called single-trajectory approach to perform the computational alanine scanning.

According to this procedure, a single molecular dynamics simulation is performed on the protein complex, then a set of snapshots is extracted from the trajectory for the ΔG_{wild} evaluation.

Subsequently on the same snapshots point mutations are introduced one at a time and the $\Delta G_{mut,X}$ is calculated for each of the amino acids at the protein-protein interface, and $\Delta\Delta G_X$ is easily obtained [72]. The single trajectory approach proved to be rather accurate considering the strong approximations on which it relies, but sometimes it gives too high $\Delta\Delta G_X$ especially for hot spots involved in buried salt bridges. In this case a two trajectory protocol can be considered if quantitatively accurate results are needed [73]. Finally, it is worth mentioning the problem of calculating the entropic contribution to the binding ΔG.

While the molecular mechanics energy term can be easily obtained from the results of a molecular dynamics simulation, the entropic term is often difficult to achieve.

The entropic term can thus be approximated to ΔS_{vib}, i.e. the contribution due to the internal vibration, whose calculation is nonetheless usually time-consuming and can be performed only on part of the protein-protein complex [74]. The relative contribution of the change in conformational entropy to the $\Delta\Delta G$ is considered to be negligible for the mutational studies, since it is supposed to cancel up when calculating it in the native and in the mutated complex [69]. In the next section we discuss an example of the computational analysis of a protein-protein interface aiming at the development of peptides inhibiting the associated PPI.

Computer aided design of tubulin targeted peptides

As an example of the development of peptides targeting PPIs we consider here the *in silico* design of tubulin targeted peptides aimed at exerting antimitotic activity. Tubulin is a heterodimeric protein formed by two monomers with a high homology degree, which are defined as the α and the β subunits.

Tubulin (α,β) dimers self-assemble in a head-to-tail fashion to form protofilaments and microtubules [75]. Microtubules are the target of several anticancer drugs that exert their cytotoxic action during the cell division process [76]. In a recent study we focused on the structure of the protein-protein interface between tubulin dimers and mapped the interactions stabilising microtubules [77]. We used a model system formed of two dimeric units aligned longitudinally (Fig. 1), as in natural tubulin protofilaments.

The protein-protein interface between the α and the β subunits of the two different tubulin dimers in our model has been defined as the ensemble of residues with a non-zero difference in solvent accessible surface area in the tetrameric versus the dimeric unit and comprised 176 residues. Computational alanine scanning was performed on each of these residues with the previously described MM/GBSA approach using 200 snapshots extracted from a 2 ns long MD trajectory. Analysing the results, it appears that the binding energy is not evenly distributed on the protein-protein interface (Fig. 1), but is confined to some critical amino acids.

Moreover, these residues show a tendency to be grouped in small clusters, close to one another in the 3D structure and often in the amino acidic sequence, forming hot regions as those described in the introductory section.

Drawing on the observation of the energetic and structural aspects of the protein-protein interface, with a special focus on the hot regions, three peptides were identified containing at least five hot spots each. Two of them, named Plug-F and Plug-H, are located on the α subunit and are composed of residues ranging from Leu_{248} to Leu_{259} and from Trp_{346} to Val_{353}.

They correspond to the T7-H8 and S9 tubulin segments respectively. The third peptide, Plug-X, involving residues from Phe_{389} to Gly_{400}, corresponds to the H11' region and is located on the β subunit (Fig. 2).

We inferred that these peptides could interfere with tubulin polymerisation via competitive binding to the microtubule plus end and/or to the isolated dimers.

These peptides correspond to tubulin subsequences, where only minimal modifications were introduced due to synthetic convenience.

The binding capability of an isolated peptide is not guaranteed in principle, even if it contains several hot spots, because it may undergo major structural rearrangements when removed from the parent protein and because of the complexity of the interactions network leading to protein aggregation. To investigate if the identified peptides retained their ability to bind to tubulin even when extracted from their protein environment, three control molecular dynamics simulations were run for each tubulin-peptide complex.

We found that in two cases, namely Plug-H and Plug-X, the structure of the peptides underwent only minor structural modifications compared to the structure of the corresponding protein segments in full length tubulin.

Moreover, we observed that the hot and warm spots characterised in the tetramer were generally conserved in the peptides. We infer from these data that either Plug-H or Plug-X could retain tubulin binding ability and therefore affect tubulin polymerisation. On the other hand, when Plug-F was simulated in complex with tubulin, it showed major structural changes, losing the helical structure observed in full-length tubulin and separating from the protein. Consequently it lost four out of the five hot and warm spots initially present. Plug-F behaviour, opposite to that of the other two peptides under consideration, suggested that it cannot strongly interact with tubulin on its own and is likely unable to interfere with its polymerisation.

The reliability of the computational alanine scanning data was further confirmed by a phylogenetic analysis on the α and β tubulin subunits. It was performed on protein sequences from 150 different organisms, and the degree of conservation of the single residues among the different species was evaluated by a normalised score function. We focused our attention on residues at the protein-protein interface by inferring that residues crucial for protein function, in this case for tubulin assembly to form functional microtubules, should be more conserved than non-influent ones. The ΔΔG of interfacial residues correlated well with the conservation score. Notably, 28 out of 32 hot spots (residues with a ΔΔG≥ 2 kcal/mol), showed a degree of conservation greater than average, and the few exceptions could be explained in terms of conservative mutations.

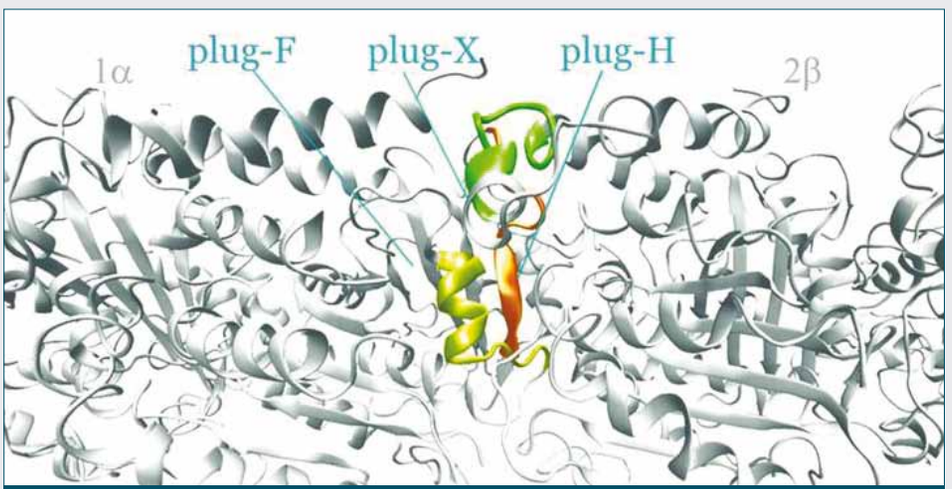

Fig. 2 - The three peptides corresponding to hot regions at the protein-protein interface are highlighted, plug-F in yellow, Plug-X in green and plug-H in orange respectively

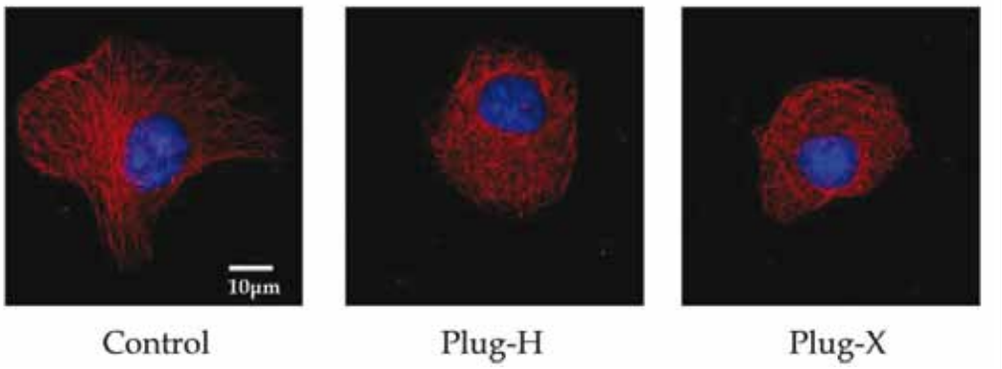

Fig. 3 - Microtubule organization in human lung carcinoma cell line A549 exposed for 24 h to different treatments. Microtubules are revealed by immunofluorescence localization of α-tubulin (red). Nuclei are stained by DAPI (blue). Control cells (left panel). Cells exposed to 50 μM Plug-H (central panel). Cells exposed to 50 μM Plug-X (right panel)

The biological activity of the designed peptides was assessed *in vitro* using purified tubulin and cultured cells. It was found that Plug-X and Plug-H, but not Plug-F, affect tubulin polymerisation. We calculated the apparent first-order rate constant of elongation and the steady-state extent of assembly.

Both the rate of elongation and the extent of assembly were heavily reduced by plug-H and plug-X, while plug-F did not modified tubulin polymerization kinetics with respect to the control.

Subsequently, two scrambled peptides derived from Plug-X and Plug-H, named Plug-Xs and Plug-Hs respectively, were tested as control, and did not affected neither the microtubules elongation rate nor the mass concentration of the tubulin polymer, as expected. Noticeably, the activity or inactivity of the peptides in inhibiting tubulin polymerisation parallels their tubulin binding ability as deduced from MD simulations. The two active peptides were further tested to evaluate their effect on tubulin critical polymerisation concentration. We determined the critical concentration of tubulin to be 9.52 μM with tubulin polymerised in the absence of peptides, but 14.54 μM and 13.67 μM with tubulin polymerised in the presence of Plug-X and Plug-H, respectively. Therefore, Plug-X and Plug-H competitively affect tubulin assembly *in vitro* by decreasing the elongation rate and increasing the tubulin critical concentration.

Finally, the ability of the selected peptides to interfere with tumour cells proliferation was tested employing lung adenocarcinoma A549 cell line as model system. IC_{50} values of 184.3±12.3 μM and 197±11 μM were derived for Plug X and Plug H, respectively. Plug-F and the two scrambled peptides Plug-Xs and Plug-Hs showed no significant cytotoxic activity at any of the tested concentrations. IC_{50} values of the order of 200 μM are obviously not enough to envisage the use of the selected peptides as drugs, but they are interesting if we consider that the plug-H and plug-X were simply "cut" from the tubulin sequence and tested, without any prior structure optimization. Due to their limited size, it is likely they can overcome the cell membrane. Indeed, confocal microscopy analysis of the treated cells showed evident microtubule network alteration, strongly supporting the peptide intake as well as the proposed mechanism of action (Fig. 3).

Plug-H and Plug-X are thus good candidates to be the basis of new optimized peptides and peptidomimetics with antimitotic activity.

Conclusions

Here we outlined a brief summary of the peculiarities of PPIs as drug targets and focused on a target centric approach to develop PPIs inhibitors. Describing the design of tubulin targeted peptides we showed that, starting from the structure of a protein-protein complex, it is possible to use molecular modeling techniques to study the network of interactions at the protein-protein interface that allow the formation of a protein complex.

Once the protein binding epitopes have been defined, if that region is a contiguous part of the protein, peptides can be synthesized to match the identified motifs.

These peptides will ideally conserve their structure and their binding ability, thus inhibiting the formation of the complex. This procedure has been defined "second nature drug design" [78].

If the structure of the peptide was not conserved, different strategies for its stabilization and restrain are available. Moreover, non peptidic molecules acting as peptidomimetics can be designed to overcome the drawbacks of molecules of peptide nature, which remain a good starting point for subsequent development.

Stefano Pieraccini was born in Genova on 10th July 1978. He got his degree and Ph.D. in chemistry at the Università degli Studi di Milano, where is currently assistant professor in the chemistry department.
His research field is related to the use of molecular modeling techniques to study biomolecules. His research activity is focused on protein-protein interactions modeling using molecular dynamics and free energy calculation techniques and on the design of peptides and small molecules able to modulate pharmacologically relevant protein-protein interactions. He is also presently involved in the computational study of protein and membrane stability in osmolyte solutions.

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RIASSUNTO

Farmaci modulatori delle interazioni proteina-proteina: un approccio modellistico molecolare

Questo articolo è dedicato al progetto di farmaci modulatori delle interazioni proteina-proteina. Alcuni aspetti generali delle interazioni tra proteine sono discussi nell'introduzione, viene poi affrontato il problema dell'utilizzabilità delle interazioni proteina-proteina come bersaglio farmacologico ed infine è descritto un esempio di disegno di peptidi attivi.