Protein–Protein Interactions

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Small-Molecule Stabilization of Protein–Protein Interactions: An Underestimated Concept in Drug Discovery?**

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The modulation of protein–protein interactions (PPIs) has been recognized as one of the most challenging tasks in drug discovery. While their systematic development has long been considered as intractable, this view has changed over the last years, with the first drug candidates undergoing clinical studies. To date, the vast majority of PPI modulators are interaction inhibitors. However, in many biological contexts a prolonged lifespan of a PPI might be desirable, calling for the complementary approach of PPI stabilization. In fact, nature offers impressive examples of this concept and some PPI-stabilizing natural products have already found application as important drugs. Moreover, directed small-molecule stabilization has recently been demonstrated. Therefore, it is time to take a closer look at the constructive side of modulating PPIs.

1. Introduction

Protein–protein interactions (PPIs) are of utmost importance for all living organisms. The underlying association of cellular proteins into functional protein complexes as well as their dissociation is a highly dynamic process which is regulated by different cellular mechanisms (Figure 1).[2] All together, PPIs in an organism form a huge and complex network known as an "interactome", which substantially contributes to the regulation and execution of the majority of biological processes. The size of the binary human interactome has recently been re-estimated to comprise about 130 000 PPIs, of which only about 8% have as yet been identified.[3] The growing knowledge in this field offers novel starting points for the development of alternative therapeutic approaches. One of the most prominent strategies is small-molecule-based modulation of PPIs, which can be achieved in two complementary ways: namely, by stabilization or inhib-

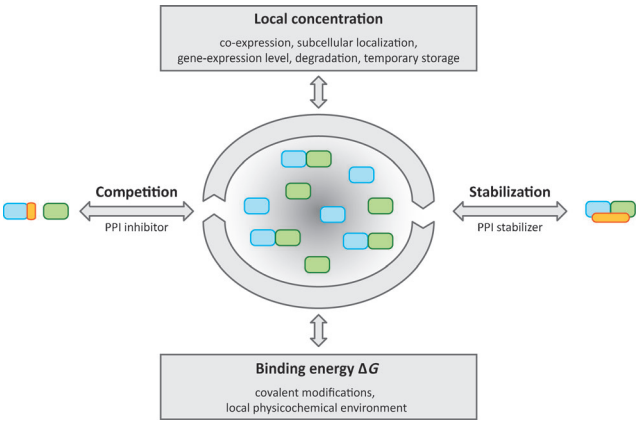

Figure 1. Important regulatory control mechanisms for the association state of interacting proteins. The equilibrium between monomeric and multimeric proteins is regulated by the local concentration of the partners and their mutual binding affinity. External factors can compete for one partner or stabilize the multimeric complex. (Adapted from Nooren and Thornton.[2])

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[**] In the general annotations to presented structure figures, SES is the solvent-excluded surface, and highlighted interaction surfaces are defined as surface patches within a distance of 3.5 Å next to the binding partner. All representations were generated with BALL-View.[1]

ition (Figure 1). To date, the inhibition of PPIs has mostly been explored, as demonstrated by numerous important publications and reviews in this field.[4,5]

In this Minireview, we want to highlight the "other side" of PPI modulation—namely, the stabilization of PPIs—with a focus on small molecules that strengthen naturally occurring PPIs by binding to the surface of protein complexes.

2. Small-Molecule Stabilization of PPIs

The mode of action of the majority of currently described PPI inhibitors is based on the direct binding of a small molecule to the interaction surface of one of the protein partners, thereby sterically preventing the binding to its interaction partner.[4,5] Nevertheless, some examples have been shown to inhibit a PPI in an allosteric fashion by exclusively binding to a surface region of one protein partner outside of the protein interaction interface itself.[4] Small-molecule stabilizers of PPIs also show two general modes of action. First, a stabilizer can bind to a single protein partner, which increases the mutual binding affinity of the protein partners in an allosteric fashion. Second, the stabilizing molecule binds to the interfacial surface of a protein complex and makes contacts to both binding partners, which also increases the mutual binding affinity. Correspondingly, the different types will in the following be termed as allosteric (one protein partner) or direct (at least two protein partners) PPI stabilizers.

2.1. Allosteric PPI Stabilizers

2.1.1. Modulators of Microtubule Dynamics

Microtubules (MTs) are built from protein heterodimers of α- and β-tubulin. The αβ heterodimers assemble into linear protofilaments, which form cylindric polymers. MTs have important functions in both nondividing and dividing cells. To fulfill the different tasks, MTs have to be rearranged regularly, which occurs by continuous polymerization and depolymerization.[6] The impairment of this complex process has dramatic consequences for the cell, especially during cell division, where MTs form the mitotic spindle that segregates the chromosomes.[7] Several natural products and derivatives thereof induce cell-cycle arrest by modulating MT polymerization and depolymerization, thereby resulting in severe cellular disturbances that can even lead to apoptosis. Thus, some of these molecules are used as antimitotic agents and belong to the most important drugs in the treatment of cancer.[8]

One of the most intensely studied MT modulators is paclitaxel (Figure 2), which was isolated from the bark of *Taxus brevifolia*.[9] Paclitaxel binds with high affinity to a hydrophobic pocket of polymerized tubulin which is exclusively located on the β subunit, thereby stabilizing polymerized MT structures (Figure 3A,B).[10] Although the exact molecular basis of its stabilizing effect is still a matter of debate, it seems that the binding of paclitaxel, amongst other effects, strengthens the lateral contacts of neighboring β subunits in the microtubule filament.[11] Thus, paclitaxel stabilizes PPIs in an allosteric fashion.

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2.2. Direct PPI Stabilizers

Two different modes of action have been observed so far for direct PPI stabilizers. 1) The stabilizing molecule first binds to one of the proteins, thereby creating or modifying the interaction surface for the second protein. This stabilizing effect can be so strong that two proteins can be induced to dimerize that do not bind to each other in the absence of these molecules. This extreme case is observed for the FKBP binding molecules FK506 and rapamycin. 2) The stabilizing molecule directly binds to the rim of an already established protein–protein interface and increases the binding affinity of the interaction partners. Such a binding mode also represents the molecular basis of action of the small molecules forskolin, fusicoccin A, epibestatin, and pyrrolidone1.

2.2.1. FK506 and Rapamycin

Perhaps the most prominent examples of bivalent PPI-stabilizing molecules are the immunosuppressants FK506 and

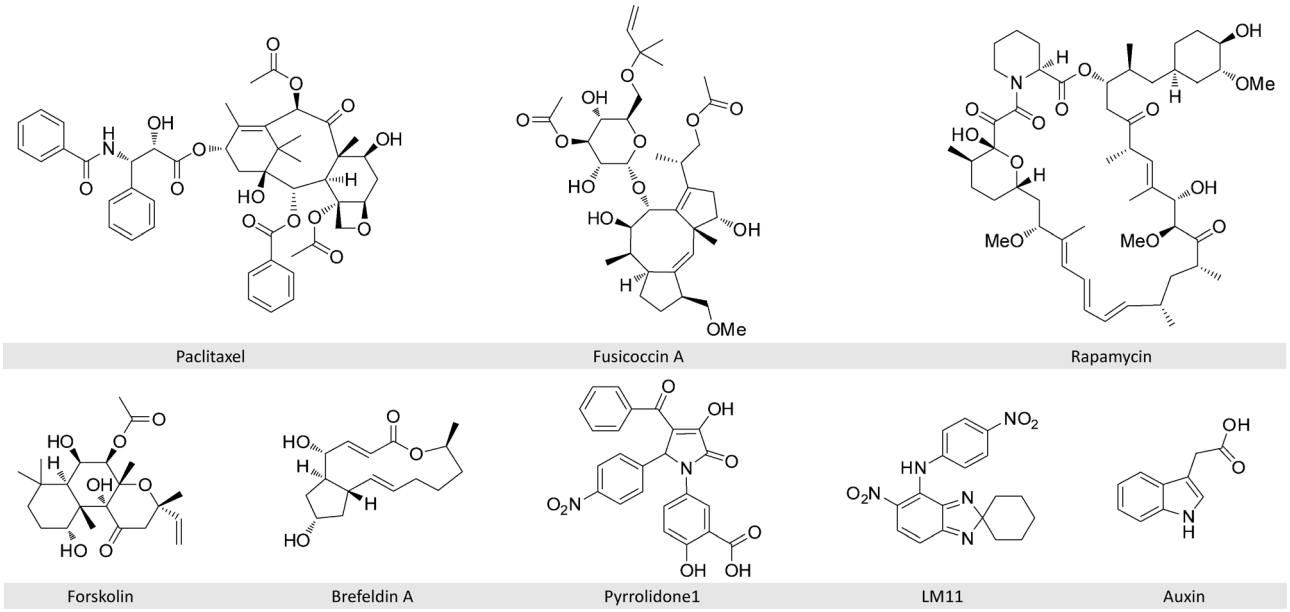

Figure 2. Examples of PPI-stabilizing small molecules discussed in this Minireview, to illustrate their wide range of chemotypes and structural complexity.

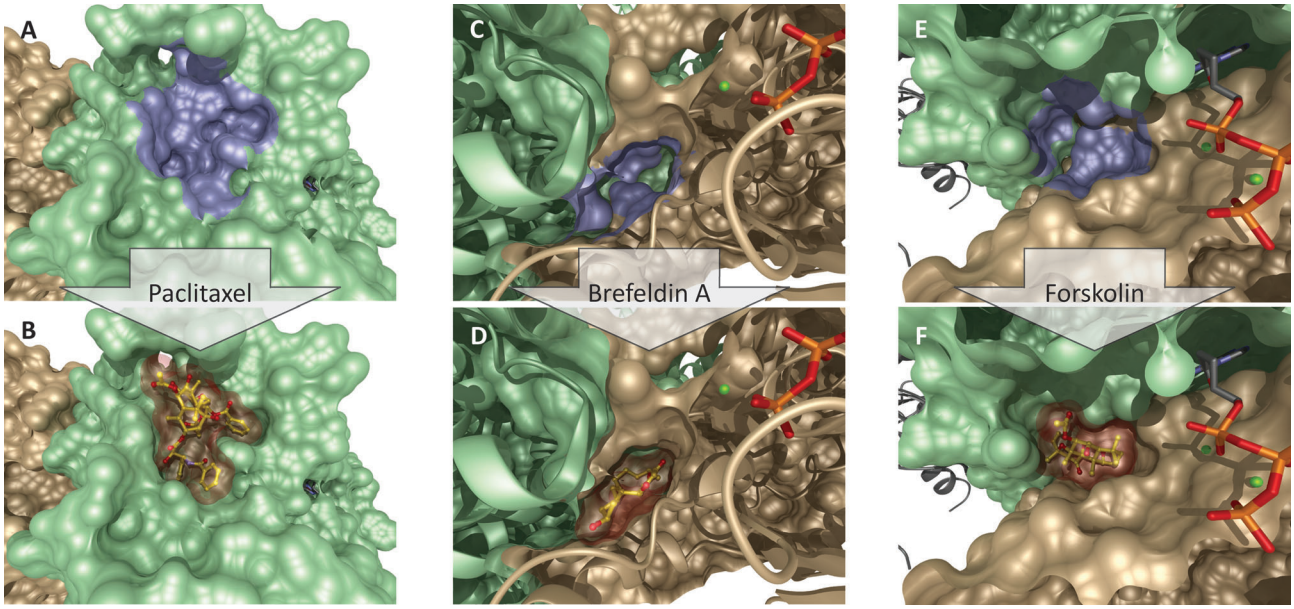

Figure 3. A) α-tubulin (gold SES) bound to β-tubulin (green SES). The binding pocket of paclitaxel, which is exclusively located on the β-tubulin subunit, is highlighted in blue. B) Paclitaxel bound to the complex (ball-and-stick model, semitransparent SES). PDB number: 1JFF.[10b] C) Section through the complex of ARF1 (gold SES and schematic representation) with the GTP analogue (stick model) and a Sec7 domain (green SES and schematic structure). The BFA binding pocket is highlighted in blue. D) BFA (ball-and-stick model, semitransparent SES) buried deep within the interface pocket. PDB number: 1R8Q.[20] E) Catalytic subunit of AC with an ATP analogue (stick model). The C_{1a} domain (green SES) and the C_{2a} domain (gold SES) are shown together with the forskolin pocket highlighted in blue. F) Forskolin (ball-and-stick model, semitransparent SES) bound to the dimer interface. PDB number: 1CJU.[29]

rapamycin (Figure 2). Despite structural differences, the in vivo effects of these natural products are remarkably similar. In two ground-breaking studies, Schreiber and co-workers identified the biological effector proteins of FK506 and rapamycin: the protein phosphatase calcineurin and the protein kinase "mammalian target of rapamycin" (mTOR), respectively.[12] The first step in the mode of action of these immunosuppressants is high-affinity binding to FKBP12 ($K_d = 0.2$ nM for rapamycin[13]), a member of the immunophilin protein family of peptidyl-prolyl isomerases.[14] Subsequently, the binary complexes FK506·FKBP12 and rapamycin·FKBP12 associate with calcineurin and mTOR through the newly created interaction surface, thereby resulting in inhibition of the catalytic activity of these enzymes. Remark-

ably, FKBP12 binds to neither calcineurin nor mTOR in the absence of FK506 and rapamycin, respectively.

The molecular basis for the extraordinary activity of these natural products was revealed by the crystal structures of the ternary complexes. In the FK506·FKBP12·calcineurin complex, the small molecule FK506 is buried deep in a binding pocket formed by calcineurin and FKBP12.[15] The majority of contacts that mediate the complex stability are formed between the proteins and FK506, while much fewer direct contacts occur between the two protein partners.

For the rapamycin·FKBP12·mTOR complex, these unequally distributed binding contributions are even more pronounced (Figure 4 A–C).[16] Here, only two minor direct contacts between the protein partners can be identified, thus explaining the low affinity of FKBP12 to mTOR in the absence of rapamycin.

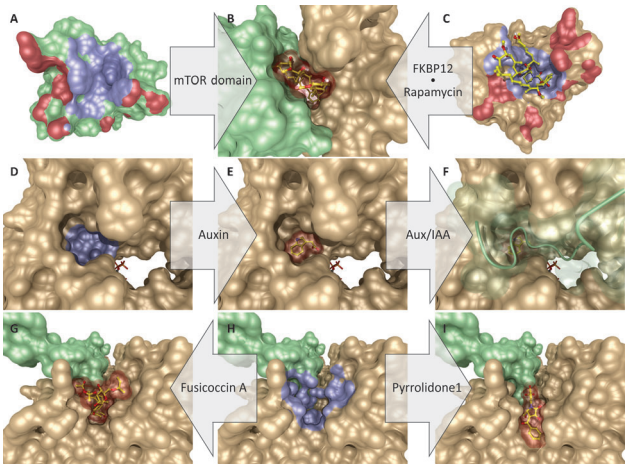

Figure 4. A) Rapamycin-binding domain of mTOR (green SES). The interaction surface to rapamycin is highlighted in blue and to FKBP12 in red. B) Ternary complex of the mTOR domain (green SES), rapamycin (ball-and-stick model, semitransparent SES), and FKBP12 (gold SES). C) Rapamycin bound to FKBP12. The interaction surface to rapamycin is highlighted in blue and to the mTOR domain in red. PDB number: 1FAP.[15] D) TIR1 (gold SES) with the auxin binding pocket highlighted in blue. The stick-model in the background shows the possible cofactor inositol-hexakisphosphate. E) Auxin (ball-and-stick model, semitransparent SES) bound to TIR1. F) Ternary complex of TIR1, auxin, and Aux/IAA peptide (green schematic representation and semitransparent SES). PDB number: 2P1Q.[35] G) Ternary complex of 14-3-3 protein (gold SES), PMA C-terminal domain (green SES), and FCA (ball-and-stick model, semitransparent SES). H) Binary complex of 14-3-3 protein and the PMA C-terminal domain, with FCA pocket highlighted in blue. PDB number: 2O98.[40] I) Ternary complex of 14-3-3 protein, PMA2 C-terminal domain, and pyrrolidone1 (ball-and-stick model, semitransparent SES). Pyrrolidone1 from PDB number 3M51 is shown in the FCA structure.[41]

2.2.2. Brefeldin A

Brefeldin A (BFA) is a fungal metabolite isolated from *Eupenicillium brefeldianum* which potently inhibits protein secretion by stabilizing the complexes of the small guanine nucleotide-binding (G) protein "ADP ribosylation factor 1" (ARF1) and several of its guanine nucleotide exchange factors (ARF-GEFs) to different extents.[17,18] This leads to blockage of the GDP/GTP exchange activity of ARF-GEF, which ultimately results in impairment of the Golgi function.[19] Despite its rather modest IC_{50} value of 15 μM and an only tenfold stabilization of the complex between ARF-GDP and Sec7, cellular effects are severe because of the underlying uncompetitive inhibition mode. Visible fusion between the Golgi and the endoplasmic reticulum already occurs within minutes after administration of the compound. The crystal structure of the ARF1-GDP·Sec7·BFA complex shows that the small molecule is deeply buried between the two proteins, with two-thirds of its contact surface with Sec7 and to one-third with ARF1.[20,21] The binding of BFA to both proteins is mostly hydrophobic in nature, with few additional polar contacts (Figure 3 C,D). Upon complexation, the conformation of BFA shows no significant deviation from its small-molecule crystal structure. BFA binds exclusively to the ternary complex; no binding to ARF1-GDP or Sec7 alone can be observed.[22]

These findings inspired the search for further small molecules that directly stabilize the ARF1·Sec7 interaction; this resulted in the in silico identification of LM11 (Figure 2), which inhibited ARF1 activation in vitro with an apparent inhibition constant of 50 μM and impaired ARF-dependent trafficking in cells.[23] Similar to BFA, LM11 also showed specificity for certain ARF isoforms, inhibiting ARF1 and ARF5, for example, but not ARF6.

2.2.3. Forskolin

The diterpenoid forskolin (Figure 2) was isolated from *Coleus forskohlii* in 1977 and used as a cardioactive and blood-pressure-lowering compound.[24] Later, it was shown that the molecular basis for forskolin action is a reversible increase in adenylyl cyclase (AC) activity, which results in a significant increase in cAMP levels in various tissues.[25] AC is a transmembrane protein with two cytoplasmic domains (C_1 and C_2), each consisting of two subdomains, with subdomains C_{1a} and C_{2a} heterodimerizing and forming the catalytic core of AC.[26] Subsequent biochemical studies demonstrated that forskolin increased the apparent affinity of the C_1 and the C_2 domains from $K_d > 10$ μM to $K_d \approx 1$ μM and resulted in a 60-fold enhanced catalytic activity of AC.[27] Since cAMP, the prototypic second messenger molecule and the catalytic product of AC, has important functions in cell physiology, forskolin has been proven to be an important tool in various cell biological studies. The underlying mode of action of forskolin has been elucidated by two crystal structures of AC domains complexed with this natural product. In the first structure, forskolin is bound to a homodimer of the C_2 domain of AC isoform II (IIC_2) in a 2:1 stoichiometry.[28] The second structure shows a forskolin derivative binding in a 1:1 stoichiometry to the ternary complex of the C_1 domain of AC isoform V (VC_1), the C_2 domain from AC isoform II (IIC_2), and the activation subunit of its stimulatory G protein ($G_{s\alpha}$, Figure 3 E,F).[29] Both structures revealed that forskolin binds in a deep and primarily hydrophobic pocket at the end of a long cleft at the interface of the IIC_2·IIC_2 and the VC_1·IIC_2 dimers, respectively. The small molecule shares

equivalent contacts to both binding proteins, burying roughly 90% of its solvent-accessible surface and closing a hydrophobic pocket between the AC domains.

2.2.4. Auxin

Auxin (indole-3-acetic acid, Figure 2) is an important phytohormone that plays a pivotal role in plant biology.[30] Seminal biochemical studies in the 1980s showed increased transcriptional activity in response to auxin.[31] Subsequent genetic analysis revealed several loci sensitive to auxin levels, and the observed effects could be mapped onto the ubiquitin-proteasome system, with the F-box protein TIR1 as a critical component.[32] F-box proteins are substrate receptors in multisubunit E3 ubiquitin ligases. Members of the transcriptional repressor family Aux/IAA have been identified as direct target proteins of TIR1, and auxin significantly stabilizes the interaction between TIR1 and Aux/IAA.[33,34] This molecular mechanism was ultimately uncovered in 2007 by Tan et al., who solved the crystal structure of the ternary complex of TIR1, Aux/IAA, and members of the auxin class (Figure 4D–F).[35] The structure identified PPI stabilization by auxin as a novel mechanism for hormone perception. Auxin binds to a pocket of TIR1 which is buried deep in a leucine-rich repeat domain that features two polar residues and an adjacent hydrophobic pocket. The polar residues interact with the carboxy group of auxin, while its indole moiety occupies the hydrophobic pocket. Together, TIR1 and auxin generate a new cavity with a joint interaction surface. Aux/IAA binds to this cavity through a coiled sequence, with a hydrophobic core motif stacking directly onto the auxin molecule. Thus, auxin and its derivatives close a mainly hydrophobic gap in the TIR1 and Aux/IAA interface which leads to the observed stabilizing effect.

In 2010, the crystal structure of a further PPI-stabilizing phytohormone (jasmonate) complexed with an F-box protein (COI1) and a transcriptional regulating protein was solved and shows a similar molecular mechanism as auxin.[36]

The mechanism of these phytohormones is particularly important because they are the first characterized examples of PPI-stabilizing small molecules which are metabolites of the organism that also harbors the respective protein target. This regulation depicts a novel concept for the mode of action of endogenous small molecules, which has to be taken into account when investigating the mechanism of other metabolic products.

2.2.5. Fusicoccin, Epibestatin, and Pyrrolidone1

The diterpene glycoside fusicoccin A (FCA, Figure 2) is a metabolite from *Phomopsis amygdali* (formerly *Fusicoccum amygdali*). In 1964, Ballio et al. reported FCA to be the toxic agent of this pathogenic, wilt-inducing fungus.[37] Studies on the molecular target resulted in the identification of a complex between the regulatory domain of the plasma membrane H^+-ATPase (PMA) and 14-3-3 adapter proteins as the primary receptor.[38] FCA binds to the interface of this complex and enhances the affinity of the two proteins by about 90-fold.[39] Whereas the affinity of FCA to the complex of 14-3-3 and a phosphorylated PMA peptide is moderate ($K_d = 700$ nM), the affinity to 14-3-3 alone is quite low ($K_d = 66$ μM) and binding to the PMA peptide is not measurable at all. In this regard, FCA displays a similar mode of action as brefeldin A and forskolin, and is distinct from FK506 and rapamycin. FCA fills a hydrophobic gap in the interface of the two proteins, with the sugar moiety exposed to the solvent (Figure 4G,H). The terpene ring is buried deep in a funnel-like pocket formed by 14-3-3 and the C terminus of PMA. In addition to its ability to enhance the apparent affinity of a short C-terminal phosphopeptide derived from PMA for 14-3-3, FCA is also able to stabilize the complex of 14-3-3 with a longer and unphosphorylated protein construct comprising the last 52 residues with an apparent affinity of 41 nM.[40]

To demonstrate the applicability and usefulness of small-molecule stabilizers of 14-3-3 PPIs, a high-throughput screening devoted to the identification of stabilizers of the 14-3-3·PMA interaction was recently performed. From a moderate-sized screening library (ca. 37000 compounds), two chemically diverse and FCA-unrelated compounds were identified.[41] Both compounds, that is, the dipeptide epibestatin and a trisubstituted pyrrolidone, named pyrrolidone1, stabilized the 14-3-3·PMA interaction, but intriguingly occupy different binding pockets at the PPI interface. Epibestatin binds to a narrow surface cleft and is tightly sandwiched between the two proteins. The molecule interacts in equal measures with 14-3-3 and PMA. In contrast, pyrrolidone1 occupies a more solvent-accessible site, which substantially overlaps with the binding pocket of FCA (Figure 4I).

2.2.6. Stabilization of Native Protein Oligomers

An impaired stability of native protein oligomers can be the source of severe diseases. Thus, a possible therapeutic approach could be the stabilization of such oligomeric complexes by small molecules. One example is the stabilization of the transthyretin (TTR) tetramer.[42] TTR is a transporter for thyroxine (T4) and retinol, and can form amyloid fibrils, which are associated with amyloid diseases. Several mutations are known which destabilize the tetramer and facilitate the formation of amyloid fibril. Miroy et al. showed that small-molecule binding to the T4 binding site is able to stabilize the TTR tetramer and inhibit fibril formation in vitro. It is noteworthy that the design of small-molecule stabilizers that address the binding pocket of a native ligand, as in the TTR example, will in other cases most probably not be a promising strategy.

A second example is the motor neuron disease amyotrophic lateral sclerosis (ALS), which is characterized by several mutations in the gene encoding for the dimeric enzyme superoxide dismutase 1 (SOD1).[43] Some of the mutations have been recognized to cause SOD1 monomerization, thereby leading to protein aggregation.[44] Ray et al. focused on a strategy to stabilize the SOD1 dimer to prevent monomerization by using small molecules.[45] To find such stabilizers they performed a virtual screening on a hydrophobic pocket at the dimer interface and identified 15 compounds which showed SOD1-stabilizing activity in vitro, and thereby prevented aggregation.

A third example targets various human cancers in which the transcription factor c-Myc is constitutively overexpressed, which leads to uncontrolled and pathogenic gene expression.[46] The activity of c-Myc is highly dependent on heterodimerization with its partner "myc-associated factor x" (Max). In addition to successful attempts to inhibit this PPI to suppress c-Myc activity, Jiang et al. made use of the ability of Max to homodimerize and tried to stabilize the Max homodimer to prevent c-Myc activity by binding to Max.[47] By means of virtual screening, they successfully identified small molecules capable of stabilizating the Max·Max dimer and preventing c-Myc·Max interaction.

3. Drugability of PPI Interfaces

The presented examples highlight different mechanisms for the stabilization of PPIs and indicate that this approach—complementary to the inhibition of PPIs—offers a valuable alternative for the discovery of novel bio-active small molecules. This, however, raises the question of whether these examples can be regarded as being representative and if this concept can be generalized. A first answer to this question was given by Block et al., who performed a systematic analysis of surface cavities at the rim of PPI interfaces and their possible drugability.[48] The results revealed 380 interfacial pockets on 198 transient PPI complexes extracted from the Protein Data Bank that displayed considerable similarities with enzymatic substrate binding pockets in terms of their hydrophilicity, cavity volume, and burial, thus suggesting a reasonable chance of successfully addressing PPIs with stabilizing small molecules.[49]

These findings are particularly interesting because small-molecule PPI inhibitors have been recognized from a classical viewpoint to be less druglike, with the possible consequence that these chemical entities are underrepresented in current screening libraries.[5] In contrast, the occurrence of rim-exposed PPI cavities with highly similar properties to known drug binding pockets suggests that there is a reasonable chance of finding stabilizing candidate molecules in current compound libraries.

Of special interest might be to address PPIs that, when stabilized, "normalize" a diseased state instead of inducing apoptosis, similar to the microtubule-stabilizing drugs. A possible target for this approach might be the cystic fibrosis transmembrane conductance regulator (CFTR), whose functions could possibly be restored upon stabilizing its interaction with protein partners such as NHERF1.[50] Another example could be the stabilization of the interaction between the tumor suppressor protein p53 and 14-3-3, which might enhance the physiological activity of p53.[51] The attenuation of detrimental or pathologic signaling might also be restored to "normal" levels by small-molecule stabilization. Examples of the latter could be the complexes of IκB·NFκB or Raf·14-3-3.[52]

4. Summary and Outlook

Nature provides us with a number of compounds that make a good case for the concept of PPI stabilization as a promising strategy in drug discovery and chemical biology. Small molecules that bind to the rim of the interface in protein–protein complexes display important advantageous features. Since they bind to their targets in a noncompetitive manner, their affinity does not need to be in the low nanomolar range to trigger a strong physiological effect. Furthermore, in contrast to the development of enzyme inhibitors, which is hampered by strong homologies within enzyme families, the binding surfaces of binary protein complexes are often more distinct, thereby facilitating the development of more-specific small molecules. The latter is, furthermore, supported by the fact that the target of a PPI-stabilizing molecule exists only temporarily. In addition, the rim of the interface of a given protein–protein complex has been demonstrated to provide multiple binding sites, thus allowing the identification of chemically diverse stabilizers, as shown with FCA, epibestatin, and pyrrolidone1 in the case of the 14-3-3·PMA2 interaction.

Despite these promises, the examples of natural products acting as PPI stabilizers, and in silico analysis of the "drugability" of the rim of protein complexes, only a few examples of PPI stabilizers resulting from target-oriented small-molecule discovery have as yet been reported. As experience increases, we are confident that PPI stabilization will soon become more popular in drug discovery, thereby following the path of success that PPI inhibitors started ten years ago.

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