Targeting proteins self-association in drug design

Léopold Thabault,[1,2] Maxime Liberelle,[1] Raphaël Frédérick[1*]

[1] Louvain Drug Research Institute (LDRI), Université catholique de Louvain (UCLouvain), B-1200 Brussels, Belgium.

[2] Pole of Pharmacology and Therapeutics, Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCLouvain),B-1200 Brussels, Belgium.

*** Corresponding Author :**

Prof. Raphaël Frédérick

Université catholique de Louvain (UCLouvain)

Louvain Drug Research Institute (LDRI)

Tour Van Helmont, B1.73.10

73 avenue Mounier

1200 Brussels

Belgium

Phone : +32 2 764 73 41

Abstract

Protein self-association is a universal phenomenon essential for stability and molecular recognition. Disrupting constitutive homomers constitutes an original and emerging strategy in drug design. Inhibition of homomeric proteins can be achieved through direct complex disruption, subunits intercalation or by promoting inactive oligomeric states. Targeting self-interaction grants several advantages over active site inhibition thanks to the stimulation of protein degradation, the enhancement of selectivity, a sub-stoichiometric inhibition and a by-pass of compensatory mechanisms. This new landscape in protein inhibition is driven by the development of biophysical and biochemical tools suited for the study of homomeric proteins, such as DSF, native MS, FRET spectroscopy, two-dimensional NMR and X-ray crystallography. The present review covers the different aspects of this new paradigm in drug design.

Keywords

Protein-protein interaction; Homo-oligomers Disruptor; Protein destabilisation; Allosteric inhibition; Self-association.

Teaser

Disrupting protein self-association bears great therapeutic promesses and could provide exciting alternatives to active-site inhibitors.

1. PPI as a well-accepted paradigm in drug design

The human interactome is nowadays widely considered as one of the keys to cell mechanistic regulation. Targeting protein-protein interactions (PPIs) has thus emerged as a new strategy to fine-tune protein activity and biological processes that are found to be dysregulated in many diseases [1,2]. From cancer therapy to anti-infectious agents, reports of successful therapeutics targeting PPIs are now numerous and can genuinely be considered as one of the great achievement of modern Medicinal Chemistry [3–5]. The emergence on the market of biologicals as well as small molecules targeting PPIs, such as cyclosporine, tirofiban or PDL-1 antibodies, are just a few examples of this ongoing revolution.

During the past 15 years, the ever-growing importance of chemical biology, as well as significant technical progresses, have unlocked the study and targeting of more and more challenging protein interfaces [5]. Among these new landscapes resides the targeting of self-assembling proteins. Protein homo-association is a widespread phenomenon whose dysregulation can lead to diseases [2]. These numerous PPI constitute an underexplored pool of potential targets for therapeutic agents. One early example of a drug targeting protein self-assembly has been the discovery of the anti-cancer drug paclitaxel which binds to β-tubulin, preventing the assembly and disassembly of microtubules [6].

Targeting homomeric complexes has been described through different mechanisms of action and can provide several benefits when compared to a more classic active site approach. Different biophysical and biochemical methods have, besides, been specifically applied to this emerging field of drug design. The present review develops this rising concept of disrupting homomeric interactions.

2. Principles of homomeric assembly

Protein self-association is a ubiquitous phenomenon present in large extent in both prokaryotes and eukaryotes organisms [7–9]. As an illustration, the majority of protein structures elucidated by X-ray crystallography adopt a homo-dimeric or higher homomeric quaternary fold [7,10]. Protein self-association is universal because it provides numerous benefits.

Homomeric complexes increase protein stability as they reduce the hydrophobic clusters exposed to solvent by burying them at the monomeric interfaces [11]. Protein stability indeed often increases along its oligomeric state, and mutations targeting the oligomerization interface are reported to significantly destabilize the mutated protein [12,13]. Homo-oligomers also allow for the production of (near) symmetrical structures, which grant several advantages, such as a reduced propensity to aggregation, higher stability, better robustness to errors in synthesis and an increase in folding efficiency [14,15]. Moreover, protein self-association constitutes an efficient way to produce large macromolecular structure by using a minimal amount of genetic space [14,16].

Furthermore, from a drug design outlook, self-assembly is fundamental for physiological functions. Indeed, self-association is often essential for molecular recognition, whether it is for protein-protein or protein-ligand interaction (**figure 1**) [17].

Figure 1.

Protein self-association can be required for functional assembly of a protein active site. Several enzymes indeed function as homo-oligomers, with active sites containing elements from different subunits [18]. Tryptophan-2,3-Deoxygenase 2, encoded by the TDO2 gene, is an oxidoreductase whose active site is constituted by the association of two different subunits. Its heme cofactor is responsible for the correct folding of the holo-protein active site as well as its oligomerization into an active tetramer [19].

Homomerization can also promote molecular recognition without directly modifying the active site architecture. In such cases, the quaternary structure often improves the complex overall stability, thus indirectly allowing the active site to maintain a proper 3-dimensional structure [10]. From crystallographic data, it appears that *E.coli* purine nucleoside phosphorylase monomer would be the smallest subunit capable of performing a catalytic activity. Nevertheless, experiments demonstrate that this protein needs to adopt a hexameric structure before exhibiting physiologically relevant catalytic activity [20].

These observations extend to membrane receptors such as G-protein-coupled receptor (GPCR) or Tyrosine kinase receptors (TKR) where hetero and homo oligomerization are essential for downstream signaling [21,22]. Transcription factors do not escape to this universal rule as well since homo- (and hetero-) association is necessary for signaling [23]. Dimerization of signal transducer and activator of transcription 3 (STAT3), a transcription factor involved in cellular proliferation and survival, allows its translocation into the nucleus and its binding to promotors of the genes it regulates [24]. Another example involves the dimerization of Epidermal Growth Factor Receptor (EGFR). Upon binding of its endogenous ligand, EGFR extra- and intracellular domains dimerize [25]. This dimerization activates EGFR tyrosine kinase function and is, therefore, essential for downstream signalling [26].

A dynamic self-association has also been demonstrated to fine-tune the activity of proteins. Indeed, some proteins can adopt several ternary folds in their lower homomeric state. These different folds then allow the proteins to oligomerize into different quaternary states, leading to multiple biological functions [27–29]. These proteins, called morpheeins, are now considered as a reservoir of potential drug targets as it appears that several enzymes adopt morpheein-like behaviour [30]. One of the very first examples relates to the porphobilinogen synthase (PBGS), an attractive target in porphyria, tyrosinemia malaria and uremia. PBGS is involved in tetrapyrrolic cofactors biosynthesis such as porphyrin. This protein exists as an equilibrium

between low activity hexamers and high activity octamers via a transition to a dimeric state. The dimers can adopt different conformations each preferentially leading to the formation of hexa- or octamers [31].

During the past years, the rise of PPI as druggable targets, as well as a broader understanding of the fine-tuning role of protein self-association, led to the emerging concept of homomeric disruption as a new strategy in drug design [32]. This new approach to protein targeting has come with several advantages and drawbacks.

3. Benefits from homomeric disruption

3.1. Extended druggability

Several advantages come with the targeting of self-association when compared to active site inhibition. As discussed above, disrupting self-association can lead to a loss of physiological function. Homomeric interfaces thus constitute a pool of potential allosteric sites whose targeting can lead to protein inhibition and destabilization. Targeting of these oligomeric interfaces can, therefore, unravels new approaches for challenging targets. STAT3 is a transcription factor that regulates proliferation, survival and other biological processes. Phosphorylation of this transcription factor promotes its homodimerization and is required for translocation into the nucleus and promotion of genes expression. A constitutive activation of the STAT3 dimer strongly contributes to carcinogenesis and tumor progression [33]. This transcription factor has long been considered as a challenging target due to the lack of an "active-site" to target. Nevertheless, development of peptidic and non-peptidic compounds disrupting the constitutively activated STAT3 dimer have unlocked the targeting of this protein otherwise considered as undruggable [33–37].

Another example involves the Rad52 protein. Rad52 is a protein involved in DNA repair and DNA homologous recombination. Rad52 adopts a heptameric ring superstructure which is essential for its binding to DNA. Disruption of the RAD52 rings by small molecules has enabled the targeting of this DNA-repairing protein previously considered as undruggable [38].

In addition to expanding our reach to new targets, targeting self-assembly can provide several advantages over active site inhibition, such as an increase in selectivity, an escape of compensatory mechanisms, the promotion of protein degradation and sub-stoichiometric inhibition.

3.2. Increased Selectivity

Proteins active-sites often share common features among the same family due to phylogenetic evolution. Selectivity issues can arise over these similarities, and active site inhibitors often display off-target effects due to inhibition of multiple proteins of the same family. However, homomeric interfaces generally tend to be less conserved than actives sites where molecular recognition of common cofactors or compounds is critical [39,40]. Therefore, targeting of these less conserved and more protein-specific sites constitute promising avenues to achieving the desired selectivity (**Figure 2**).

Figure 2.

A well-known example of a selectivity issue among a protein family involves protein kinase inhibition. Protein kinases constitute a protein family playing critical roles in cellular signalling. These proteins thus form an essential class of therapeutic targets, and numerous protein kinases inhibitors are available on the market. However, these enzymes share a highly conserved ATP-binding domain. Therefore, Achieving kinase selectivity through active site inhibition constitutes a challenge, and many inhibitors display a lack of selectivity among the kinome [41,42]. Recent reports of protein kinase oligomeric disruptors thus constitute a promising advance into achieving more selective inhibition of this family of proteins [12,43–45].

Another therapeutic area where selectivity could be achieved through the targeting of self-association is the field of anti-infectious agents. Indeed, targeting of a pathogen protein needs to be selective over its human host equivalent. With this perspective, targeting the oligomeric interface of parasites proteins such as plasmodium falciparum tetrameric malate dehydrogenase (PfMDH) or Trypanosoma cruzi dimeric triosephosphate isomerase (TcTIM) has been suggested as a new approach in achieving selectivity over their human counterparts [46,47].

3.2. Escape of compensatory mechanisms

Targeting oligomeric interfaces can also lead to a by-pass of compensatory mechanisms sometimes encountered with active site inhibitors [43,44,48]. These adaptations can include an increase in substrate biosynthesis, promotion of the target expression, or mutations of the active site. The inhibition of protein self-association whose active-site targeting lead to an adaptive response can provide promising therapeutic results.

A recent example involves the inhibition of the RAF kinase family in cancer therapy. BRAF, a dimeric protein member of this RAF family, is an essential component in the mitogen-activated protein kinase (MAPK) cascade [49]. BRAF is a very frequently mutated kinase in human cancer. These oncogenic mutations lead to a constitutive hyperactivation of the MAPK pathway and thus promote tumorigenesis [49]. ATP-competitive inhibitors, such as vemurafenib, target BRAF most common variant, V600E, which function as a monomer. These inhibitors have demonstrated profound clinical effects for patients presenting this mutation [50]. However, while vemurafenib potently inhibits the monomeric V600E variant, it also paradoxically leads to activation of the wild type protein. This activation stems from an enhanced dimerization between the drug-bound and the drug-free protomers, resulting in transactivation (at low concentration) of the drug-free subunit [50]. The development of peptide targeting the BRAF dimeric interface have demonstrated promising result in overcoming this paradoxical BRAF activation induced by vemurafenib [43,44].

Human thymidylate synthase (hTS), a key enzyme in the folate pathway, is targeted in cancer therapy with 5-fluorouracil (5-FU), a prodrug of the catalytic site inhibitor 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP). hTS can regulate its own expression through interaction with its mRNA [51]. Unfortunately, FdUMP inhibition of hTS can impair this regulatory pathway, hence leading to resistance mechanisms through protein overexpression [52]. Interestingly, the destabilization of hTS using peptides interacting at the protein dimer-interface inhibits hTS successfully without leading to its subsequent compensatory

overexpression and thus allowed to escape this resistance mechanism [48].

Targeting self-association of viral proteins can also lead to a reduced viral adaptation to the drug when compared to active-site inhibitors (**Figure 3**). Viral resistance to drugs is often linked to single point mutations of the active site, leading to a decreased affinity. Regarding human immunodeficiency virus type 1 (HIV-1) protease, mutations under drug pressure often occur close to one another to compensate each other's while granting drug resistance. These concerted mutations are unlikely to happen in the complex dimeric interface where variations are less tolerated [53–55]. In this context, intense efforts were devoted to the development of protein disruptors of various HIV proteins [56]. Another example involves the development of disruptors of the dimeric human Kaposi's sarcoma-associated herpesvirus (KSHV) protease, an essential protein for viral replication. These inhibitors are thought to be broad-acting due to the high conservation of the protease interface among human herpesvirus as well as less prone to mutation-driven resistance, although resistance phenomenon’s can also eventually occur[54]. From a practical standpoint, the use of these allosteric inhibitors, in combination with active site inhibitors, could lead to a lower resistance rate, as it is for instance the case with combination therapy against HIV proteins [57,58].

Figure 3.

3.3. Promotion of protein destabilization and misfolding

Homomeric disruption can also lead to an increase in protein degradation. Indeed, protein disruption leads to protein instability which can be anticipated as a promoter of protein misfolding [12,20,46]. Molecular chaperones can further recognize and degrade these misfolded proteins [59]. Destabilizing compounds have thus already been suggested to promote protein degradation through an inducing of their misfolding [12,60].

Targeting self-association can moreover lead to an increase in protein turnover through

enhanced proteasome-protein degradation. Protein turnover is a highly regulated mechanism involving the exposition of short amino-acids sequence, named degron, that can act as a signalling sequence and lead to protein degradation by the cell machinery [61]. Several degrons can be found at proteins oligomeric interfaces [62]. More specifically, N-terminal acetylation, which occurs in 90% of human proteins, drives a specific degradation signal [63]. These N-terminal acetylated residues are often sterically shielded within the hydrophobic core of oligomeric proteins and thus inaccessible to ubiquitin ligases [64]. Targeting homomeric interfaces can lead to degrons exposition and promote proteasome-dependent degradation [43,65].

Consequently, disrupting self-interaction can lead to protein misfolding as well as an exposition of signal sequence promoting protein degradation (**Figure 4**).

Figure 4.

For instance, targeting of the dimeric interface of survivin, a member of the inhibitor of apoptosis (IAP) gene family, significantly reduces the cellular protein level. Co-treatment with bortezomib, a proteasome inhibitor, rescued this protein loss and demonstrated that targeting the dimeric interface of this oncoprotein induces its degradation through a ubiquitin-proteasome dependant pathway [65].

As discussed previously, inhibition of hTS using peptides targeting its interface converts the dimeric protein into a still dimeric but inactive form. This conversion of hTS into its inactive form further decreases its cellular protein level by around 25%. [48].

Another example involves the disruption of dimeric p8, a subunit of the human transcription factor TFIIH that participates in DNA repair activities. Gervais et al. report that p8 targeting by small molecules binding to its dimeric interface reduces its thermal stability from 88 to 40°C. They further expect this thermal destabilization to shorten the lifetime of p8 and promote its

misfolding *in vivo*, altering its recognition by p52 and subsequent incorporation to TFIIH [66].

Stimulation of protein degradation has recently gained considerable interest through proteolysis- targeting chimaeras (PROTAC) strategy [67]. However, the impact of protein disruption on homomeric degradation has not yet been studied extensively. Therefore, the scientific community could benefit from comparative studies between orthosteric inhibitors and oligomer disruptors.

3.4. Sub-stoichiometry

Finally, targeting of homomeric interface could lead to sub-stoichiometric protein inhibition. Two main mechanisms can account for this effect.

First, related to the paragraph above, homomeric disruption can lead to protein misfolding and degradation. Promoting protein degradation can lead to sub-stoichiometric effect as one inhibitor can induce the degradation of several proteins. As a comparison, promoting protein degradation using PROTAC technology commonly leads to sub-stoichiometric protein inhibition [67].

Interacting at the interface of multiple subunits can also lead to a single molecule inactivating a complete protein complex (**Figure 5**). This full complex inactivation can constitute an advantage when compared to a classical 1:1 inhibition through interaction at an active site [68]. Indeed, inhibition curves reported for oligomeric disruptor are often very steep with hill coefficient well above 1, suggesting a cooperative effect for oligomeric disruptors [38,54,69,70].

Figure 5.

A well-known example includes the mode of action of paclitaxel, which is known to bind to β-tubulin and thus allosterically inhibit the disassembly of microtubules [6]. At low concentration

(low nM), taxol can bind to microtubules in a substoichiometric fashion with up to 1 molecule of paclitaxel for 1000 molecules of β-tubulin in the microtubules [69].

Another example includes the disruption or the Tumor Necrosis Fator (TNF) cytokines family where one inhibitor binds at the interface of the trimeric protein and inactivate the complex (1:3 stoichiometry) [71,72].

Achieving a sub-stoichiometric effect could moreover unlock cellular inhibition of proteins otherwise considered as undruggable due to their high cellular concentrations. Indeed, with a 1:1 stoichiometry, the cell-based inhibition of active site inhibitor often cannot reach below the threshold of their target cellular concentration. This is a well known issue in the context of Lactate dehydrogenase (LDH), a tetrameric enzyme that is an attractive target for cancer therapy [73]. LDH cellular concentration ranges between 1 and 10 μM, depending on the tissue and cancer cell line [74]. Active site inhibitors, even when demonstrating affinity in the low nM range, struggle to cross this μM threshold for *in cellulo* inhibition due to this very high protein concentration [74,75]. Recent advances in LDH disruption through targeting of its tetramerization site offer new avenues to LDH inhibition in cancer therapy [76].

Targeting self-association can, therefore, bring several advantages when compared to active site inhibition. However, several obstacles have to be overcome before achieving homomeric disruption.

4. Challenges in targeting Homomeric association

4.1. Targeting homomeric protein-protein interfaces

The first challenge in targeting self-association is related to the nature of homomeric interfaces. Indeed, homomeric interfaces are protein-protein interactions and thus share the same topological attributes. These interfaces are often characterized by large and shallow regions with hydrophobic "hot spots" clustered around polar clamps [1,7,32,77]. This high complexity often negatively affects the druggability of oligomeric interfaces [1]. The epitope of interaction can also be large and discontinuous, explaining, at least in part, why there are numerous reports of peptides interacting at these interfaces [48,78]. Therefore, targeting protein interfaces constitutes an arduous task and general strategies exploited to target PPI are readily relevant to homomeric interactions [1,79].

Still, inhibitors targeting protein self-association do not always interact at the homomeric interface. Indeed, allosteric sites can sometimes regulate a protein's oligomeric state [80]. Those allosteric sites can share more druggable features and hence were reported to be advantageous over the targeting of protein-protein interfaces [81].

4.2. Complexity of self-association

Self-Association can follow complex patterns, and strong structural knowledge is required to identify key components in subunits associations. Mechanisms of self-association are often sequential or sometimes concerted and depend on the nature of the homomer [14,82,83]. Therefore, different oligomeric interfaces with different binding strengths can thus coexist within the same protein complex. The efficient and rational targeting of these interfaces thus requires extensive preliminary studies.

Protein subunits can also assemble following other patterns. For instance, entire domains of

proteins can symmetrically swap between one another to constitute functional oligomers [82]. As discussed before, morpheein proteins can adopt different conformations leading to diverse oligomeric states, with altered physiological functions [30,84]. Theses different patterns of association further increase the complexity of targeting homomeric interfaces and have already been comprehensively studied and reviewed [7,80,83].

4.3. Disruption of highly stable complexes

Another challenge in achieving the disruption of homomeric complexes resides in their intrinsic stability. Indeed, except for allosteric targeting, inhibitors targeting self-association are subjected to competition with the subunits of the protein complex. Therefore, the probability of success depends on subunits cellular concentration as well as the affinity of the protein complex. The stability of homomeric structures varies within a wide range of affinity [32]. This parameter strongly influences the experimental set-up dedicated to the study of ligand targeting self-association as well as the very nature of their mode of inhibition. Overall, inhibitors of self-association are more common for low-affinity homomers such as viral enzymes [32,54,85–87]. In this regard, a distinction can be made between "weak" and "tight" homomeric complexes.

Homomeric complexes can be considered as "weak" when dissociation occurs at subunits concentration between the mid-nanomolar to the micromolar. This range of concentration is usually suitable for most biochemical and biophysical assays and allow for a direct study of equilibrium in solution.

Study of the dissociation of "tight" oligomers, with dissociation constant (K_d) in the low nanomolar range or below, is often more challenging. Indeed, the low subunit concentration at which dynamic exchange between the oligomeric states occurs precludes the use of most methods widely used for the study of oligomeric states. Moreover, high dilution can destabilize proteins due to issues such as adsorption [88]. Consequently, the study of highly diluted proteins

can be challenging and usually requires technical adaptations. Besides, high-affinity homomeric complexes can challenge the identification of low-affinity interface ligands due to unfair competition with the subunits. Hit discovery, especially of low-affinity compounds, can, therefore, be masked by this intrinsic competition [12,76]. Therefore, a general strategy in the identification of new ligands to tight homomeric interfaces resides in the design of weaker homomers or "forced monomer" by mutating key residues or "hot spots" for homomers association [12,44,89,90].

Proteins inhibition through targeting of their self-association thus constitutes an appealing but challenging concept. Targeting such protein dynamics can give rise to original mechanisms of protein inhibition. The following part of this review will exemplify these different mechanisms.

5. Mechanisms of homomeric disruption

Different mechanisms can account for inhibition through targeting of protein self-assembly. Stability of the homomeric complex, discussed above, is often a crucial parameter and can greatly influence the type of inhibition that can be expected for a given target. Furthermore, these diverse mechanisms can be interconnected, with inhibitors sharing multiple mode-of-action.

5.1. Direct homo-oligomeric disruption

The most direct and intuitive approach to the targeting of self-association is a ligand-induced oligomeric disruption. In this model, ligand binding to its target induces the direct disruption of the protein complex. To that end, the ligand can intercalate at the interface of the homomeric complex, thus weakening the overall cohesion and promoting subunits dissociation (**Figure 6a**) [38,71,91,92].

TNF-α is a pro-inflammatory cytokine involved in immunity, inflammation and antitumor

response. The targeting of the interaction between TNF-α and its receptor is of great therapeutic interest, and many anti-TNF-a antibodies are available on the market. A mechanism of inhibition of TNF-α involves the targeting of this trimeric protein with small molecules and peptides that interact at the trimeric interface and lead to subunit dissociation (**Figure 6b**) [71,93,94].

Figure 6.

Oligomeric disruption can also be the result of interaction at the active site that leads to conformational changes and oligomeric disruption. This specific event can generally occur when the active site is composed of different subunits. Inducible Nitric oxide synthase (iNOS) catalyzes the production of nitric oxide and is a therapeutic target in various inflammatory, immunological, and neurodegenerative diseases. iNOS is active as a dimer with the active site located at the interface of the two monomers. iNOS dimer disruptors act by complexing the iNOS heme cofactor, thereby disrupting the catalytic site architecture and promoting dimer dissociation [95,96].

Another specific case of direct disruption includes the formation of a covalent bond at the protein interface. Phosphoglycerate dehydrogenase (PHGDH) is a tetrameric protein of importance in cancer metabolism. Disruption of PHGDH tetrameric structure has recently been demonstrated as a new mechanism of inhibition through covalent interaction at the oligomeric interface [97].

Formation of a covalent bond at the active site of a protein can also lead to a perturbation of its oligomeric state. Homomeric disruption through covalent inhibition was for instance reported for the ClpP protease. ClpP is a protease of significant importance in *S.*aureus virulence. This protein is active as a tetradecameric unit and inactive as a heptamer due to misalignment of its catalytic triad. Sieber and co-workers reported several irreversible inhibitors that form covalent

bonds in ClpP catalytic site, leading to conformational changes that shift this protease oligomeric state from a 14 to a 7-mer [70].

5.2. Subunit intercalation

Interaction at a tight oligomeric interface is not always sufficient to lead to a disruption of the homomeric complex. Ligand insertion between subunits can indeed happen without inducing dissociation. Subunit intercalation is thus associated with conformational change, which translates into a loss of protein function [47,48,66,72,98]. Inhibition of CD40L, a trimeric cytokine from the TNF family, has been described with a small molecule intercalating deeply between the subunits of the trimeric complex. This intercalation resulted in an inactivation of the CD40L trimer but did not induce dissociation of the complex (**Figure 7**) [72].

Figure 7.

5.3. Capture of an inactive monomeric conformation

Other mechanisms of inhibition can exist for homo-oligomers undergoing a dynamic exchange in solution. Inhibitors can indeed block a monomer into an inactive conformation through a specific interaction with this subunit (**Figure 8a**). This capture of a monomer requires a substantial amount of free monomeric subunit in solution to achieve an efficient inhibition. Such an approach is, therefore, more suited to the inhibition of weak homomeric complexes such as viral proteins [80,87,96,99].

As an illustration, Craik and co-workers studied the targeting of a dynamic region of the KSHV Protease, essential for its dimerization process. Inhibitors interacting at this binding site "locked" an essential tryptophan residue into an "open" conformation, thus preventing subunit dimerization (**Figure 8b**) [54,87].

iNOS dimer disruptors, already discussed above, have also been reported to complex the heme

cofactor of the iNOS monomer subunit. These inhibitors thereby act as direct dimer disruptors together with a monomer sequestration mechanism [96]. This last example illustrates the inter-connexion between the different mechanisms that can account to inhibition through targeting of self-assembly processes.

Figure 8.

5.4. Promotion of inactive multimeric complexes

In opposition to the previous mechanism, ligands can target self-association by stabilizing aberrant or inactive multimeric conformations [100–103]. This mechanism of inhibition involves the interaction at allosteric sites not directly involved in the oligomerization process. The targeting of these allosteric sites is presented as advantageous as they do not compete with the other subunits (**figure 9a**) [27,81].

For instance, targeting of HIV-1 integrase dimeric interface, a key enzyme for viral replication, induces its premature multimerization, thereby preventing its interaction with DNA and subsequent integrase function [81,86,104].

Another example involves the targeting of the serum amyloid P component (SAP) in the treatment of Alzheimer disease. SAP is a pentameric protein playing several roles in the formation of the αβ amyloid fibril deposits. Treatment of SAP with hexanoyl bis(D-proline) (CPHPC) promoted an aberrant decameric complex through non-covalent cross-linking of two pentamers. Promotion of this decameric complex induced a profound depletion of the SAP in the cerebrospinal fluid due to enhanced clearance of this aberrant multimer (**Figure 9b**) [102,103].

Figure 9.

Allosteric inhibition of proteins displaying a morpheein-like behaviours can also lead to the promotion of inactive multimers [27,84]. As already discussed above, morpheeins can adopt

several oligomeric states with different activities. In this context, Jaffe and co-workers reported inhibitors that bind preferentially to inactive hexameric PBGS. Binding to hexameric PBGS stabilized the protein into this conformation and prevented it from going back to its dimeric state and then further oligomerizing into active octamers [31].

5.5. Prevention of association

Targeting of homomeric interfaces can also lead to an inhibition of the multimerization process. This mechanism is described for the inhibition of transmembrane receptors such as G protein-coupled receptors (GPCRs) or tyrosine kinase (TKR) receptor [45,91,105,106]. Prevention of the association of membrane receptor is similar to the mechanism of subunit allosteric capture described above. However, in this situation, oligomerization can be induced by the endogenous ligand, and the dynamism of subunits associations and dissociations is impacted by the fact that these receptors are membrane-bound. Receptors oligomerization can influence downstream signalling, trafficking as well as their stability [107,108]. Therefore, targeting these receptors association constitutes a promising therapeutic opportunity, as well as a mean to obtain pharmacological effects distinct from active-site agonists and antagonists.

For instance, endothelial growth factor rector (EGFR) is a tyrosine kinase receptor whose binding to Endothelial growth factor (EGF) induces its dimerization and activate its tyrosine kinase function [25]. Inhibition of EGFR dimerization has been achieved by peptide targeting either the intra- or extracellular interface, as well as by antibodies sterically disturbing its extracellular association (**Figure 10**) [45,109,110].

Figure 10.

On the other hand, dissociation constants of homomeric complexes are sometimes too low to observe dynamic exchanges in solution. Compounds interacting at the interface of these complexes often require a pre-dissociative step to bind the oligomeric interface. In this

situation, these inhibitors can only prevent or slow down the sub-units association rather than disrupt the protein complex [76,111]. This approach requires denaturating conditions such as low pH or chaotropic agents, to denature the homomer into a lower oligomeric state [112]. An example involves inhibition of the Human glutathione reductase (GR) association into dimers. Dissociation constant of the monomer-dimer equilibrium is estimated to be in the pM range for this enzyme. Identification of peptidic ligands interfering with GR dimerization thus required a pre-dissociation step to observe an inhibition [111]. These harsh conditions can promote aggregation and irreversible denaturation of the protein complex. Therefore, engineering of weaker homomers, as discussed above, is nowadays widely preferred to this homomeric pre-dissociation approach.

6. Specific methods to monitor targeting of self-assembly

Developing homomeric disruptors involves several technical challenges and researchers have thus relied on the use of specific experimental tools that allow for the monitoring of protein self-association. Comprehensive reviews on the several methods available for the monitoring of oligomerization states are available [16]. The present discussion will focus on biophysical and biochemical techniques that have been adapted to the identification and characterization of ligands targeting protein self-assembly. The suitability of these methods will depend on the strength of the homomeric interaction studied. A range of concentration close to the dynamic exchange is usually desirable at least for the identification of new ligands. Therefore, a significant limitation will be the detection and stability of the proteins at these concentrations. **Table 1** hereafter summarizes the different inhibitors discussed in this review with their relative target, mechanism of inhibition and methods used for their study.

6.1. Analytical centrifugation

Analytical centrifugation involves the measurement of a gradient of protein through the

application of a centrifugal field. This method allows the measurement of the sedimentation velocity and the sedimentation equilibrium. First, sedimentation velocity data are useful to study the homogeneity of solutions containing molecular species with different sedimentation coefficient. On the other hand, sedimentation equilibrium provides an accurate determination of the Mw of these proteins [113]. Together, these experiments allow for the characterization of the different species of oligomers in solution [20,114,115]. However, as protein measurement is performed through optical methods (usually following the 280nm absorbance along the radial-gradient), the sensibility of this technique depends on the extinction coefficient of the protein studied. Studies of oligomerization state using analytical centrifugation generally report protein concentrations ranging between mid nanomolar to micromolar.

While this method provides interesting insights into the oligomerization equilibrium of macromolecules, its low throughput makes it challenging for the identification of small molecules disruptors and only few studies exploiting this method are available [99].

6.2. Differential Scanning Fluorimetry

Homomeric dissociation often parallels protein destabilization, and molecules interacting at homomeric interfaces can reduce the thermal stability of the studied oligomers [20,46]. Differential Scanning Fluorimetry (DSF) has thus emerged as a powerful tool for the screening and identification of molecules targeting self-association. Upon heating, protein denaturation leads to exposistion of its hydrophobic core to the solvent. This exposure can be followed either directly by monitoring the intrinsic protein fluorescence (stemming from the tryptophans and tyrosines residues, using nano-DSF); or indirectly by the use of fluorescent probes (such as SYPRO Orange) that will interact with these hydrophobic residues upon exposure. Homomeric disruptors can thus reduce the target thermal stability upon ligand titration [12,46,66,76]. On the opposite, a ligand-induced multimerization into an inactive conformation, as discussed before, can increase protein thermal stability [100,104]. The high throughput of this method

renders it especially suited for the screening of large number of molecules and was applied successfully for the discovery of homomeric disruptors [12]. This technique relies on fluorescent change upon denaturation and µM to nM (when using nanoDSF) amount of protein are usually required. DSF thus allows for the following of the protein stability upon a wide range of concentration. Nevertheless, careful considerations have to be taken when studying thermally destabilizing molecules as promiscuous binders can bind to and stabilize unfolded fractions of the protein, leading to an apparent reduction of the melting temperature, leading to false-positive results [116].

6.3. ELISA

ELISA is a conventional biochemical method for antibody-antigen detection that has been adapted to the study of oligomeric states disruptors. This adaptation requires specific subunit-labelling and relies on the loss of signal after washout upon addition of the disruptor. Disruption of the protein complex will induce a loss of subunits after washout. The read-out stems from molecular recognition of either a non-coated subunit or the full protein complex. ELISA has proven to be a useful tool to quantify ligand-induced complex disruptions of various targets [71,72,78,94,117]. However, since this technique quantifies the loss of subunit after washout upon ligand addition, the protein complex studied must be stable enough, so no significant amount of subunit is lost upon washout in control experiments.

6.4. FRET

Förster resonance energy transfer (FRET) is a useful tool for the study of proteins complexes dynamics. FRET relies on short distances (50 to 100 Å) energy transfers between two light-sensitive (fluorophores) molecules. A FRET assay is composed of at least two components, a donor and an acceptor. Laser-induced excitation of the donor molecule results in a fluorescent photon emission that further excites the acceptor fluorophore which, in turn, emits a

photon[118]. Proteins fluorescent labelling can be either performed by chemical labelling or by fusion with fluorescent proteins (YFP, CFP, for *in-cellulo* FRET).

However, specific subunits-labelling of homomeric complexes can be challenging. Indeed, standard labelling procedures usually cannot control the ratio of donor and acceptor molecules which produces "non-FRET" pairs and reduces the signal-to-noise ratio. Despite this challenge, FRET constitutes a useful tool in the study of ligands targeting homodimeric systems [48,98,119,120]. Moreover, the use of Single-molecule FRET (smFRET) can overcome the poor signal-to-noise ratio that arises when studying homomeric complexes. SmFRET can suppress the background noise stemming from labelling heterogeneity by following a single homomeric complex at a time thanks to single-molecule microscopy [118,121].

Performing FRET experiments require the use of powerful instruments to monitor low-intensity fluorescence. The sensitivity of this method depends therefore on the nature of the fluorescent labelling in regard to its fluorescence intensity and resulting signal to noise ratio.

6.5. Intrinsic tryptophan fluorescence

Intrinsic tryptophan fluorescence (ITF) constitutes a powerful tool for the study of oligomeric state. Indeed, the relative scarcity of tryptophan combined with the high sensitivity of the indole fluorophore to its environment makes this residue a valuable probe for the monitoring of protein oligomerization state [122]. Tryptophan residues are often buried at the hydrophobic interfaces of homomeric complexes. Therefore, changes in the protein quaternary state usually induces a substantial shift in fluorescence intensity as the tryptophan quantum yield decrease in a polar environment, *i.e.* when exposed to surrounding water molecules. Various proteins can thus display a significant change in their intrinsic fluorescence spectrum depending on their oligomerization state [123]. When studying homomeric interfaces, ITF can either be useful for a direct study of interaction at an oligomeric interface containing a tryptophan residue, or for

the monitoring of a change in the oligomeric state induced by an inhibitor [44,76,124]. As ITF relies on protein fluorescence, the sensibility of this technique will depend both on the nature of the oligomeric protein and the instrument's sensitivity.

6.6. Isothermal Titration Calorimetry

Isothermal Titration Calorimetry (ITC) is a reliable tool to measure dissociation and association of a protein. ITC relies on the detection of endo or exothermic pulses that occur upon a binding event. An isothermal titration calorimeter consists of two thermally isolated cells containing the same buffer. Addition of small amounts of a concentrated protein solution into one the cells will promote a dilution-related dissociation of this protein that translates into a heat pulse (generally endothermic). Further addition will yield smaller heat pulses due to an increase in protein concentration which will promote protein association. The calorimeter will monitor these different heat pulses, and the data will then be fitted to obtain a dissociation constant [125]. This method is also a valuable tool for quantitative studies of ligand-protein interactions. ITC has proven to be useful when studying self-association, either for direct measurement of a binding affinity between a disruptor and a protein interface or for monitoring homomeric association upon addition of a protein disruptor [12,38,44,47,48,100]. However, the sensitivity of most calorimeters for the monitoring of protein association usually limits the use of this technique to proteins having a Kd in the µM range as lower concentrations can give rise to heat pulses that are harder to detect.

6.7. Native Mass spectrometry

Native Mass spectrometry (MS) allows for the precise determination of the molecular weight of large macromolecular complexes. This method relies on the non-denaturing ionization of a protein complex using electrospray (ESI). This ionized protein complex keeps its quaternary structure and will be detected by its m/z ratio [126]. Native MS has been very beneficial for

accurate determination of a protein oligomerization state upon addition of oligomers disruptors and can provide both quantitative and qualitative information on protein complex disruption [12,38,70,71,93,100,101].

6.8. Nuclear Magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) experiments and especially two-dimensional HSQC NMR is a valuable tool to monitor the binding of ligands at oligomeric interfaces [54,66,87,127,128]. This method follows the variation of the chemical shift of key residues of a ^{13}C- or ^{15}N- labelled protein through HSQC 2D NMR upon addition of a ligand. Protein-based 2D NMR is a low throughput method that required µM amount of pure labelled protein as well as strong structural knowledge of the target to identify and assign the chemical shift of key interface residues. While technically challenging, protein-based NMR provides valuable structural information about the ligand-binding site as well as direct information about the ligand-induced protein conformational changes.

6.9. Polyacrylamide gel electrophoresis

PolyAcrylamide Gel Electrophoresis (PAGE) is a common biochemical tool used for qualitative and semi-quantitative information about a ligand-induced oligomeric disruption. PAGE can be performed under native (native PAGE) or denaturating conditions by using cross-linking.

Native PAGE relies on the separation of protein complexes along with a polyacrylamide gel according to their electrophoretic mobility. Under native conditions, a protein electrophoretic mobility will depend on its masse-to-charge ratio as well as on its size and shape. After migration, numerous methods are available for further protein complex detection, such as activity assays, western-blot or non-specific staining. These various methods of detection allow for the use of native PAGE under a broad range of concentration. Native PAGE has proven to

be useful for semi-quantitative studies of ligands interfering with self-association [31,38,65,117].

Cross-linking can provide a static picture of a protein oligomeric-state. This biochemical method relies on the addition of a covalent reagent that links interacting subunits together. The separation of the cross-linked subunits will then depend on their electrophoretic mobility under denaturating conditions. Under these denaturating conditions, this electrophoretic mobility will depend only on the masse to charge ratio. Cross-linking offers qualitative and semi-quantitative information about the oligomeric state of a protein upon addition of a ligand and has been successfully applied to it [91,93,97,124]. A wide range of protein concentration are available when using cross-linking as protein concentration can be increased after addition of the covalent reagent and before the loading of the protein onto the SDS PAGE.

6.10. Size exclusion chromatography

Size exclusion or gel filtration chromatography (SEC) follows the migration of macromolecules through a column whose stationary phase is composed of inert polymeric porous beds. This method allows for the measurement of the size of macromolecules. SEC constitutes, therefore, a direct probe of the oligomeric states of proteins in solutions. Monitoring of ligand-induced change in oligomeric-state using SEC was reported for various targets such as viral proteases[54,90], inducible nitric oxide synthase (iNOS) [95], proteases [70] or GPCR [100]. SEC provides direct and qualitative information about the ligand-induced disruption of protein complexes.

SEC sensitivity depends on the method used for protein detection. UV absorption is the most common read-out for protein detection and thus requires µM amount of protein. However, the exploitation of other read-outs such as enzymatic activity, radioactivity, mass spectrometry or even ELISA, can extend the sensitivity of this method to more diluted or complex media

[95,129].

6.11. X-ray crystallography

X-ray crystallography provides a high-resolution 3D-structure of a protein-ligand complex. X-ray crystallography was successfully exploited for the obtention of structural data of ligands interacting at protein complex interfaces [47,48,71,72,87,95,101,130]. However, several parameters can challenge the acquisition of X-ray data for oligomer disruptors. First, the obtention of protein crystals often relies on the optimization of stabilizing conditions for the protein complex [131]. Molecules interacting at protein homomeric interfaces often destabilize the protein target, and the presence of these destabilizing ligands can thus challenge the identification of suitable conditions for crystallization [132]. Protein homomeric disruption can also be dependent on subunits concentration, and a high concentration can shift the equilibrium towards the formation of the protein complex. As crystallization studies require very high protein concentration, these experiments could shift the equilibrium towards the formation of a protein complex, excluding the ligand.

Table 1.

7. Summary and Outlook

Targeting homomeric proteins is a stimulating but largely underexplored area of drug design. Over the past decades, significant technical progress has provided Medicinal Chemists with a large toolbox of biophysical and biochemical methods and strategies to succesfully target protein self-associations. As reports of ligands breaking constitutive protein self-interactions become more common, one begins to understand the promising advantages that this strategy can afford over "active-site" targeting.

Breaking homo-oligomers bears great therapeutic promises with molecules able to induce protein degradation and inhibition in a sub-stoichiometric fashion. As the potential of target-induced degradation is currently greatly emulating the scientific community, inhibitors of self-association could provide exciting alternatives to the PROTAC strategy.

Furthermore, inhibiting protein oligomerization can enable the targeting of proteins deemed as "undruggable". Indeed, homomeric interfaces constitute a reservoir of allosteric sites that could unlock the inhibition of these challenging targets. Targeting of these interfaces can thus unravel new mechanisms of protein inhibition and provide original compounds. This "out of the box" strategy also provides great prospects in avoiding common pitfalls of active site targeting such as selectivity issues and development of compensatory mechanisms.

We believe, therefore, that homomeric disruptors have yet to demonstrate their full therapeutic potential.

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Conflicts of interest.

The authors declare no conflicts of interest.

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Figure legends

Figure 1. Protein self-association is a key parameter for protein function and stability. Schematic representation of the different aspects in which self-association drives molecular recognition.

Figure 2. Targeting the homomeric interface can unlock the inhibition of challenging proteins and yield more selective ligands. (a) Conventional orthosteric inhibitors are unable to target proteins lacking an active-site while (b) the targeting of homomeric interfaces can unlock the inhibition of these challenging targets. (c) An active-site ligand can display poor selectivity profile due to the high conservation of the active site among the protein family. (d) A homomeric interface ligand can display increased selectivity due to the high specificity of its target's interface.

Figure 3. Inhibitors targeting homomeric interface are less likely to be subject to mutation-driven resistance. (a) Concerted mutations can occur at the active site due to drug-pressure and provide resistance while (b) mutations at the complex protein interface are less likely to be tolerated.

Figure 4. Homomeric disruption can promote protein degradation and misfolding. Disruption of a homomeric complex can lead to proteasome-dependent protein degradation due to degron exposition or protein misfolding due to intrinsic destabilization and chaperone-mediated degradation.

Figure 5. Targeting homomeric interfaces can lead to substoichiometric protein inhibition. (a) Representation of an active-site inhibitor inhibiting a protein complex in a 1:1 stoichiometry. (b) Representation of a substoichiometric inhibitor targeting a protein complex interface.

Figure 6. Inhibition of homomeric protein through complex disruption. (a) Schematic representation of the general mechanism. (b) (Left) Crystallographic structure of the apo trimeric TNFα cytokine (PDB ID: 1TNF). (right) Crystallographic structure of dimeric TNFα cytokine in complex with a ligand that induces subunit dissociation (PDB ID: 2AZ5).

Figure 7. Inhibition of CD40L through subunit intercalation. (Left) Crystallographic structure of the apo trimeric CD40L cytokine (PDB ID: 1ALY). (right) Crystallographic structure of the CD40L cytokine in complex with a ligand that intercalates deeply at the interface of the trimer without inducing subunit dissociation (PDB ID: 3LKJ).

Figure 8. Inhibition of homomeric proteins through monomer capture. (a) Schematic representation of the general mechanism. (b) (left) Crystallographic structure of the monomeric truncated Δ196 KSHV protease in complex with inhibitor DD2 that traps this protein into an inactive monomeric conformation (PDB ID: 3NJQ). (right) Crystallographic structure of the KSHV protease in its apo dimeric conformation (PDB ID: 2PBK).

Figure 9. Inhibition of homomeric proteins through the promotion of aberrant multimers. (a) Schematic representation of the general mechanism. (b) (left) Crystallographic structure of the apo pentameric SAP (PDB ID: 2W08). (right) Crystallographic structure of the SAP non-covalently cross-linked by CPHPC into an aberrant decameric conformation (PDB ID: 4AVT).

Figure 10. Representation of the different strategies exploited to prevent EGFR association into a dimer. (a) Targeting of extracellular domain with antibodies or peptides. (b) Targeting of the intracellular asymmetric dimer with peptides.

Figure 11. ELISA can be adapted to monitor ligand-induced protein complex disruption. Washout of the disrupted subunit leads to a loss of recognition by specific antibodies and subsequent diminution of ELISA signal. HRP: horseradish peroxidase.