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Intrinsically disordered proteins and biomolecular condensates as drug targets

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Summary

Intrinsically disordered domains represent attractive therapeutic targets because they play key roles in cancer as well as in neurodegenerative and infectious diseases. They are however considered undruggable because they do not form stable binding pockets for small molecules and therefore have not been prioritized in drug discovery. Under physiological solution conditions many biomedically relevant intrinsically disordered proteins undergo phase separation processes leading to the formation of mesoscopic highly dynamic assemblies, generally known as biomolecular condensates, that define environments that can be quite different from the solutions surrounding them. In what follows we review key recent findings in this area and show how biomolecular condensation can offer opportunities for modulating the activities of intrinsically disordered targets.

Introduction

Protein domains that do not fold into well-defined structures are said to be intrinsically disordered [1]. The conformations of this class of domains cannot be represented by a single structure and are best depicted by conformational ensembles that describe their structural heterogeneity [2]. The widespread nature of intrinsic disorder and the often important functions of intrinsically disordered domains challenge our understanding of how protein sequences encode biological functions. It also represents a challenge for the field of drug discovery because the tools used to target globular domains with small molecules may not entirely suit intrinsically disordered ones [3].

This class of domains can have a propensity to phase separate into biomolecular condensates often formed by liquid liquid phase separation [4,5]. This phenomenon leads to the formation of dynamic mesoscopic assemblies, stabilized by a large number of weak transient non-covalent interactions, that are liquid and generate unique chemical environments [6].

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Our understanding of how biomolecular condensation may allow specific functions to emerge is likely still incomplete; it is nevertheless already clear that they can act as reservoirs of primed inactive protein [7], as scaffolds to facilitate protein protein interactions [8,9] and as molecular sieves to regulate molecular traffic through biological membranes [10,11], among other functions [12].

It is thus natural that this phenomenon has raised substantial interest in the field of drug discovery, as evidenced by the foundation of a number of biotechnology companies with this focus [13]. Indeed, understanding how small molecules partition in biomolecular condensates and whether they can be used to modify the composition, stabilities, rates of formation and physical properties of these assemblies holds substantial promise for challenging indications [14] and may allow using small molecules to modify the activity of therapeutic targets currently considered *undruggable*.

The free energy landscape of intrinsically disordered proteins

Free energy landscapes are useful to describe the conformational properties of intrinsically disordered proteins and of the multimeric assemblies that they can form [15]. They represent the free energy of the protein as a function of its conformation and are usually plotted in 3D as contour plots. The free energy is plotted in the z axis and the conformational space available to the protein in the xy plane, projected on two structural descriptors such as, for intrinsically disordered proteins, the degrees of structuration, s , and multimerization, n (Fig. 1). Stable states correspond to free energy minima and the frequency of transitions between them is given by the height of the free energy barriers.

The landscape expected for an intrinsically disordered protein undergoing biomolecular condensation is shown in Figure 1. In the region of the landscape corresponding to no multimerization ($n=1$) the minima correspond to the various states that may be populated by monomeric intrinsically disordered proteins, such as disordered (D), partially (PS) and fully structured (FS) states. Since they have similar free energies and the barriers connecting them are low, the structural properties of the intrinsically disordered protein can abruptly change upon population shifts caused for example by changes in solution conditions [16], post-translational modifications [17] or by interactions with other molecules [18,19].

In the region corresponding to multimers ($n \gg 1$) minima may correspond to biomolecular condensates (BCs), glassy solids or hydrogels (HGs) and fibrils (Fs). Intrinsically disordered proteins that form condensates by liquid-liquid phase separation are thought to remain disordered [20,21] and can diffuse across the liquid-liquid interface and in the bulk of the condensate. Upon fibrillization, by contrast, protein molecules change conformation to form quaternary structures stabilized by intermolecular hydrogen bonds (F) in which they occupy permanent positions [22]. Hydrogels are in an intermediate situation both in terms of structuration and dynamism [12].

Contrary to what is the case for monomeric intrinsically disordered proteins, the stable states of biomolecular condensates can have quite different thermodynamic stabilities. Biomolecular condensates formed by liquid-liquid phase separation are in fact thought to

be kinetically but not thermodynamically stable relative to fibrillization. Indeed amyloid fibrils represent the most thermodynamically stable state available to protein sequences and their formation is in general irreversible [23]. The barriers connecting such states are high due to their relatively high density and the polymeric nature of their components [24].

Small molecules can reshape the energy landscapes of intrinsically disordered proteins that form biomolecular condensates

At equilibrium the populations of the various states accessible to a protein depend on their relative free energies. Decreasing the free energy of a specific state, for example by selectively targeting it with a small molecule, can be used to reshape the energy landscape for therapeutic intervention [3]. For a simple two-state system such as a globular protein in exchange with the corresponding unfolded state, for example, targeting the globular state with small molecule *chemical chaperones* stabilizes the protein against proteolytic degradation and aggregation by decreasing the population of the unfolded protein that is a precursor of both processes [25,26].

In a generalization of this mechanism of action, targeting a specific state of an intrinsically disordered protein, including multimeric states, with small molecules can be useful for different therapeutic purposes, as shown in Figure 2a. For intrinsically disordered proteins that fold upon interaction with a binding partner, for instance, small molecules that stabilize an alternate, binding-incompetent conformation will act as allosteric inhibitors of the interaction [27]. For intrinsically disordered proteins that can phase separate into biomolecular condensates stabilizing the monomer state with small molecules can inhibit the condensation process [28], whereas stabilizing the condensate will have the opposite effect, thus inhibiting the functions of the monomeric protein. Finally, although it is unlikely that small molecule binding to an intrinsically disordered protein will abolish amyloid formation, it can kinetically stabilize the monomer and thus decrease its aggregation propensity [29]. Given that these processes play key roles in the biological functions of intrinsically disordered proteins [4], as well as in disease [30], it is necessary to develop tools, both conceptual and technical, allowing us to direct small molecules to all the relevant states that they populate.

We direct the reader to other reviews to gain a good understanding of the state of the art in targeting small molecules to orthosteric and allosteric sites in fully structured states of intrinsically disordered proteins (FS) [31] and to sites of both primary and secondary nucleation in amyloid fibrils (F) [32]. Although targeting such states, even when they exist at low population, can allow modulating structural and functional properties of intrinsically disordered proteins, we here focus our attention on the challenging and relatively unexplored goals of directing small molecules to their disordered and partially structured states (D, PF) (Fig. 2a,b) as well as to biomolecular condensates (Fig. 2c).

Targeting disordered states with small molecules

Targeting intrinsically disordered regions with small molecules represents a formidable challenge for drug discovery [33–35]. These proteins do not display stable, well-defined

binding pockets and as a consequence they are generally considered *undruggable* therapeutic targets and thus not prioritized by the pharmaceutical industry. Despite this, due to their potential as targets for highly relevant disease areas, including neurodegeneration and oncology [36], a number of drug discovery programs have explored this enticing prospect in different ways, with promising results (Table 1).

As structure-based drug discovery cannot in principle be used to target intrinsically disordered proteins one approach is to rely on screens of inhibitors of protein-protein interactions and on phenotypic screens. Examples of this for oncology include the discovery of a small molecule that targets the intrinsically disordered region of p53 interacting with MDM-2 [48], of direct binders of c-Myc that inhibit its interaction with Max [40,49–52], of a small molecule targeting EWS-FLI1 that inhibits its interaction with RNA helicase A [53,54], of an allosteric inhibitor of PTP1B that targets its intrinsically disordered domain [44] and of a direct inhibitor of the transactivation domain of the androgen receptor (AR) [55,56].

Nuclear magnetic resonance (NMR) is a powerful tool to study weak interactions and has recently been used to identify small molecules that bind to protein p27 [19,57]. Although the affinity was weak, in the mM range, the authors were able to show how titrating the inhibitor destabilized the interaction between p27 and Cdk2/cyclin A [57], establishing a proof of concept. Identifying small molecules stabilizing the structural properties of intrinsically disordered proteins against thermal denaturation has also been used for screening, leading to a compound interacting with NUPR1 that after optimization has shown promising anticancer activity [43,58].

Although, on average, intrinsically disordered proteins are devoid of the structural features associated with *druggability*, they may transiently populate collapsed/structured conformations that instead may be *druggable*. The situation is reminiscent of the formation of cryptic binding pockets in globular proteins [59] and, as in this case, molecular simulations can help to reveal the relevant conformations, which can then subsequently be studied with tools of structure-based drug discovery such as molecular docking. In its first implementations this approach was used to investigate the druggability of the intrinsically disordered proteins A β 42 [60], α -synuclein [61] and has more recently been used to discover inhibitors for c-Myc, MBD2 and p53 with some success [62–64].

Studying in detail how intrinsically disordered proteins interact with small molecule inhibitors identified by screening can help to unveil the intermolecular interactions that stabilize the complexes, the nature of the conformational changes that the small molecule can induce in the protein and thus the molecular basis for selectivity. Several academic laboratories have focused their attention on inhibitors identified by two-hybrid screening to inhibit the interaction between c-Myc and Max by targeting c-Myc [49]. By using biophysical techniques such as NMR and circular dichroism (CD) it was found that different inhibitors appeared to recognize independently different motifs with partial helical secondary structure [65,66] suggesting that small molecules can be targeted to specific intrinsically disordered sequences.

Similarly, a study of the interaction between a small molecule inhibitor, EPI-001, and the transactivation domain of AR by using solution NMR showed how this small molecule interacts with a sub-domain formed by three partially folded helices (Tau-5) but not with a similar one formed by two such helices (Tau-1), also supporting the idea that it is possible to target small molecules to intrinsically disordered proteins with some degree of selectivity. In this specific case it was found that this small molecule did not interact with the three partially helical sequences independently, suggesting the formation of a binding pocket involving residues found in at least two of them [67].

Although small molecule binding can change the conformation of intrinsically disordered targets [65] an analysis of the biophysical properties of these complexes clearly indicates that they do not lose their disordered character upon binding, precluding the obtention of a structure of the complex by using conventional structural biology methods. In this scenario molecular simulations represent powerful tools to complement the information obtained experimentally. The most studied system is again that formed by c-Myc and its small molecule inhibitors and the results obtained by different laboratories [68–70] consistently indicate that the inhibitors do not have a single binding pose, that they can interact with different motifs in the disordered target, in different conformations, and that they have a moderate effect on its conformational ensemble (Fig. 2b); the study of a similar system such as p27 led to equivalent results [71]. A very recent investigation of the binding of fasudil and 49 of its analogs to α -synuclein [37] showed good agreement with the results of NMR experiments, highlighting that molecular simulations may be useful in the future to guide the optimization of hits for drug discovery [72].

Covalent inhibition can be attractive for intrinsically disordered proteins because it can alleviate weak affinity issues. It requires the presence of a nucleophilic side chain in the target, typically a Cys or Lys, and of a warhead moiety in the inhibitor, typically an electrophilic group that may be generated *in situ* [73]. The modification of the structure of the intrinsically disordered target produced by covalent modification can be easily detected by mass spectrometry and lead to target inhibition by the mechanisms shown in Figure 2a. In addition it can directly inhibit the target if it modifies a residue in a motif mediating its interaction with a binding partner. This orthosteric mechanism of inhibition is less plausible with non-covalent inhibitors due to the non-druggable nature of the extended conformations involved in protein-protein interactions. A number of small molecule inhibitors of intrinsically disordered proteins appear to act as covalent inhibitors such as oleocanthal, that inhibits tau aggregation [74], baicalein, that inhibits α -synuclein aggregation [75], EPI-001, that binds to transactivation domain of AR [56] and nimbolide, that inhibits RNF114, a E3 ubiquitin ligase, and therefore stabilizes its substrates [76]. Very recently a systematic search for covalent inhibitors of the interaction between c-myc and Max lead to the identification of EN4, a compound bearing an acrylamide warhead, that reacted with some selectivity with Cys 171 in c-Myc and showed promising antiproliferative properties [41].

Targeting biomolecular condensates with small molecules

One defining feature of biomolecular condensates produced by liquid-liquid separation that differentiates them from conventional protein aggregates such as amyloid fibrils is their liquid character (Fig. 2c) [4]. Similarly to what is the case in the liquid-liquid extraction procedures used in the chemistry laboratory to isolate reaction products, small drug-like molecules can distribute between specific condensates and the surrounding solution depending on their partition coefficient P (Fig. 2c) [6,77]. In doing so they may change the properties of the condensate in ways that could be used for drug discovery in various disease areas such as neurodegeneration, [24] oncology [77] and infectious diseases [30,78].

Like intrinsically disordered proteins, biomolecular condensates are unconventional drug targets, out of the reach of conventional structure-based drug discovery tools. In this scenario cell-based or phenotypic assays may be again used to identify small molecules that appear to target them [79]. In an example of this approach a library of drug-like molecules was screened for their ability to dissolve stress granules by using a cell-based multiparametric imaging assay. The resulting hits, lipoamide and lipoic acid, could potentially be used to treat amyotrophic lateral sclerosis (ALS) after further development. This currently incurable disease can be caused by mutations in intrinsically disordered proteins such as FUS [80] or TDP-43 [81] that accelerate liquid to solid transitions in stress granules [82].

In these initiatives it is important to characterize in as much detail as possible the mechanism by which small molecules modulate the relevant phase transition. This will be crucial to investigate whether the approach can be generalized to other relevant targets, to design robust assays amenable to high throughput mode and, finally and most importantly, to guide the optimization of the structure of the small molecules to maximize potency. This is a highly challenging endeavour, however, due to the difficulties associated with reproducing *in vitro* key properties of biomolecular condensates. Despite recent progress, enumerating their components, and their stoichiometries, for example, is non-trivial [83], as is reproducing the highly dynamic character that they can have in cells due to energy consumption processes [84].

In considering what the effect of small molecule partitioning in biomolecular condensates will be, it is relevant to invoke the phenomenon called polyphasic linkage, that links the phase separation properties of a specific protein with its interaction with ligands [85–87]. Specifically, when the strength of the interaction between a ligand and a protein is not the same in the various phases that the protein may form, addition of the ligand will change the phase diagram. Polyphasic linkage, similarly to allostery, does not put forward a specific mechanism for the correlation but it is plausible that it occurs due to differences in the strength of intermolecular interactions in the various phases, that can have both energetic and entropic causes as revealed by both experiments and simulations [87–89] and to changes in molecular structure upon phase separation [90] among other factors [14,82].

Outlook

Intrinsically disordered proteins and biomolecular condensates represent unconventional therapeutic targets, which will require the development of new concepts and new tools for drug discovery. Phenotypic or high-throughput screens have led to the identification of a number of small molecules that appear to target these protein states, which has led to great interest in understanding their detailed mechanisms of action. The results from the limited number of studies available clearly indicate that the interaction between small molecules and both intrinsically disordered proteins and biomolecular condensates has specific features that should be taken into consideration in designing screening assays, choosing libraries for screening and, especially, in optimizing the chemical structures of hits and leads during drug development.

As far as intrinsically disordered proteins are concerned much of the emphasis has been put on applying the tools of structure-based drug discovery, in an ensemble fashion, for virtual screening. Although intuitive, this approach assumes that drug action relies on the establishment of highly specific interactions between the drug and a *druggable* conformation of the target identified by clustering a conformational ensemble. It appears, however, that small molecules bind to disordered targets in different poses and conformations, by establishing different non-covalent interactions. As a consequence, the effect of changes in the chemical structure of hits and leads on the stability of the complex and the structure of the target can only be rationalized by considering the energy landscape of the monomeric disordered protein (Fig. 1B).

Even in cases where the interaction between small molecules and intrinsically disordered targets can be modeled for drug development, for example by using advanced sampling techniques of molecular simulations [71], the optimized small molecules are likely to interact with intrinsically disordered targets with weak affinity. Indeed the size of small molecules precludes by definition the establishment of a large number of simultaneous interactions with the target, or allows it only with a substantial entropic penalty upon partial folding of the target. It has been proposed that in certain cases the interaction between small molecules and disordered proteins could increase the entropy of the intrinsically disordered target, perhaps by releasing long range interactions [91] but it remains to be seen whether this mechanism is compatible with selective targeting [29].

It is becoming increasingly clear that, for a given intrinsically disordered protein forming biomolecular condensates, collapse and biomolecular condensation are favoured by similar solution conditions because they are stabilized by equivalent interactions, that are intramolecular in one case and intermolecular in the other [92] (Fig. 2c). Since the collapsed conformations of disordered proteins are those easiest to target with small drug-like molecules *i.e.* more druggable, it is possible that biomolecular condensates produced by liquid-liquid phase separation feature a high density of binding sites for small molecules with the appropriate functionalities. As we gain a more detailed understanding of the specific interactions that stabilize the biomolecular condensates representing therapeutic targets [93–96] we will be in a better position to design and optimize the structures of small

molecules targeting these assemblies to modify their properties in ways that are useful for drug discovery.

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Highlights

- Intrinsically disordered domains are challenging therapeutic targets because they do not form stable binding pockets for small molecules
- Small molecules that bind intrinsically disordered domains typically interact with different motifs, in different poses and with low to moderate affinity and selectivity
- Biomolecular condensates generate environments that can selectively accumulate small drug-like molecules
- Targeting monomeric proteins and biomolecular condensates with small molecules can lead to populations shifts that alter protein phase equilibria

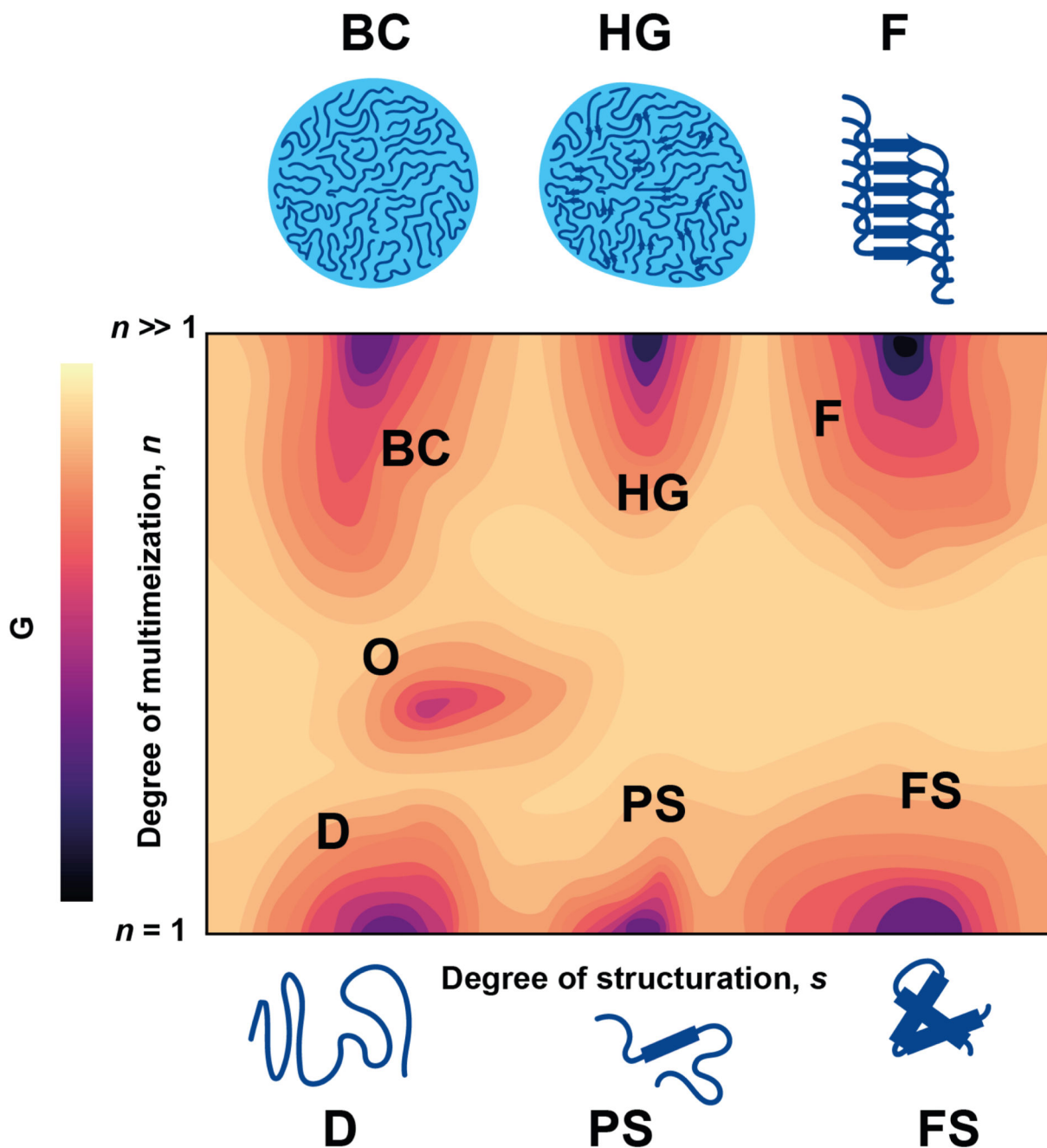


Figure 1.

Generic free energy landscape of an intrinsically disordered protein with a propensity to condensate where the free energy of a protein molecule (G) is represented as a function of its degree of structuration (s) and multimerization (n). The letters represent the different minima that may be populated by such molecule, that are illustrated by representative conformations and include highly disordered state (D), partially and fully structures states (PS, FS), an oligomeric state (O), a biomolecular condensate (BC), a hydrogel (HG) and a fibril (F). Changes in the free energy of any state caused by interaction with a small molecule can lead

to population shifts or changes in kinetic stability that can be used to alter the propensity of the protein to interact with a binding partner, form condensates or form fibrillar aggregates as shown in Figure 2.

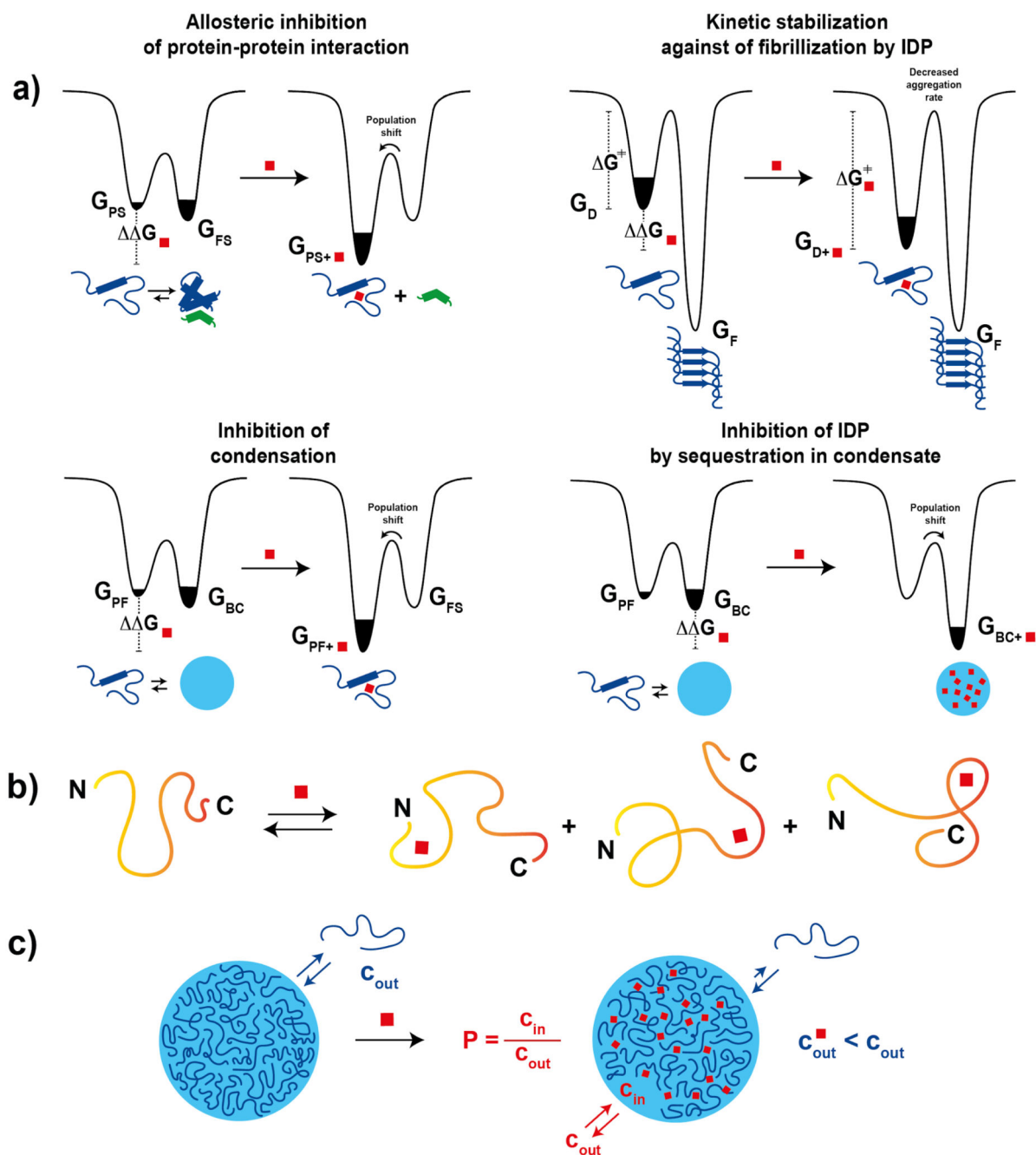
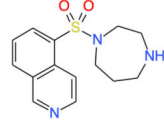
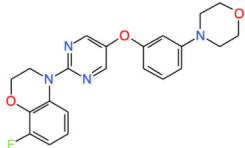
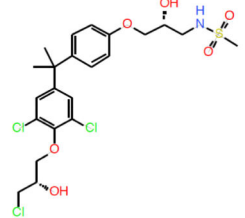
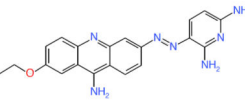
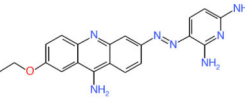
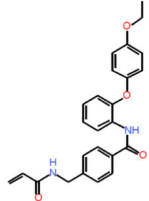
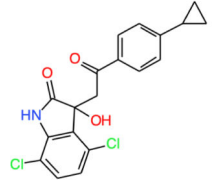


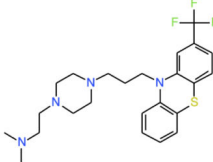
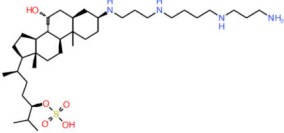
Figure 2.

a) Energy landscape of an intrinsically disordered protein before and after small molecule binding illustrating how it can inhibit protein-protein interactions, provide kinetic stability against fibril formation as well as cause population shifts that promote or suppress biomolecular condensation. b) Schematic illustration of a generalized mechanism for the interaction between small molecules and intrinsically disordered proteins derived from both experimental and computational studies. c) Schematic representation of a biomolecular condensate, of the exchange of protein molecules from the biomolecular condensate to

the surrounding solution and of the effect of small molecule binding to an intrinsically disordered protein undergoing biomolecular condensation according to the polyphasic linkage framework.

Table 1
Small molecule inhibitors of disordered states of proteins with proven *in vivo* efficacy.

Target	Disease family	Compound	Identification	In vivo efficacy	Clinical trials (Phase)	Structure	Ref.
α -synuclein	Parkinson's disease	Fasudil	Drug repurposing	Improved motor and cognitive functions at 10 and 30 mg/kg	Approved for use in humans but not assayed for Parkinson's disease		[37]
A β	Alzheimer's disease	SEN1576	Rational design	Reduced deficits in in vivo long-term potentiation and memory at 0.3 and 1 mg/kg	-		[38]
AR	Castration resistant prostate cancer	EPI-7170	Second generation lead compound from a phenotypic screen	Tumor growth inhibition at 25 mg/kg	NCT04421222 ¹ (1)		[39]
c-Myc	Cancer	MYCMI-6	Cell-based protein interaction screen	Apoptosis induction and reduction of tumor cell proliferation and microvasculature at 20 mg/kg	-		[40]
		EN4	Covalent ligand screen	Tumor growth inhibition at 50 mg/kg	-		[41]
EWS-FLI1	Ewing's sarcoma	TK216	Rational design	Tumor growth inhibition at 100 mg/kg	NCT02657005 (1)		[42]
NUPR1	Pancreatic adenocarcinoma	ZZW-115	Ligand-based design	Tumor growth arrested at a 5 mg/kg	-		[43]

Target	Disease family	Compound	Identification	In vivo efficacy	Clinical trials (Phase)	Structure	Ref.
PTP1B	Diabetes, obesity	MSI-1436	Serendipity	Suppresses appetite, reduces body weight and improves plasma insulin at 5-10 mg/kg	Completed: NCT00806338 NCT00606112 NCT00509132 Discontinued: NCT02524951 (1)		[44, 45]
Tau	Alzheimer's disease	TRx0237	In vitro aggregation assay	Reduces the brain atrophy rate at 4 mg/kg twice a day.	NCT03446001 (1)		[46, 47]

¹Clinical trial of EPI-7386 (structure not available).