Inhibiting Protein-Protein Interactions as an Emerging Paradigm for Drug Discovery

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Abstract: Association of proteins into homo- and hetero-oligomers plays an important role in a plethora of biological phenomena. Inhibition of these interactions is increasingly recognized as a valuable new direction in drug design. In this mini-review we consider inhibition of protein misfolding and aggregation, molecules that disrupt enzyme quaternary structure, and signaling inhibitors, as emerging drugs.

Key Words: Protein-protein interactions; inhibitor; amyloid; HIV; drug design; small molecule-protein interaction.

INTRODUCTION

Protein hetero- and self-association are increasingly recognised as playing essential roles in biology, as many proteins successfully exert their biological function only when a component of a correctly assembled complex [1,2]. Failure of proteins to assemble into fully functioning higher-order assemblies plays a role in the increasing number of pathophysiological states known to be associated with protein misfolding. The enormous importance of protein misfolding to an increasing number of disease states has been extensively reviewed elsewhere [3-10]. In the first part of this mini-review, we highlight the way in which our increasing knowledge of this area has inspired recent approaches to reverse aggregation in diseases of protein misfolding.

In the second part of this review, we change the focus to problems associated with misassembly of proteins into higher order species and discuss the potential for disruption of enzyme self-association to become a general paradigm for drug design. Although these two fields are emerging independently in the literature, they share a common theme: the use of small molecules to disrupt protein-protein interactions to achieve a therapeutic outcome. Other instances in which protein-protein interactions are being explored as drug targets include some of the cell signaling pathways involved in cancer and inflammatory responses. These will also be highlighted, along with key methodological advances that have facilitated the emerging focus on protein-protein interactions in the design of novel therapeutic approaches.

BACKGROUND: PROTEIN-PROTEIN INTERACTIONS IN HEALTH AND DISEASE

Protein-protein interactions are fundamental to living organisms, and much has been written on their role in such crucial processes as self assembly, e.g. of viruses, signal transduction and apoptosis [11]. Furthermore, many human diseases are the result of abnormal protein-protein interactions, either between misassembled endogenous proteins, or due to an unwanted interaction between proteins of pathogen and host. Examples of the latter include cervical cancer, bacterial infections, leukemia and neurodegenerative disease, as recently reviewed [12]. Thus, protein-protein interactions are attracting increasing attention as targets for a new generation of drugs.

Acceptance that small molecules can be employed to modulate protein-protein interactions *in vivo* is growing, and has been reviewed elsewhere [1, 13-18]. As our knowledge of the key roles played by protein-protein interactions in a wide range of cellular processes increases, the potential for such small molecules to be employed to manipulate these interactions to a therapeutic end continues to grow. This is a much less mature drug discovery paradigm than active site targetted approaches, but several recent advances show great promise [11]. These advances are the subject of this review.

DISRUPTING PROTEIN AGGREGATION – TOWARDS THERAPIES FOR AMYLOIDOSES

In recent years, there has been an explosion of biochemical data regarding the key events of protein misfolding and the fibrillogenic process [3-10]. This has inspired the search for small molecule inhibitors that disrupt the amyloid formation process, and several groups have shown that such molecules can indeed be found. These small molecules have been classified on the basis of their target and mechanism of action, as follows: (1) molecules that stabilize the amyloidogenic protein precursor; (2) molecules that prevent fibrillogenesis by acting on partially folded intermediates of the folding process as well as on low molecular weight oligomers populating the initial phase of fibril formation; (3) molecules that interact with mature amyloid fibrils and weaken their structural stability; and (4) molecules that displace fundamental co-factors of the amyloid deposits like glycosaminoglycans and serum amyloid P component and favor the dissolution of the fibrillar aggregate [7]. Some molecules appear to act by more than one of these mechanisms.

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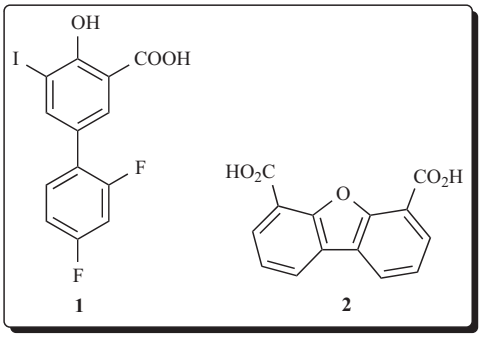
The prevailing hypothesis that for illnesses associated with amyloidoses, it is not the amyloid fibrils themselves, but pre-fibrillar assemblies that represent the toxic species would appear to constrain therapeutic intervention aimed at reversing diseases of protein aggregation by disassembling the aggregates. Clearly, an increase in the concentration of toxic pre-fibrillar species must be avoided in any successful therapy [5]. Much attention is therefore being focused on the first category of small molecule inhibitors, those that stabilize the amyloidogenic protein in a non-amyloidogenic form, inhibiting unwanted protein-protein interactions indirectly. Proof of principle has been successfully demonstrated for transthyretin, a tetrameric protein that has been well characterized and requires dissociation from the native quaternary structure, a tetramer, to the monomer prior to unfolding and formation of amyloid associated with disease. Molecules such as iododiflunisal (**1**) [19] and those derived from dibenzofuran-4,6-dicarboxylic acid (**2**) [20] have been demonstrated, by X-ray crystallography, to bind to tetrameric transthyretin and also inhibit dissociation to monomer, explaining their ability to prevent amyloid fibril formation.



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The self-recognition ability of protein fragments seems to be crucial to amyloidogenesis, providing a simple design principle for peptide-based inhibitors of this process. Amyloid fragments can be mutated or chemically modified in such a way as to impair the assembly of the fibril, for example by mutation of a key residue to proline, or methylation of key amide residues in the peptide backbone, or use of D-analogues of certain amino acids [21]. Several neurodegenerative diseases are associated with aggregated amyloidogenic protein deposits, many containing proteins rich in polyglutamine repeats. Polyglutamine aggregates associated with Huntingdon's disease have been successfully disrupted using polyglutamine peptides containing proline, reducing cell death [12, 22-23]. Yan, *et al*. [23] reported the design of nanomolar affinity inhibitor of islet amyloid polypeptide (IAPP) cytotoxic fibrillogenesis to prevent the formation of pancreatic amyloid, which is associated with type II diabetes. An IAPP analogue completely blocks IAPP cytotoxic self-assembly and also dissociates cytotoxic IAPP oligomers and fibrils, reversing their cytotoxicity. This research is thus yielding promising drug candidates for the treatment of diabetes, and the inhibitor design concept, which relies on binding of the analogue to the amyloidogenic region *in vivo*, may be applicable to other protein aggregation diseases.

Recent research points towards a common feature of amyloidoses being the existence of particular trigger sequences or amyloid stretches that are self-complementary. This opens up further opportunities for anti-amyloidoses therapeutics that target these sequences and prevent, cap, or disrupt the amyloid spine [24]. For example, drugs can be designed that stabilize the native form of a protein, so that trigger sequences are less likely to be exposed, as illustrated by the characterisation of cholyl-leu-val-phe-phe-ala-OH as a potent and selective inhibitor of amyloid β-peptide polymerization that blocks the formation of neurotoxic species of Aβ [25], and more recently, as a general strategy to neutralize neurotoxic peptide sequences implicated in Alzheimer's disease [26,27]. The interaction between fibrillising proteins and specific inhibitors has been examined in depth by several methods including thioflavin T fluorescence assays, electron microscopy, and solid state and solution nuclear magnetic resonance (NMR) techniques [27,28].

Pharmacological concentrations of the HMG-CoA reductase inhibitor, lovastatin, decrease the formation of the Alzheimer β-amyloid peptide *in vitro* and in patients [29]. Kapurniotu *et al*., 2003 have reported the conversion of natively amyloidogenic sequences into inhibitors of amyloid formation by conformational restriction of β-amyloid peptide, *via* cyclisation. New classes of amyloid inhibitor continue to be reported [33] and syntheses of anti-amyloid compounds are emerging in the literature [34,35]. Aggregation inhibitors have also been isolated *in vivo, via* the screening of bacterial metabolite libraries [36]. The possibility of using chaperones for therapeutic intervention (category 2) is also being explored [37].

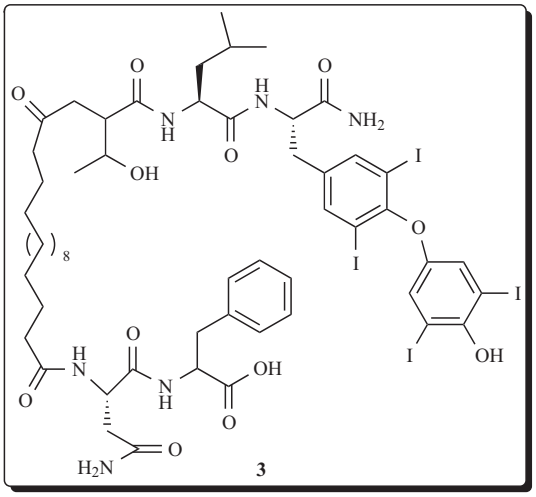
TARGETTING PROTEIN-PROTEIN INTERFACES IN METABOLIC ENZYMES

In addition to avoiding the problems associated with misassociation and misfolding, the critical importance of proteins assembling into their native quaternary structure is increasingly acknowledged. The modulation of protein function by oligomeric state is emerging as a common theme in the literature. The classic role of protein oligomerisation in allosteric regulation, as illustrated with haemoglobin and oxygen binding [38], and in the control of critical rate-limiting steps with enzymes such as phosphofructokinase [39] is now known to be only one of many potential biological functions of self-association. A change in quaternary structure is often associated with a change in function, and transient protein-protein interactions can thus be important biological regulators [40]. For some enzymes, such as UDP-glucose pyrophosphorylase, the monomer is the active species and the oligomer is apparently inactive [41]. With other enzymes, such as HIV-1 protease, the opposite is the case. This opens up new opportunities in drug design, based on the use of small molecules to manipulate protein-protein interactions *in vivo.* Such opportunities are only just beginning to be realized, and some representative case studies are reviewed here.

HIV Protease Inhibitors

HIV-1 protease is a dimeric enzyme encoded from the viral genome and is an important target in the development of therapeutic agents to counter the effects of HIV infection. Most current drugs focus on the active site, but the absolute requirement for the dimeric structure of the enzyme has led

to a new focus on the monomer-monomer interface of the enzyme [14,42]. New drugs are being developed that disrupt the quaternary structure and inactivate the enzyme [1, 43,44]. Dimerisation inhibitors target the monomer-monomer interface which is comprised of the N- and C-termini of each subunit. Early research demonstrated proof of principle with a tetrapeptide that inhibited the enzyme *via* an interface disruption mechanism, with a variety of compounds being designed from this lead, as well as the discovery, by screening, of natural product inhibitors that are non-peptidic in nature [1,14]. Using a focused library approach, a range of potent and specific compounds have been developed, including a recently developed dimerisation inhibitor referred to as Thx-1 (**3**), which has a K_i of 71 ± 5 nM [44].



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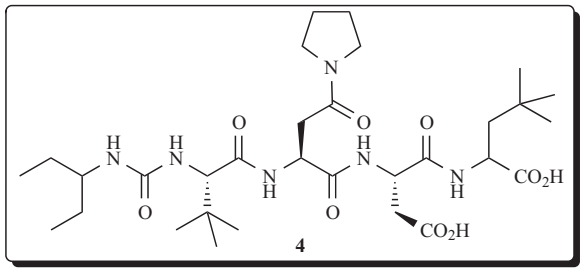
Ribonucleotide Reductase

Ribonucleotide reductase from *Herpes simplex* is the focus of drugs such as acyclovir, a nucleoside analogue that targets the active site of the enzyme [1]. The active form of the enzyme is comprised of two sub-units, with an interface that has also been targeted for interface disruptor drugs [14]. Again, a lead compound was designed *via* a short peptide, which has subsequently been elaborated to yield a late generation compound (**4**) with an IC_{50} of < 1 nM. This has been shown to be successful in a mouse model [15].



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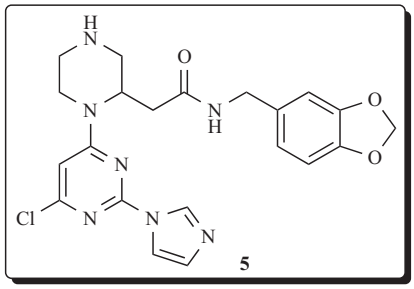
Triose Phosphate Isomerase

Since the enzyme active site tends to be very highly conserved across species, drug targets have been historically restricted to metabolic pathways that are unique to the pathogen of interest, in order to ensure that toxicity to the host is minimized. A shift in focus to protein-protein interfaces, which are often less conserved, offers the possibility of targetting enzymes that are also present in the host. This principle has been applied to the design of inhibitors of *Plasmodium falciparum* triose phosphate isomerase, with a view to disrupting the homodimeric enzyme to effect inhibition [46].

Olivares-Illana *et al*. [47] undertook a detailed study of the structures of triose phosphate isomerase from eight species determined by X-ray crystallography. They found conservation of the interface residues in the enzyme from trypanosomatids *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Leishmania Mexicana* and significant differences between these interfaces and those from triose phosphate isomerase isolated from humans, yeast and chicken. This opens up the possibility of specific inhibition of triose phosphate isomerase, not previously considered a drug target since it has a ubiquitous presence in all cells. Proof of principle was established with the molecule 6,6′-bisbenzothiazole-2,2′diamine, which irreversibly inactivates the enzymes from the three trypanosomatids, but does not effect the enzymes from other species [47].

Nitric Oxide Synthase

Inducible nitric oxide synthase has been implicated in the pathogenesis of a number of diseases, including autoimmune conditions and inflammatory responses. It is a dimeric enzyme that is inactive in the monomeric state [1]. Phenylimidazoles (e.g. (**5**) [48]) inhibit inducible nitric oxide synthase activity in cell-based assays, but not in crude extracts of the dimeric enzyme. It is thought, based on size exclusion chromatography of cellular extracts and X-ray crystallography of the inactive, monomeric enzyme with the compound bound, that this is because the compounds bind to the monomeric form upon synthesis, and inhibit dimerisation [48]. This is an example of allosteric inhibition of protein-protein interaction [16].



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EMERGING TARGETS

As more successful lead compounds emerge for drug development based on disruption of protein-protein interactions, more targets amenable to this approach are coming to light, including known antibiotic targets for which potent active site inhibitors have not been found. For example, dihydrodipicolinate synthase (DHDPS) plays a critical role in the biosynthesis of lysine and *meso*-diaminopimelate (*meso*-DAP) in microbes, and is therefore an important antibiotic target [49]. The recent observation that the enzyme is only fully active in its tetrameric state [50,51] suggests that

protein-protein interface disruptors may be designed as lead molecules for new antibiotics.

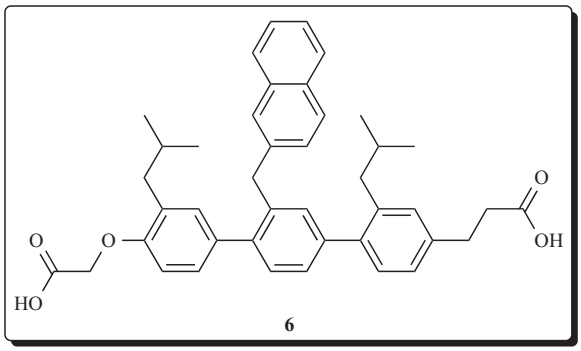
Similarly, GDP-mannose pyrophosphorylase (GDP-MP) is an enzyme that plays a critical role in mannose metabolism in *Leishmania* species. GDP-MP is responsible for catalyzing the conversion of mannose-1-phosphate to GDP-mannose using GTP as the nucleoside-diphosphate donor [52]. GDP-mannose is subsequently used as a building block for the production of glycoconjugate molecules. These glycoconjugates, including GPI-anchored lipids, are found on the outer glycocalyx of the parasite and are important virulence factors in leishmaniasis [53]. GDP-MP has also been shown to require full assembly of its hexameric state for full activity, again suggesting that molecules capable of disrupting the hexamerisation interface of the enzyme may provide lead drug candidates [51].

INTERRUPTING SIGNALING PATHWAYS

Small molecules that interrupt key protein-protein interactions in signaling pathways have significant potential as drugs [17]. There are many examples of the successful design of effective non-peptide ligands for different types of receptors, including vascular endothelial growth factor receptor, somatostatin receptor, neuropeptide Y receptors, thromboxane A2 receptor and protease-activated receptors [14]. Examples of known drugs that act *via* modulation of protein-protein interactions [15] have spurred research into using this mode of action as a design principle. Homodimerisation of proteins, such as histidine kinase, is an essential step in bacterial signal transduction and has been the target of novel drug discovery systems [54]. Much research has focused on the interruption of protein-protein interactions implicated in cancer [18]. The approved anti-cancer agent Taxol, and related compounds, have been shown to act by binding to the β-subunit of the tubulin heterodimer and stabilizing the complex, leading to accelerated formation of microtubules and cell cycle arrest [1]. Vinblastine, another anti-cancer agent, acts by inhibiting formation of the α–β tubulin heterodimer [15].

Much interest has been reported on the interactions of tumour suppressor p53, a protein that is activated in the majority of human tumours, often *via* binding to oncogenic proteins [55]. Oncoprotein Hdm2 inhibits p53 by forming a protein complex that mediates nuclear export of the tumour suppressor and subsequent degradation by proteasomes, and inhibition of this complex using dodecapetides optimised by phage display has been shown to be potent, with IC_{50} values lower than 5 nM [1]. Helical β-peptides have also been explored as inhibitors of this interaction [56].

Using chemical library screening, a range of small molecules have been identified that interfere with a variety of clinically relevant protein-protein interactions. Examples, reviewed by Berg [1], include several which offer new leads in cancer chemotherapy as follows. (1) The association of Bcl-X_L and Bcl-2, which are anti-apoptotic proteins known to be over-expressed in solid human tumours, has been linked with drug resistance to cancer chemotherapy and is a validated tumor target attracting considerable attention [57-60]. Small molecule inhibitors of this interaction, such as compound (**6**) with an IC_{50} of 114 nM [16], have been identified using an elegant fluorescent labeling approach, which identifies successful inhibitors by disrupting fluorescence between the protein complex [1]. These interactions have also been characterized by isothermal titration microcalorimetry and NMR spectroscopy [16] (2). Lead compounds that inhibit dimerisation of Myc/Max, a transcription factor complex implicated in a high percentage of terminal cancer patients, have also been identified using a fluorescence based assay [1]. (3) Inhibition of the interaction of integrins with matrix metalloproteinases has been explored as a therapeutic avenue for the prevention of angiogenesis and potentially cancer [1,16].



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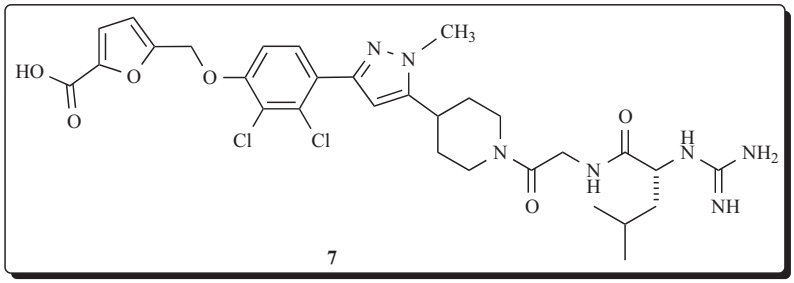
Other examples emerge from the anti-inflammatory field including: (1) Inhibition of the interaction between cytokine tumour-necrosis factor alpha, and its receptor, as an approach to inhibiting unwanted inflammatory responses, such as rheumatoid arthritis, to various viral and bacterial infections. (2) Inhibition of the interaction between leukocyte function-associated antigen-1 (LFA-1) and its adhesion molecule, intercellular adhesion molecule-1 (ICAM-1, CD54), which mediates migration of leukocytes from the bloodstream to the surrounding tissue during the early stages of inflammation and the activation of T-cells. (3) Peptidomimetic analogues of interleukin-2 have been identified that interrupt binding to its receptor, e.g. compound (**7**) (IC_{50} 60 nM) [16]. In all cases, small molecules have been identified with clinical promise [1].

ARE SMALL MOLECULES REALISTIC INHIBITORS OF LARGE PROTEIN INTERFACES?

Inhibition of specific protein-protein interactions using antibodies with high specificity and affinity is well established. Therapeutic antibodies constitute 30% of biopharmaceuticals in clinical trials [12], and with an estimated US$5–7 billion annual expenditure on antibody-based antagonists, they constitute the fastest growing portion of the prescription drug market [16]. Although proof of principle, in terms of efficacy, has been clearly established using these large molecules, they are unsuitable for delivery to intracellular targets and attention is therefore turning to smaller molecules.

Natural molecules that are known to disrupt protein-protein interfaces are rare [16] and the proposed use of small molecules to disturb protein-protein interactions that vary from 550–4900 $Å^2$ [14] and are typically greater than 1100 $Å^2$ [61] has, in the past, been greeted with considerable skepticism [1,12,15,16]. However, while the number of small



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molecule lead compounds that inhibit protein-protein interactions is relatively small to date, it is nonetheless growing rapidly, and covers a range of potencies [11,16,17] including some nanomolar inhibitors, as described in the previous sections.

The success of the approach, despite the apparent insurmountable challenge of disrupting such large interfaces, is in accord with recent research demonstrating the importance of "hot spots" in protein-protein interactions (*i.e.* an examination of the physical chemistry of the interface demonstrates that interactions between a small number of amino acids that account for a large percentage of the binding energy across the surface [16,62]). This has increased optimism that small molecules can indeed be rationally designed to disrupt protein-protein interactions spanning large surfaces, acting as a molecular merkin to mask the crucial region of the interface. Such hot spots often contain arginine, histidine, asparagine, tryptophan, tyrosine and serine residues [14,15,63].

When designing small molecules to interfere with a protein-protein interaction, peptides and peptidomimetics make the most intuitively obvious starting point. Although peptides themselves are not necessarily ideal drug candidates [12], cross-linked interfacial peptides and helix mimetics have been employed to target various protein-protein interfaces, including those of anti-apoptotic protein Bcl-X_L and estrogen receptors, with some success *in vivo* [11]. Helix mimetics have also been employed to mimic the p53 helix in anti-cancer strategies [11,56]. Problems associated with the aggregation of peptidic drugs are being addressed [64].

General strategies for design of compounds to interfere with specific protein-protein interactions are emerging [65]. *In silico* approaches using shape comparison programmes are starting to be employed to generate lead compounds to dock onto protein-protein interactions, building on crystallographic data [66]. Synthetic methodology is also being developed which enables modular, parallel library synthesis of compounds designed to mimic α-helical scaffolds [67]. Acknowledgement that binding of small molecules may involve a certain degree of conformational flexibility of the protein broadens the possibilities of molecular design beyond those that can be discerned from a single crystal structure, because protein hot-spots appear to be especially adept at binding [16].

Screening remains an important method for discovering new drugs leads. Computational approaches and virtual screening [1] coupled with new bioinformatic and proteomic methods [14] allow powerful predictions of protein complexes *in vivo* to inform inhibitor design. Specific screens for protein-protein interaction inhibitors are emerging, including those based on fluorescence techniques [13]. Cell-based assays that monitor the intracellular behavior of target molecules, rather than binding or catalytic activity of purified proteins, can now be used in high-throughput screens to discover and profile molecules that act primarily by modulating protein interactions [17]. Furthermore, phage display technologies have facilitated the discovery of many peptide modulators of protein-protein interactions [1].

Focused libraries capture the advantages of both design and screening strategies, and are meeting with success [68]. As described earlier, for example, dimerisation inhibitors of HIV protease were identified using a focused library [44]. Combinatorial modification of peptide scaffolds, beginning with a low affinity consensus sequence peptide, has been shown to produce libraries of compounds that inhibit protein signaling [69]. Fragment assembly is also proving useful to probe large areas of chemical space with minimum compound synthesis to improve efficiency [16,17].

TECHNIQUES FOR MEASURING EFFICACY OF PROTEIN-PROTEIN INTERACTION INHIBITORS

In addition to simple activity screens, advances in the field of drug design aimed at targeting protein self- and hetero-association are going to be critically dependent on monitoring changes in protein activity as a function of oligomeric state. A number of simple techniques have been employed to measure oligomerisation of proteins. Qualitatively, the common methods are analytical gel permeation liquid chromatography [70] and blue native PAGE [71]. These techniques are relatively simple and inexpensive for preliminary screening of small molecules, but can lead to aberrant results when analysing asymmetric macromolecules and rapidly diffusing or dissociating systems.

More advanced techniques being employed in this area include X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, surface plasmon resonance [72] and analytical ultracentrifugation [16,20,51], coupled with isothermal titration microcalorimetry, antibody inhibition experiments and site-directed mutagenesis [16]. Advances in bioinformatics and proteomic technologies are also of paramount importance to identify new targets that may be crucial to cell viability and amenable to drug design using this approach [14].

Quantitatively, analytical ultracentrifugation is an excellent method for measuring self- or hetero-association [73-75]. The development of continuous size-distribution [*c(s)*] analysis using the program SEDFIT [76] has provided

an efficient approach to generate size-distribution profiles of heterogeneous macromolecular systems [52, 73-75] allowing the identity of multiple species in the analytical ultracentrifuge to be determined from a simple sedimentation velocity experiment, without the need for time-laborious sedimentation equilibrium data [73,75]. The programme SEDPHAT [77] has enabled efficient global analyses to be performed with sedimentation equilibrium data with and without sedimentation velocity data sets, as was recently successfully demonstrated for GDP-MP [52] and DHDPS [51].

CONCLUSION

The field of drug design based on inhibition of protein-protein interactions is still in its infancy, but a number of promising examples suggests that the combination of new proteomic technologies to identify new drug targets, *in silico* analyses, focused or high throughput screening and *de novo* organic synthesis, will continue to open up new possibilities in this exciting and rapidly developing area. The combination of increasingly detailed structural information on protein-protein interactions, advances in proteomic analyses, and inhibitor design and screening, augur well for developments in this field.

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