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REVIEW

Stabilization of protein-protein interactions in drug discovery

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ABSTRACT

Introduction: PPIs are involved in every disease and specific modulation of these PPIs with small molecules would significantly improve our prospects of developing therapeutic agents. Both industry and academia have engaged in the identification and use of PPI inhibitors. However in comparison, the opposite strategy of employing small-molecule stabilizers of PPIs is underrepresented in drug discovery.
Areas covered: PPI stabilization has not been exploited in a systematic manner. Rather, this concept validated by a number of therapeutically used natural products like rapamycin and paclitaxel has been shown retrospectively to be the basis of the activity of synthetic molecules originating from drug discovery projects among them lenalidomide and tafamidis. Here, the authors cover the growing number of synthetic small-molecule PPI stabilizers to advocate for a stronger consideration of this as a drug discovery approach.
Expert opinion: Both the natural products and the growing number of synthetic molecules show that PPI stabilization is a viable strategy for drug discovery. There is certainly a significant challenge to adapt compound libraries, screening techniques and downstream methodologies to identify, characterize and optimize PPI stabilizers, but the examples of molecules reviewed here in our opinion justify these efforts.

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Chemical biology; druggable genome; medicinal chemistry; PPI stabilization; PPIs; small molecules; tool compounds; X-ray crystallography

1. Introduction

Protein–protein interactions (PPIs) are one of the core processes by which cells function and interact with their environment, making them very interesting targets for modulation by small molecules. Consequently, the ongoing development of PPI inhibition as a therapeutic strategy is a great leap forward for the field of medicinal chemistry, greatly increasing the druggable genome. However, the opposite strategy of PPI stabilization is still underrepresented in the scientific literature, despite promising early results in the field. The most compelling argument in favour of small-molecule PPI stabilization stems from the numerous examples of natural products that convey their physiological activity by stabilizing specific protein complexes. Some of them have been used for many years as therapeutic agents, like the immunosuppressants cyclosporin (Sandimmun®, Novartis Pharmaceuticals), FK506 (Prograf®, Astellas Pharma) and rapamycin (Rapamune®, Pfizer), or the anti-cancer agent paclitaxel (Taxol®, Bristol Myers Squibb). Others, like brefeldin A, forskolin and the diterpene glycosides fusicoccin A and cotylenin A, are tremendously useful biological tool compounds. As these natural product PPI stabilizers have been known for a long time, they have already been extensively reviewed on multiple occasions [1–4] and will not be covered in depth in this review. This review will focus on synthetic PPI stabilizers after a brief update on natural product actin stabilizers that have not been reviewed before, and a reflection on natural product PPI stabilizers in drug discovery. An overview of all reviewed compounds can be found in Table 1.

2. Natural product PPI stabilizers

2.1. Natural products mimicking actin-binding protein gelsolin: swinholide A, rhizopodin, and lobophorolide

Actin and actin polymerization are essential components of the cellular cytoskeleton [5–7]. Actin remodeling is often associated with malignant phenotypes such as cancer, which renders actin and actin dynamics a potential drug target [8–10]. Many natural products have been identified as actin stabilizers [11], which hints at a possible evolutionary role for these natural products in their host organism as defense molecules, given the abundance and central role played by actin in cell survival.

Swinholide A (Figure 1) is a cytotoxic marine macrolide produced by the marine sponge *Theonella swinhoei* [12], which disrupts the actin cytoskeleton by severing actin filaments and stabilizing G-actin as an unphysiological homodimer complex in a 2:1 G-actin-ligand stoichiometry [13]. The

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Article highlights

- Stabilization of PPIs has been exploited by nature who has evolved highly active and selective compounds for targets otherwise difficult to reach.
- The drug discovery community has not yet embraced PPI stabilization as a viable strategy to modulate biological targets.
- Most known synthetic PPI stabilizers have been discovered by accident, without PPI stabilization being the intended strategy.
- Lead generation is the limiting step in the systematic exploitation of PPI stabilization as a strategy to address biological targets.
- Computational methods have had minor successes in PPI stabilizer lead generation, but still little is known about preferable screening conditions, desirable molecular scaffolds and compound libraries for the generation of PPI stabilizers.
- Developing methodology for PPI stabilization lead development could grant access to compounds that are capable of modulating new, previously undruggable targets such as transcription factors.

This box summarizes key points contained in the article.

44-membered macrocyclic structure of swinholide A exhibits a twofold axis of symmetry, which is key to the compound's mode of action. The structure of the swinholide A-G-actin ternary complex, solved to 2.0 Å resolution by Rayment and co-workers in 2005 [14], shows that while the two actin monomers do not make contact with one another, both halves of the ligand make simultaneous, mostly hydrophobic, contacts with both actin monomers. The binding site of swinholide A on G-actin overlaps with that targeted by the cytotoxic trisoxazole macrolide toxins Kabiramide C and jaspisamide A, which in contrast to swinholide A, form a 1:1 G-actin-ligand complex [15]. Another C_2-symmetric macrocyclic natural product, rhizopodin (Figure 1) [16], was recently found to also inhibit actin polymerization via stabilization of a G-actin homodimer complex [17]. Similar to the swinholide A-G-actin structure, the two actin monomers within the rhizodopin-G-actin structure make minimal contacts with one another. In contrast to the swinholide A-G-actin complex, however, each half of the C_2-symmetric structure of the rhizodopin macrocycle makes contact with the hydrophobic surface of only one actin monomer. Intriguingly, lobophorolide (Figure 1), a 22-membered macrolactone secondary metabolite isolated from the brown algae *Lobophora variegate* [18], was also found to stabilize the G-actin homodimer complex, only this time via a cooperative 2:2 stoichiometry in which each lobophorolide molecule can be seen making simultaneous contacts with both actin monomers and the second lobophorolide [19]. This mode of action is reminiscent of that seen for the Roche p53-activator, RO-2443 (quod vide), which acts by stabilizing an MDMX homodimer complex [20].

2.2. Natural products targeting actin filaments: phalloidin, jasplakinolide, and dolastatin

The heptapeptide mushroom toxin, phalloidin has long established itself as a stabilizer of actin filaments [21,22]. In fact, the staining of fixed cells with dye-labeled phalloidin derivatives is

Table 1. Overview of the PPI stabilizers in this review.

Compound	Protein complex	PDB code	K_D (μM)	EC_{50} (μM)
Natural product Actin stabilizers				
Swinholide A	G-Actin	1YXQ	0.025 [13]	–
Rhizopodin	G-Actin	2VYP	–	0.005 (cell assay) [141]
Lobophorolide	G-Actin	3M6G	n/d	n/d
Phalloidin	F-Actin	–	0.010 [21]	–
Jasplakinolide	F-Actin	–	–	0.035 [28]
Dolastatin 11	F-Actin	–	–	9.5 [34]
Post hoc identified synthetic PPI stabilizers				
RO-2443	HDMX/HDM2	3U15/3VBG	0.078 [20]	0.041/0.033 [20]
NS309	SK-CaMBD/CaM	4J9Z	–	0.44 [47]
Tafamidis	Transthyretin	3TCT	K_{D1} = 0.003; K_{D2} = 0.27 [71]	2.7 [71]
ICRF-187 (Dexrazoxane) /ICR-193	Topoisomerase II	1QZR (ICRF-187)	–	0.1 (ICRF-193) [142]
Trifluoroperazine	S100A4	3KO0	–	150 [87]
Compound 3	Influenza A virus nucleoprotein	3RO5	–	0.04 [89]
Ifenprodil	GluN2B/GluN1b	3QEL	0.32 [91]	–
Pleconaril	Rhinovirus 14	1NA1	–	0.16 [99]
Lenalidomide	CRL4/CK1α	5FQD	0.242 [100]	–
CC0651	Cdc34/ubiquitin	4MDK	–	1.72 [103]
(R,R)-2a	GluR2	3BBR	–	0.73 [109]
CK-636	Arp2/3 complex	3DXK	–	24 [111]
BMS-8	PD1/PD1-L	5J8O	–	0.146 [115]
BMS-202	PD1/PD1-L	5J89	–	0.018 [115]
BiBET	BRD4	5AD3	–	0.000100 [118]
BMS-493	RAR/NCoR	3KMZ	0.18 [124]	–
GW6471	PPARα/SMRT	1KKQ	–	0.24 [125]
4-hydroxytamoxifen	ERRγ/SMRT	1S9Q	0.035 [136]	–
Asoprisnil	PR/NCoR	2OVM	–	0.0002 [126]
Intentionally found synthetic PPI stabilizers				
Epibestatin	14–3-3/PMA2	3M50	1.8 [143]	–
Pyrrolidone 1	14–3-3/PMA2	3M51	80 [143]	–
Isoproterenol	SOD1	4A7T	n/d	n/d
5-Fluorouridine	SOD1	4A7S	n/d	n/d
Compound 24	Aldolase/TRAP	4TR9	n/d	n/d

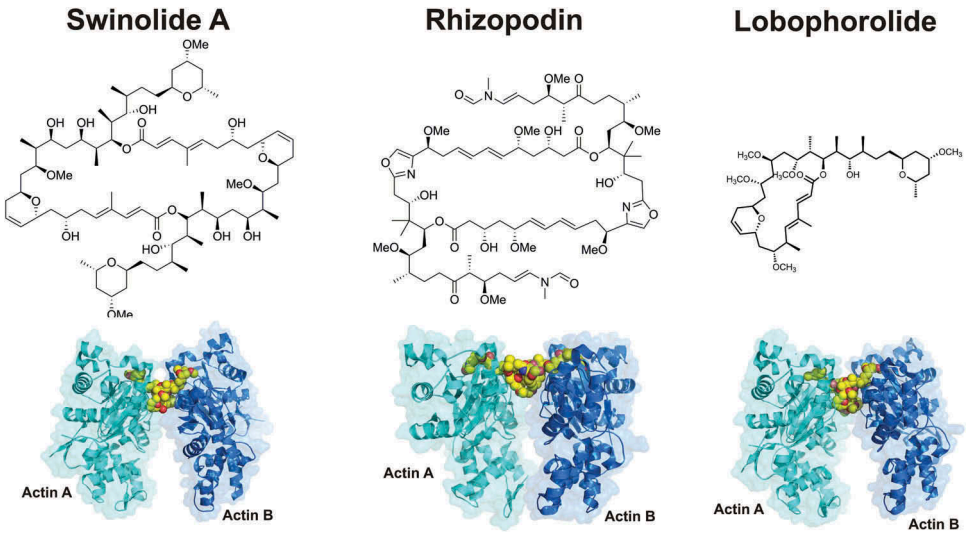

Figure 1. Natural-product actin stabilizers.

a routine technique in cell biology to visualize the actin cytoskeleton by fluorescent microscopy, specifically actin filaments [23,24]. However, attempts at the structure elucidation of phalloidin's binding mode at high resolution have been hindered by the practical difficulties of working with dynamic polymerizing actin. Early biochemical and cellular studies concluded that phalloidin stabilizes actin filaments [21] and induces actin polymerization [25], while later scanning transmission electron microscopy studies on undecagold-tagged phalloidin [26] and X-ray fiber diffraction analysis of rhodamine-labeled phalloidin [27] revealed the position and orientation of the molecule within actin filaments, which is at the interface of three actin monomers in a 1:1 stoichiometry with the dye/undecagold-tag protruding away from the binding cleft into solvent.

Jasplakinolide, a cyclic natural product of mixed polyketide/peptide origin isolated from the marine sponge, *Jaspis johnstoni*, competes for the same binding site on filamentous actin as phallodin [28]. Consequently, jasplakinolide stabilizes actin filaments *in vitro*, and disrupts actin filaments *in vivo* [29]. Analogous macrocyclic secondary metaboliltes, chondramide C [30–32] and doliculide [33] have been shown in both biochemical assays and in cells to interact with actin filaments with a similar mode as phalloidin and jasplakinolide. However, there is currently no structural evidence to corroborate these findings. The marine depsipeptide, dolastatin 11 has also been reported to stabilize F-actin *in vitro* [34]. However, contrary to phalloidin and jasplakinolide, dolastatin 11 was shown by X-ray fiber diffraction analysis to bind at an alternative site to phalloidin on actin filaments, in the cleft between the two long-pitch F-actin strands [35].

2.3. Natural product PPI stabilizers and drug discovery

The pool of natural product PPI stabilizers provides us with a wealth of inspiration and examples, and should thus not be overlooked. First, there is a strikingly large variation in molecular structures, ranging from large macrocycles – such as cyclosporin A, lobophorolide and ustiloxin D – to small *hydrophobic* compounds – like brefeldin A – to small highly hydrophilic compounds – for example, inositol tetraphosphate. Being natural products, many of these compounds are terpenoid or peptidic in nature, often displaying complex three dimensionality, yielding high shape complementarity with their corresponding targets. With the notable exception of taxol, which exerts its function in an allosteric fashion, these natural products bind at the interface of the protein binding partners in the complex that they stabilize. This means that shape complementarity is in respect to a pocket that is formed by two or more protein partners, yielding the potential for very high selectivity against this specific combination of proteins. This is indeed illustrated by rapamycin being an inhibitor of exclusively the kinase mTOR through stabilization of a PPI with FKBP12 [36].

Natural product PPI stabilizers have demonstrated the validity of this strategy to treat disease and the potential for high selectivity. Similarly to the revolution after the establishment of PPI inhibition as a viable therapeutic strategy, the ability to stabilize specific PPIs would mean a huge increase in the number of druggable targets, allowing drug discovery to intervene in pathways or targets that were not previously considered in drug development. However, despite these promising characteristics and examples, studies focusing on PPI stabilization are relatively scarce.

3. Synthetic PPI stabilizers

Despite being underrepresented, the number of known synthetic PPI stabilizers in the literature is steadily growing. In this review, we will attempt to provide an exhaustive overview of all these synthetic compounds that stabilize a complex of two or more proteins for which structural information (i.e. biochemical data showing a stabilizing effect and an X-ray crystal structure showing the compound binding the protein complex) is available. That being said, we acknowledge that there are other compounds in the literature which effectively stabilize single protein entities [37,38] or for which biochemical assays suggest PPI stabilization as the mechanism [39,40], but which have not yet been confirmed by structural data.

In their 2016 review, Zarzycka et al. classify different PPI stabilizers according to the type of complex that they stabilize [2]. In the light of drug development we have, however, chosen to divide the compounds into (I) compounds for which *post hoc* mechanism determination showed them to be PPI stabilizers and (II) compounds which were identified in studies specifically looking for PPI stabilizers.

3.1. Synthetic PPI stabilizers identified post hoc

3.1.1. RO-2443

The activation of the tumor repressor protein p53 by inhibiting its PPI with the negative regulator MDM2 is a promising approach for the development of novel anti-cancer drugs, the most famous example of which are the Nutlin class of compounds [41]. However, this approach is ineffective in cells with normal expression levels of MDM2, as MDMX seems to take over MDM2's role of suppressing p53 anti-tumor activity. It is therefore desirable to develop compounds that inhibit both the p53/MDM2 and the p53/MDMX interaction. Thus, researchers at Roche screened a library of small molecules to identify such dual inhibitors and were able to identify a series of indolyl-hydantoin based molecules that indeed inhibit the interaction of p53 with both suppressor proteins in an *in vitro* HTRF assay [20].

Surprisingly, the mechanism of inhibition involves the stabilization of the formation of homodimers and, while not experimentally proven, possibly also heterodimers of MDM2 and/or MDMX. Co-crystal structures showed that RO-2443 (Figure 2) occupies a groove of MDM2/MDMX with three pockets where normally p53 is shown to bind, referred to as the Phe19, Trp23 and Leu26 pockets [42]. Upon 2:2 binding of RO-2443 to MDM2/MDMX, dimerization to a quaternary complex is induced, where each of the two drug molecules makes contact with both protein partners in an antiparallel fashion. The indolyl-hydantoin moiety of one molecule RO-2,443 occupies the Phe pocket of one of the dimer partners and the difluoro-phenyl moiety occupying the Trp pocket of the other dimer partner. Together, the two drug molecules provide a dual bridge between the two proteins. Additionally, a large part of the interaction interface is provided by the two drug molecules, indicating that this is indeed an interaction that is mediated and stabilized by the drug.

3.1.2. NS309

The small conductance K^+ channel (SK) is regulated through Calmodulin CaM, which is a calcium sensing protein that opens SK in the presence of high concentrations of Ca^{2+} [43]. The SK itself is associated with a broad range of diseases, including several cancers and hypertension [44]. The 1-EBIO (1-ethyl-2-benzimidazolinone) class of compounds were reported to open the SK K^+ ion channels as early as 1996. They are part of a family of benzimidazolinones that were found to open up several K^+ channels in whole-cell patch clamp ion current assays [45,46, p.2]. These assays showed increased ion current through the cell upon compound addition, but the mechanism of action remained unknown. The co-crystal structure of CaM in complex with the CaM binding domain of SK (CaMBD) showed that 1-EBIO binds to the interface of these two proteins, acting as a

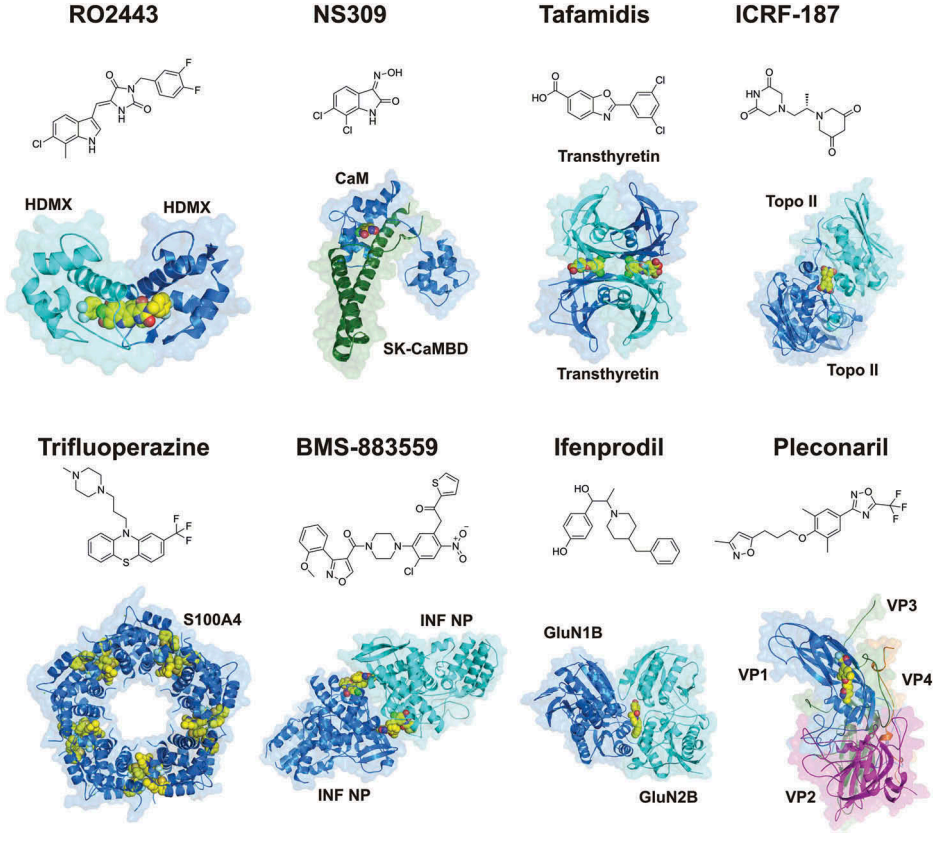

Figure 2. Synthetic compounds I that stabilize PPIs.

stabilizer of the interaction [47]. In this way it, mimics constitutively high Ca^{2+} concentration and binding of CAM to SK, causing the channel to be permanently opened. While the stabilizing potency of 1-EBIO is rather low (EC_{50} = 395 μM), molecular docking showed that the much more potent analog NS309 (EC_{50} = 0.44 μM, Figure 2) is also capable of binding the same pocket [47]. Indeed, crystallography with NS309 was able to show that it binds in the same binding pocket and in doing so also stabilizes an intrinsically disordered part of the protein, making it visible in the electron density where it was previously not [48]. Additionally, a new series of structurally similar compounds has been suggested to bind in the same location as NS309 and 1-EBIO, as seen through mutational and docking studies. However, no structural data has been obtained for these compounds as of yet [49].

3.1.3. Tafamidis

Amyloid diseases are characterized by the deposition of protein aggregates known as amyloid fibrils [50]. One such condition is transthyretin amyloidosis (ATTR), which is believed to be caused by the aggregation of transthyretin (TTR, previously known as prealbumin) and is often fatal within 10 years [51]. Under healthy, physiological conditions TTR is a tetramer, but disassembly into smaller oligomers, misassembly and misfolding have been identified as possible causes of TTR aggregation and ATTR development [52]. The natural thyroid hormone (S)-Thyroxine (T_4) was found to be an inhibitor of TTR aggregation and a stabilizer of the TTR tetramer. This finding was the basis for the therapeutic strategy to prevent TTR aggregation by small molecule induced stabilization of the tetramer [53], which has led to the development of a vast number of T_4 mimicking compounds [54–70]. One specific compound, tafamidis (Figure 2) has been approved by the European and Japanese medicines agencies and is currently being considered for approval by the US Food and Drug Administration (FDA). This promising compound indeed stabilizes the TTR tetramer by binding to the interface of two component proteins as shown by X-ray crystallography [71], in this way inhibiting the formation of fibrils. More importantly, it also stabilizes the clinically relevant mutant forms V30M and V1221I, making it a broadly applicable drug in early stage ATTR [71].

In addition to tafamidis, very recently another compound, tolcapone, has been reported to bind to the same TTR dimer interface pocket [72]. Tolcapone has already been FDAapproved for treatment in Parkinson's disease, making it a useful additional candidate for ATTR treatment. It has a higher TTR tetramer stabilizing potency, which seems to be due to a better fit into the interface pocket, as shown by X-ray crystallography [72].

3.1.4. ICRF-193 and ICRF-187 (Dexrazoxane)

The family of anthracyclines is one of the most effective chemotherapeutics available for the treatment of various tumor types [73]. Despite their efficacy, cardiotoxicity due to the generation of reactive oxygen species (ROS) limits their use in the clinic [74,75]. The FDA-approved cardioprotective drug bisdioxypiperazine dexrazoxane (ICRF-187) (Figure 2) is therefore being used in combination with anthracyclines. This compound is hydrolyzed *in vivo* to form ADR-295, which reduces ROS formation by scavenging free iron ions [76]. More interestingly, ICRF-187 derivatives, ICRF-193, were found to be inhibitors of topoisomerase II (topo II), an enzyme which actively facilitates DNA disentanglement, using *in vitro* decatenation assays [77]. It exerts its action by locking the dimeric enzyme in an inactive closedclamp state, which has been shown to also contribute to oxidative stress relief [78,79]. In later crystallographic studies with the *S. cerevisiae* enzyme, it was shown that this molecule directly stabilizes the closed-state dimer by binding to a symmetric pocket formed by the two topo II monomers, forming a molecular bridge between the two proteins [80]. Even though the compound is rather polar and can potentially form up to 12 hydrogen bonds, its binding mostly relies on hydrophobic contacts to a 'dome of tyrosines' and the expelling of six out of eight water molecules from the pocket. While this mode of action makes the compound a stabilizer of a specific conformation of a protein complex rather than the overall formation of a complex, it does so by directly stabilizing the interacting surface of the two interacting proteins, qualifying it as a PPI stabilizer.

3.1.5. Trifluoperazine

The S100 proteins were named after their ability to stay soluble in 100% ammonium sulfate [81]. More importantly, high S100A4 expression has been found to contribute to metastasis in several cancers, neurodegeneration, rheumatoid arthritis, kidney fibrosis, and cardiac hypertrophy [82–85], making it a very interesting drug target. It is mainly homodimeric, and exerts its action through binding Ca^{2+} and subsequent binding to other proteins [84]. Through screening of a library of FDA-approved compounds, a series of phenothiazines was identified to inhibit the Myosin-II related activity of S100A4 [86]. Subsequent crystallography showed that one of these compounds, trifluoperazine (TFP) (Figure 2), not only binds to S100A4 and inhibits Myosin-II activity, but also induces the formation of a pentameric oligomer, where most contacts between the monomers in the pentamer are mediated through two copies of TFP [87]. Next to crystallography, sedimentation and crosslinking experiments showed that oligomerization not only occurs in the solid state, but also at physiological solute conditions in the presence of TFP. This shows that this is indeed a physiologically relevant example of PPI stabilization by a small molecule.

3.1.6. Compound 3 & BMS883559

The influenza nucleoprotein (INF-NP) is a crucial protein in viral replication through the encapsidation of viral genetic material and binding of single-stranded RNA. It has therefore previously been used as a drug target [88]. High throughput screening for compounds that inhibit viral replication in a whole cell assay led to the identification of a series of compounds that inhibit this protein [89]. Subsequent crystallographic studies showed how two copies of one of these compounds, compound 3, bind in an anti-parallel fashion to the interface of NP_A and NP_B, two subunits of the NP assembly. Upon binding of the compounds, the formation of this complex is stabilized, which subsequently induces higher order oligomerization to form inactive hexameric structures. Some more structurally similar compounds, such as BMS-883559 (PDB ID 4DYB) (Figure 2), have been deposited into the PDB showing a similar binding mode, but no publication has been associated with them as of yet.

3.1.7. Ifenprodil

Ifenprodil (Figure 2) is an anti-hypertension drug in phase II clinical trials that has, along with a family of compounds known as phenylethanolamines, also been shown to possess neuroprotective activity by inhibiting *N*-methyl-D-aspartate (NMDA) receptors *in vivo* and using whole cell assays [90]. These receptors are ion channels that consist of heterodimers of mostly GluN1 and GluN2, and are activated upon binding of glycine and glutamine, leading to neurotoxic effects [90]. The mode of action of ifenprodil had long been studied using mutational studies, which indicated that it binds to the GluN2 and GluN1 Amino-Terminal Domain (ATD). However, crystallographic studies with the ATDs showed that it binds to the interface of two GluN1 and GluN2 ATDs instead of just one [91]. This is supported by a 20-fold stabilization of the complex as determined using sedimentation experiments and detectable complex formation in ITC exclusively in the presence of the compound.

In the crystal structure ifenprodil is sandwiched between two ATD domains, binding mainly through hydrophobic interactions and a hydrogen bond to each ATD monomer. While the overall conformation of the proteins themselves is not greatly affected, the compound locks the two protein partners into a closed-clamp structure, forming a complementary bridge between the two monomers. Recent crystallography and electron cryomicroscopy have shown that stabilization of this closed clamp state by ifenprodil is also maintained in the full-length protein, which in turn allosterically stabilizes the closed state of the ion channel [92,93].

3.1.8. Pleconaril

Pleconaril (Figure 2) is a compound in phase II clinical trials that belongs to a family of isoxazole-derived picornavirus inhibitors, initially discovered in an anti-viral screening campaign using a whole-cell infection assay [94]. Their anti-viral activity against various viruses was later shown to stem from binding to and stabilization of the virus capsid [95,96], as well as inhibition of ICAM-1 mediated virus to host attachment [97]. The viral capsid structure consists of repetitions of four viral proteins (VP1–VP4) that together form the building block for the viral icosahedral encapsidation. This tetrameric protein complex also contains the binding site for attachment to ICAM-1. Crystallographic studies with EV-D68 and HRV14 viruses have shown that the pleconaril family of molecules binds to this tetrameric VP1–VP4 protein complex, in a pocket underneath the ICAM-1 binding site known as 'the canyon' [98,99]. The compound lies deeply buried inside a hydrophobic barrel within VP1, making a single contact with VP3 through Ala24 (HRV14) or Val24 (EV-D68). It has, however, not been shown whether this direct contact with VP3 is needed for stabilization. Some studies show the same pocket in VP1 being occupied by fatty acids or other small molecules, making the same contact with VP3 as pleconaril, but these do not show such strong stabilizing activity [99]. Despite a wide variety of compounds that have been developed for this pocket, no systematic study on their effects on the binding of the protein partners to each other has been performed. Nevertheless, it is clear that the overall complex is stabilized in the presence of pleconaril.

3.1.9. Lenalidomide

Lenalidomide (Figure 3) is structurally related to pomalidomide and thalidomide, which are together known as immunomodulatory drugs (IMiDs). All of their therapeutic

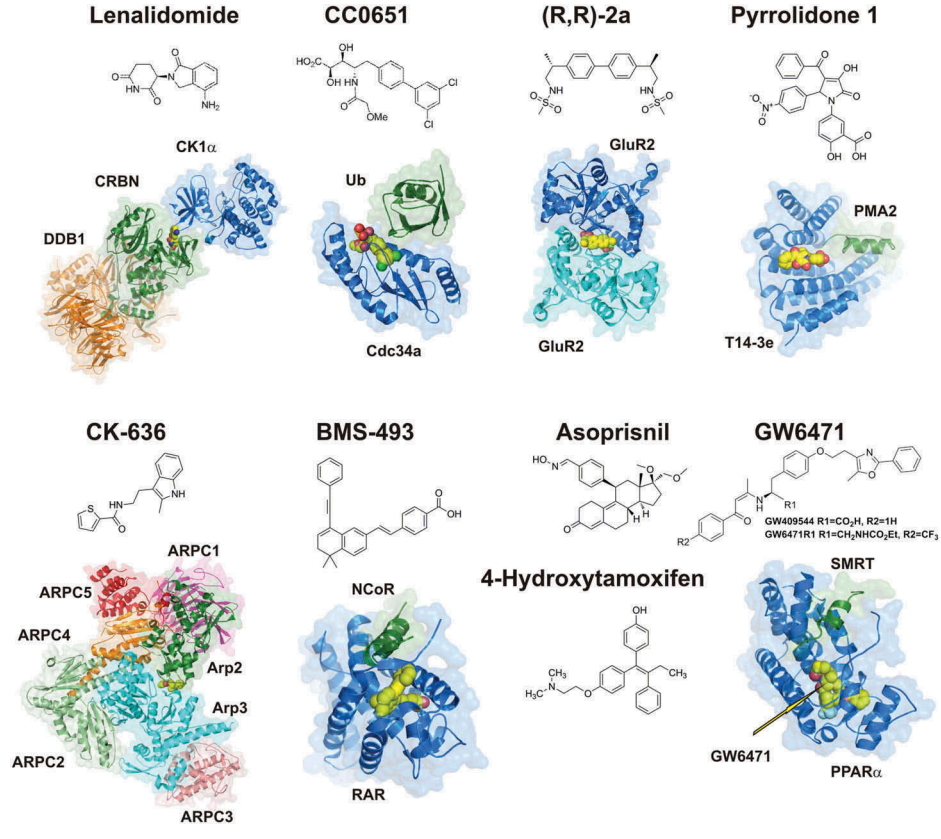

Figure 3. Synthetic compounds II that stabilize PPIs.

development was achieved without a clear understanding of their mode of action, but in the past few years they have been shown to function by redirecting the E3-ubiquitin ligase cereblon (CRBN). CRBN is the substrate adaptor of the $CRL4^{CRBN}$ E3-ubiquitin ligase, a cullin-ring ligase composed of damaged DNA-binding protein 1 (DDB1), cullin 4a (CUL4A), and regulator of cullins 1 (ROC1). In multiple myeloma, ubiquitination through CRL4 and subsequent proteasomal degradation of two members of the B-cell IKAROS family transcription factors, IKZF1 and IKZF3, effectively kills multiple myeloma cells. In the rarer del(5q) myelodysplastic syndrome (MDS), the substrate marked for degradation is the casein kinase 1A1 (CK1α), which leads to the subsequent death of the del(5q) cells.

Crystallography has clearly shown that IMiDs stabilize the interaction between $CRL4^{CRBN}$ and CK1α while at the same time blocking the binding of the endogenous substrates (MEIS2) thereby modulating the ubiquitin ligase by simultaneous up- and down-regulation of the ubiquitination of proteins [100]. The glutarimide ring of the IMiD makes hydrophobic and hydrogen-bonding interactions with the CRBN domain while the phthalimide end of the molecule also makes one hydrogen-bond to CRBN with the remainder of its interactions being hydrophobic to the CK1α protein. There are also direct interactions between the CRBN and CK1α proteins, making this a three body synergistic interaction, which is supported by the fact that there is no detectable binding of CRBN to CK1α (or to IKZF1) in the absence of an IMiD [100].

Interestingly there is little sequence conservation between CK1α and IKZF1 with the exception of Gly151 in IKZF1, which is equivalent to Gly40 in CK1α. However there appears to be an important conformational homology in these binding domains allowing $CRL4^{CBRN}$ to recognize a number of proteins in the presence of IMiDs. This liganddependent interaction is all the more surprising as the hydrophobic phthalimide C5-C7 region contributes only ca. 100 $Å^2$ of binding surface to the overall complex, although the resulting overall Protein-IMiD-Protein complex does have a typical PPI interaction surface of 1051 $Å^2$.

A further example of the mediation of this class of drugs via CRBN recruitment involves a new modulator, CC-885, which has potent anti-tumor activity and has been shown to stabilize the interaction of CRBN with the translation termination factor protein G1 to S phase transition 1 (GSPT1) [101]. The stabilization of this complex by this new modulator results in ubiquitination and degradation of GSPT1. The stabilization is again dependent on a very similar Gly-containing loop; however specificity for CC-885 over other IMiDs is achieved by interaction of the longer CC-885 molecule with specific residues unique to GSPT1.

3.1.10. CC0651

In addition to the IMiDs, small-molecule modulation of the enzymatic activities of the ubiquitin-proteasome system (UPS) is garnering interest as a therapeutic strategy for the treatment of a whole range of diseases, among them cancer and neurological disorders [102]. Sicheri and co-workers conducted a screen for novel small molecule inhibitors of SCF-catalyzed protein ubiquitination, specifically, a multi-protein high-throughput HTRF assay against ubiquitination of the human CDK inhibitor p27Kip1 by the enzymatic cascade of E1 enzyme Uba1, E2 enzyme hCdc34, and the SCFSkp2 E3 complex [103]. One compound, CC0651 (Figure 3), was reported to inhibit p27Kip1 ubiquitination in a dose-dependent manner with an IC_{50} = 1.72 μM. In subsequent *in vitro* mode-of-action studies CC0651, and thermal denaturation assays found to specifically target the E2 enzyme hCdc34.

The precise binding mode was determined by solving the X-ray crystal structure of CC0651 bound to hCdc34 at 2.3 Å resolution where it was revealed that the compound binds 19 Å distal from Cys93 – the active site cysteine residue – at a surface-exposed pocket dominated by hydrophobic residues. Furthermore, the pocket is only partially present in the apo hCdc34, and only forms to accommodate the binding of the ligand. While clearly explaining the ability of CC0651 to target hCdc34, these data alone could not satisfactorily explain the inhibitory effect of CC0651 on hCdc34-mediated ubiquination of p27Kip1 in the HTRF assay. In subsequent work by the same research group, 2D 1H-15N-HSQC spectroscopic studies on 15N-labeled ubiquitin in the presence of both Cdc34A and CC0651 identified peak shifts and peak broadening of ubiquitin resonances, which were suggestive of a stabilization of the Cdc34A–ubiquitin complex by CC0651.

To justify this suggestion, and elucidate the compound's precise binding mode, the X-ray crystal structure of CC0651 bound to the Cdc34A–ubiquitin complex was solved at 2.6 Å resolution [104]. Crucially, beside the contacts observed in the CC0651–hCdc34 binary complex, which were unperturbed by ubiquitin binding, CC0651 was also observed to make numerous van der Waals contacts with hydrophobic amino acid residues on the ubiquitin protein, thus leading to stabilization of the ternary complex. The functional consequences of this stabilization are to suppress Cdc34A-ubiquitin thioester hydrolysis, without disrupting the binding of Cdc34A to cognate docking sites on the E3 complex. Ubiquitin engages in multitudes of protein–protein interactions within the ubiquitin-proteasome system. The specificity of CC0651 toward Cdc34A, taking into account the heterogeneity of the donor ubiquitin surface across the E2 family, suggests that small-molecule stabilization may be a fruitful strategy to selectively address other ubiquitin-E2 complexes.

3.1.11. (R,R)-2a

The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPA-receptor) is a glutamate responsive ligand-gated ion channel (iGluR) that is characterized by its sensitivity to AMPA activation [105]. This receptor is a key player in synaptic neurotransmission over the cell membrane, where glutamate binding induces opening of the ion channel, membrane depolarization and receptor desensitization. The ligand binding domain consists of an intramolecular dimer of two protein domains that fold into a clamp like structure with C_2 symmetry [105]. Many agonists have been found for the AMPA receptor, such as cyclothiazide (CTZ), aniracetam, CX614 and a series of biarylpropylsulfonamides, which originate from a combination of *in silico* approaches and library screening [106–108]. A particularly interesting series of biarylpropylsulfonamide compounds was found using a whole-cell AMPA

activity assay [106]. Crystallography showed that these compounds bind to and stabilize the dimer interface, locking the receptor in a state that cannot be desensitized [107,108]. Due to the C_2-symmetry of the protein complex there are two identical ligand binding sites, and indeed two copies of the compounds are observed in the crystal structures. This feature was used to their advantage by Kaae and co-workers, who used the biarylpropylsulfonamide series of compounds to rationally design a dimeric stabilizer compound using *in silico* verification techniques. The resulting compounds are the most potent AMPA stabilizers so far and one of them, *R,R*-2a (Figure 3) has also been crystallized in complex with the AMPA-receptor. The structure shows that the compound simultaneously binds to both biarylpropylsulfonamide pockets, as expected from *in silico* modeling [109]. This makes (*R,R*)-2a one of the few compounds that has been developed into a strong PPI stabilizer by rational design, demonstrating that it is possible to apply medicinal chemistry design approaches to generate improved PPI stabilizers.

3.1.12. CK-636

The actin-related protein 2/3 (Arp2/3) complex is a large protein assembly that consists of seven protein chains. Under the influence of nucleation-promotion factors (NPFs) it plays a major role in the regulation of actin polymerization, specifically the formation of branching points [110]. High-throughput screening of a 400,000-member compound library for activity against Arp2/3 dependent actin polymerization identified two inhibitory compounds[111]. Crystallization studies showed how one of these compounds, abbreviated CK-636 (Figure 3), bound to the interface of the Arp2/Arp3 protein partners of the protein complex. Binding to this interface appears to stabilize the protein in its inactive conformation, with the effect that upon binding of the activating protein, conformational change does not occur and thus protein activity is inhibited.

3.1.13. BMS-8 and BMS-202

Immunotherapy is a widely discussed therapeutic strategy in oncology in which a patient's own immune system is triggered to fight a tumor. In the past decade the approval of several antibody-based drugs have made it clear that this is a valuable and promising approach in cancer treatment [112]. A leading role in these developments has been played by cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1) and its ligand-programmed cell death protein 1 Ligand (PD1L) [113]. These checkpoint proteins inhibit T cell activation and are used by tumors to protect themselves against attack by the immune system. Because they are ligand activated, these proteins have been the target of antibody based 'checkpoint inhibitors', which have shown very promising results in the clinic.

The first series of small molecule inhibitors of PD1 was reported in a patent and is the result of an HTRF-based screening campaign on the PD1/PD1-L interaction [114]. Only later did crystallographic and biophysical studies with two of these compounds, BMS-8 and BMS-202 (Figure 4), show that the compounds inhibit the interaction by inducing dimerization of PD1-L [115]. Each dimer binds one molecule of the stabilizer at its interface, effectively burying the PD1 interaction surface and thus inhibiting the interaction with PD1.

3.1.14. AZD3514 and BiBET

The family of bromodomain (BRD)-containing proteins is responsible for reading acetylated lysines on histones, and inhibition of BRD proteins has recently become a very popular target mechanism in drug discovery [116]. Upon binding and recognition of acetylated histone lysine residues, BRD containing proteins facilitate the assembly of transcriptional machinery, causing them to be effectors of acetylation-mediated gene up regulation [117]. In particular, the subfamily of bromodomain and extraterminal (BET) proteins, composed of BRD2, BRD3, BRD4 and BRDT, has drawn considerable attention, since BRD4 is associated with translation of the critical oncogene c-Myc, which is a master regulator of cell proliferation [117].

The compound AZD3514 originated from a program aimed at identifying androgen receptor (AR) down regulators. During its development, a discrepancy was observed between the effects of the compound in *in vitro* assays employing purified protein constructs, and in cellular assays [118]. The authors identified structural similarities to known BRD4 inhibitors and investigated BRD-containing proteins to be the primary target of the compound. Indeed, subsequent biochemical assays showed that the compound binds BRD4 and, unexpectedly induces dimerization. Crystallography showed that AZD3514 induces and binds to a dimer of BRD4 domains by reaching out to the acetyl lysine binding pockets of the two BRD monomers [118]. The observation that both acetyl pockets were exploited by the compound was used to further evolve the compound into a 'bivalent' inhibitor, BiBET (Figure 4), optimized to bind to BRD dimers, thus yielding the most potent BRD4 inhibitor reported so far [118].

3.1.15. BMS493

Members of the nuclear receptor (NR) superfamily of ligand-dependent transcription factors can discriminate between activating and repressing modes of action for target gene transcription by recruiting either corepressors or coactivators. Even though most NR modulators occupy a binding site in the ligand binding domain (LBD), some are considered direct PPI modulators as they make direct binding contact with coregulator proteins of their target NR.

Such direct NR modulators have been reported for the retinoic acid receptor (RAR). RAR exerts its physiological functions in the control of development, reproduction and homeostasis by acting as a heterodimer with retinoid X receptor (RXR) [119]. In the absence of a RAR agonist, inhibition of RAR-RXR controlled gene transcription is mediated by recruitment of the corepressors SMRT and NCoR. RAR agonist binding results in release of corepressors, formation of coactivator complexes, and activation of transcription [120,121]. Alternatively, the basal transcriptional activity of RAR can be down regulated further by the use of inverse agonists [122]. One such inverse agonist is BMS493 (Figure 3), which has been identified as a pan-RAR inverse agonist, and strongly enhances corepressor binding [123]. Crystallographic studies with BMS493, RAR and NCoR have shown that BMS493 binds within

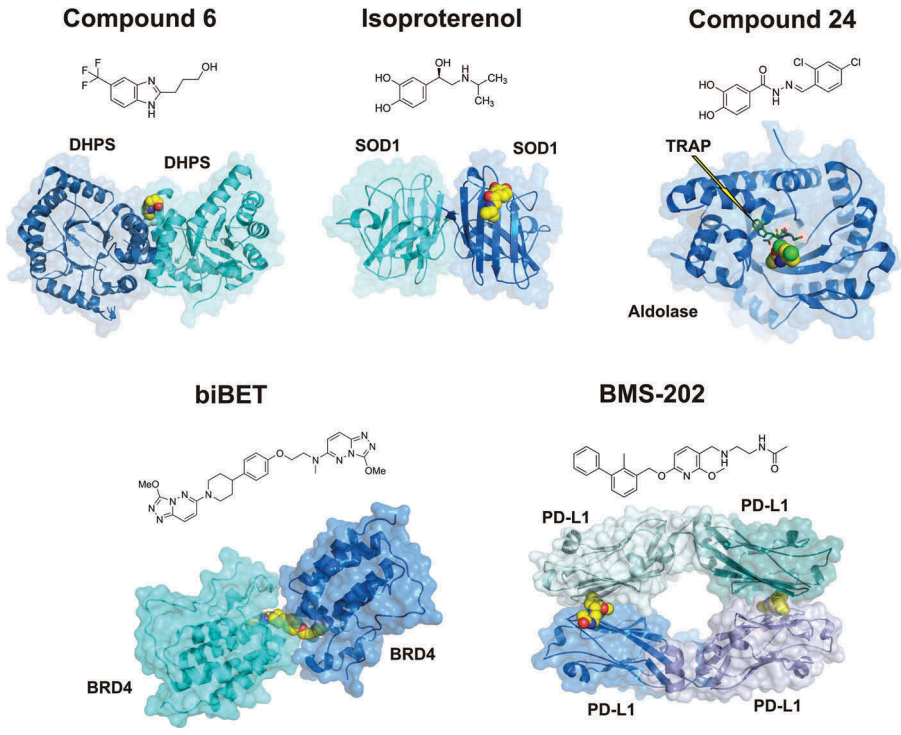

Figure 4. Synthetic compounds III that stabilize PPIs.

the orthosteric ligand binding site of the LBD, but extends out of the site to directly contact the NCoR peptide [124]. The same studies show that the inverse agonism can be explained through the folding of the C-terminal helices H11 and H12 being strongly disrupted by ligand binding, which in turn forces H10 to fold into a well-defined β-sheet instead of its expected helical folding. This extended β-sheet in H10 is the driving force behind the strengthening of the RAR/NCoR interaction by forming an anti-parallel β-sheet with the N-terminal part of the repressor [124], yielding a unique co-repressor interaction compared to other described NR/co-repressor interactions [125–127]. Additionally, there are direct hydrophobic contacts between the BMS493 ligand and the NCoR residues Leu2051, His2054, Ile2055 and Ile2058, making this compound a hybrid allosteric and direct PPI stabilizer.

3.1.16. GW6471

Another ternary structure of a NR-LBD in complex with a corepressor and a direct PPI stabilizer is described for the peroxisome proliferator-activated receptor α (PPARα) [125]. GW6471 (Figure 3) is a PPARα antagonist, which is derived from the PPARα agonist GW409544 and potently inhibits the GW409544-induced activation of PPARα in a PPARα-GAL4 chimeric receptor reporter gene assay. GW409544 is a typical PPAR agonist in that a carboxylic acid moiety of the agonist ligand makes a direct hydrogen-bonding interaction with Tyr464 on the conformationally mobile C-terminal helix 12 (AF-2 domain) of the LBD [128]. This stabilizes helix 12 in an agonist conformation, creating a hydrophobic cleft on the surface of the receptor that is the docking surface for the α-helical LXXLL coactivator motif. In GW6471, the carboxylic acid of GW409544 is replaced by a bulkier ethyl amide moiety. A crystal structure of the PPARα/GW6471/SMRT ternary complex shows that the key agonistic hydrogen-bonding interaction between ligand and Tyr464 on helix 12 is not present. In addition, the ethyl side chain of the amide head group occupies the same space that is occupied by the sidechain of Tyr464 in the agonist-bound structure. Both these features prevent helix 12 from docking in an agonistic conformation and force it to adopt an alternative antagonist conformation where it is loosely stacked on helix 3. This creates a large hydrophobic cleft that can accommodate the much larger LXXXIXXXL corepressor motif. GW6471 was shown *in vitro* to destabilize the binding of the PPARα LBD to peptides derived from coactivator proteins and enhance the binding of peptides derived from the corepressors SMRT and NCoR approximately five-fold [125]. Thus, GW6471 acts as a stabilizer of the protein–protein interactions between PPARα and corepressors NCoR and SMRT.

3.1.17. 4-Hydroxytamoxifen

The discovery of the anti-estrogenic drug tamoxifen (Figure 3) in the 1960s was made without directly understanding its mode of action [129]. The various targets and mechanism were unraveled over the following years, through identification of the significance of the 4-hydroxy metabolite [130,131], and subsequently by revealing the dual nature of 4-hydroxytamoxifen (4HT) with either agonistic or antagonistic properties depending upon the tissue, species and conditions [132]. As observed in a crystal structure of the estrogen receptor (ER) LBD, 4HT binds to both the ligand binding pocket, acting as an agonist, and in an alternate site with a lower affinity, inducing a conformational change in helix 12 and the F-domain, which disrupts binding of coactivators, thereby resulting in functional antagonism of ER-signaling [133,134]. Additionally, 4HT was found to stabilize the binding of a 11-mer peptide, αII, which resulted from a phage display exploration of peptides binding to an induced surface of ERα [135]. The binding

surface is situated on the face of the LBD opposite to the AF-2 region and is unique to ERα as minor changes in residues for ERβ are sufficient to prevent binding completely. The peptide binds to the ligand-bound receptor in an AF-2 independent manner and provides an alternative and previously unknown interaction site to control transcriptional activity of a NR. Finally, 4HT was also identified as an inverse agonist for the estrogen related receptor γ (ERRγ) [127,136]. ERRγ is a constitutively active NR. Binding of 4HT induces a major conformational rearrangement of the ERRγ LBD resulting in the displacement of helix 12 and thus favoring the recruitment of co-repressors [137].

3.1.18. Asoprisnil

Asoprisnil (Figure 3) is a selective progesterone receptor (PR) modulator that was developed for the treatment of gynecological conditions. Unlike the antagonist mifepristone (RU486), asoprisnil has been shown to exhibit partial agonism in some *in vivo* assays [138]. Crystallographic studies of the PR-LBD in complex with asoprisnil and corepressor peptides showed that the receptor adopts essentially the same conformation as when bound to mifepristone [139]. The crystal structure also shows the compound reaching out to the corepressor peptide, directly stabilizing its interaction with the protein. A later study showed that by soaking crystals of the PR-LBD containing a non-steroidal agonist with asoprisnil it was possible to obtain crystal structures with asoprisnil in an agonist conformation [140]. Interestingly, the binding mode of asoprisnil is comparable in both the agonist and antagonist conformations. The most significant difference for the ligand in the structures observed is in the positioning of the oxime group which in the agonist complex is displaced slightly compared to the antagonistic state to accommodate a methionine residue (M909) in helix 12. Additionally, the hydroxyl of the oxime in asoprisnil is able to form a hydrogen bond to the terminal amide of E723, which in turn hydrogen bonds to two backbone amides of helix 12. This stabilizes the agonist conformation, in contrast to the full antagonist mifepristone, which has an *N*,*N*-dimethylamino group in the corresponding position. From these structures, it is clear that minor changes to ligand functional groups can have profound effects on the LBD conformational states and the PPIs that determine the transcriptional activity of the receptor.

3.1.19. Perspective for drug development

The examples of synthetic PPI stabilizers discussed so far are the product of efforts to find protein-target modulators while being agnostic to the molecular mechanism of action. A closer look at the assays used for their discovery reveals that they have often been identified through functional cell-based assays or assay systems composed of multiple proteins. This is hardly surprising as many protein based *in vitro* assays are clearly biased toward looking at modulation of a single clearly defined target – for example, assays with purified protein constructs. The use of whole-cell phenotypic assays eliminates this bias toward a predefined target and mode of action, allowing the serendipitous detection of PPI stabilizers.

Despite their serendipitous discovery, there is a wealth of medicinal chemistry behind some of these compounds describing their structure activity relationships (SAR). Some of these compounds have been optimized without having either a clear understanding of their mechanism of action or structural guidance, while others were optimized in a very systematic and rational way. This illustrates that, in principle, known optimization strategies can be applied to PPI stabilizers as well. As a consequence, these highly designed small-molecule PPI stabilizers have very 'drug-like' properties. This is to be expected as current compound libraries and medicinal chemistry efforts are focused on the generation of such drug-like molecules. This is however in sharp contrast to what is observed for natural product PPI stabilizers, which feature a much more varied set of structural motifs such as macrocycles and compounds with a high degree of three-dimensionality. A final and crucial observation is that these optimization studies all share the premise that there is indeed a chemical starting point available, which is dependent on the availability of assays to identify such starting points.

3.2. Ab initio synthetic stabilizers

Cases of intentional discovery of PPI stabilizers are indeed very scarce. This is probably a consequence of PPI stabilization not being a strategy often considered when looking for new biological targets and mechanisms. Nevertheless there are some examples where *ab initio* PPI stabilization was successful, which will be discussed now.

3.2.1. Pyrrolidone 1 and epibestatin

Since the discovery of the mode of action of fusicoccin A and its derivatives as stabilizers of 14–3-3 PPIs, stabilization of 14–3-3 PPIs has been a relatively well-studied topic [144]. This has resulted in a search for other more selective and synthetically accessible alternatives. The application of *in silico* screening methods has been described, but has not yielded any active compounds [145]. High-throughput screening on the tobacco plasma membrane H^+-ATPase in complex with 14–3-3 yielded pyrrolidone 1 (Figure 3) and epibestatin as a novel stabilizer compounds [143]. This provided fusicoccin mimics with a significantly simplified scaffold using a highly focused assay consisting of only the two protein partners. Subsequent attempts to improving the pyrrolidone 1 scaffold yielded an improved Compound 37, with approximately double the potency of pyrrolidone 1 [146]. While these compounds represent new PPI stabilizer scaffolds, their discovery was aided by the knowledge that other highly complex natural products – for example, fusicoccanes – are capable of stabilizing this PPI, providing the knowledge that a pocket exists that can be targeted by small molecules. Nevertheless, these examples showed that screening campaigns focused on PPI stabilizers can provide novel chemical starting points.

3.2.2. Isoproteronol and 5-fluorouridine

SOD1 is an enzyme that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Familial amyotrophic lateral sclerosis (FALS) is caused by mutations in SOD1 that destabilize the native SOD1 homodimer and stimulate its aggregation *in vitro* [147]. The symmetrical dimer interface cavity of SOD1 is composed of hydrophobic residues such

as Val148 and Val7, with a small number of peripheral charged or polar residues such as Lys9 and Asn53. In an attempt to find a therapeutic approach for FALS, stabilizers of the SOD1 dimer were sought using an *in silico* screening. This approach yielded 15 compounds that experimentally stabilized SOD1 against aggregation and unfolding [148]. Site-directed mutagenesis studies were performed in order to understand the mode of action of the compounds and replacement of interface cavity residues Val148 and Asn53 by Ala led to complete loss of compound activity. The relation between those point mutations and the loss of the biological activity of compounds was confirmed by SOD1 aggregation assays [149]. In order to improve the toxicity profile of the stabilizers, additional *in silico* screening was performed. The resulting compounds were based on entirely different scaffolds and also showed inhibition of SOD1 aggregation.

At this time point, the binding mode was only tested through mutational studies [149]. Three years later the group of Hasnaian co-crystallized two of the stabilizers from the follow-up study, isoproteronol (Figure 4) and 5-fluorouridine, with the WT and mutated forms of SOD1 [150]. None of the compounds exhibited binding at the dimer interface, but rather in a shallow pocket on the protein surface. In their review, Zarzycka et al. classified these compounds as allosteric protein–protein stabilizers [2], however, in our view the experimental data from two different groups are contradictory and there is no clear agreement that those compounds are SOD1 dimer stabilizers. This example underlines the crucial importance of experimental structural data in unraveling the binding mode of small molecules.

Despite the uncertainty surrounding their binding mode, these results possibly represent the earliest deliberate *ab initio* generation of a PPI stabilizer without any pre-existing knowledge of a compound that would stabilize such an interaction.

3.2.3. Compound 24

In 2015, the group of Bosch published a molecule (Compound 24) that stabilizes the complex between an aldolase from *Plasmodium palcifarum* (*Pf*Aldolase) and thrombospondin-related anonymous protein (TRAP) [151]. The malaria-causing protozoan parasite *Plasmodium palcifarum* uses an actin/myosin motor complex located beneath the parasite's plasma membrane for cellular invasion and gliding [152]. The bridging enzyme *Pf*Aldolase connects the actin/myosin motor to transmembrane adhesins of TRP, which is expressed in a life-cycle stage specific manner [153,154]. To interfere with the dynamics of the gliding process the authors aimed at identifying small molecules that stabilize the *Pf*Aldolase/TRP complex. To this end a virtual library of 315,102 compounds was screened *in silico* against the *Pf*Aldolase–TRAP complex to select candidate compounds for biochemical and cellular testing that could stabilize and prevent the disassembly of the glideosome. Compound 24 (Figure 4) was found to disrupt the invasion of Plasmodium parasites in *in vitro* parasite assays with 95% reduction in liver cell invasion in the presence of 500μM of Compound 24. The 2.11Å ternary X-ray crystal structure of Compound 24 in complex with *Pf*Aldolase and a TRAP-derived peptide confirms the PPI-stabilizing mode of interaction. The identification and development of compound 24 is another example of computational techniques successfully yielding a PPI stabilizer.

4. Conclusion

The number of compounds reviewed in this work illustrates a growth in the number of synthetic PPI stabilizers reported in the literature. Nevertheless, the number of publications involving PPI stabilization remains far lower than for the opposite strategy of PPI inhibition. Encouragingly, some of these synthetic compounds have advanced to such a state that they are in the process of moving toward, or already being used in the clinic. For example ifenprodil (phase II clinical trial ongoing), pleconaril (phase II clinical trial ongoing) and tafamidis (approved in EU & Japan) illustrate that PPI stabilization as a therapeutic strategy is not an approach exclusively executable by nature. The development of these compounds has led to a substantial number of reports describing the SAR of some of these compounds. In many cases the compounds indeed seem to act as molecular glues, exhibiting interactions with both protein partners at the PPI interface and optimizing these individual interactions through medicinal chemistry approaches improves stabilizer performance.

Unfortunately though, there are very few reports on PPI stabilizer lead generation, which illustrates the main hurdle the field of PPI stabilization has to solve in order to effectively apply this approach in drug discovery. The choice of assay appears to be an important factor and there has been some mixed success for computational approaches, but there is currently too little literature to provide a reliable method to overcome this problem. Consequently, PPI stabilization cannot yet be used to its full potential if the scientific community relies on *post hoc* identification of PPI stabilizers.

5. Expert opinion

With an estimated number of around 300,000 PPIs occurring in human cells and with these involved in every disease and physiological process, PPIs are an extremely interesting, but as yet underexplored, target class for drug discovery. Inhibition of PPIs is a fast developing field, but is often rendered difficult due to the large surface areas that need to be addressed, which is often incompatible with the requirements for orally-bioavailable small molecule drugs (with a few notable exceptions). Another complicating factor for developing PPI inhibitors is that the relevant binding partner in a presumed PPI is often not identified, making the development of assays to assess inhibition impossible without significant effort to identify the binding partner. Generally, the development of direct binding assays (where it has been possible) for one component of a PPI has translated poorly to functional inhibition of the PPI due to the difficulty in identifying binding pockets or indeed the relevant surface of the protein that should be targeted by small molecules.

Stabilization of PPIs, on the other hand, represents a potentially small-molecule compatible approach to the modulation of PPIs, in that druggable pockets can be formed at the interface of two proteins, ligand binding to such a pocket can lead to stabilization of the protein–protein complex and potentially

result in interesting pharmacological effects. Additionally, designing compounds against a pocket formed by two protein partners yields the potential for increased selectivity against this specific combination of protein partners, in contrast to PPI inhibitors where any other protein binding to the targeted site is affected. This intriguing possibility leads to the conceptual opening up of a number of targets and related pharmacology previously considered to be outside the realm of small-molecule drug discovery.

As we have described in this review, several compounds exert their action via the stabilization of PPIs, but this has generally been shown by *post hoc* mechanistic analysis. The *ab initio* design of stabilizers of specific PPIs is as yet in its infancy, which is illustrated by the fact that out of the compounds reviewed here, only five were discovered by efforts directed toward intentional discovery of PPI stabilizers: pyrrolidone1 and Epibestatin – found by HTS –, isorpoteronol and 5-Fluorouridine and Compound 24 – found using *in silico* screening. It is expected that lead generation for PPI stabilization will be difficult given our lack of knowledge of the mechanisms and underlying principles of PPI stabilizers, and a lack of any specific chemotypes that may be enriched within PPI stabilizer chemical space. As discussed in this review, many compound libraries are heavily biased toward orally available 'drug-like' chemical space, but the natural-derived compounds that represent the bulk of PPI stabilizers exhibit very different structural properties. This may limit the success of screening approaches to PPI stabilization. The inclusion of a broader chemical space in PPI stabilizer screening sets, for example macrocycles, peptide secondary structure or natural product mimetics or compounds specifically enriched in sp^3 carbon atoms may increase the likelihood of finding PPI stabilizers. However, it is unknown whether compounds with different properties also call for a different strategy to identify and/or optimize these compounds. It is therefore crucial to gain more insight into PPI stabilizer function, such that our screening methods and library compositions can be focused toward the identification of PPI stabilizers. Cellular assays seem to be overrepresented in the serendipitous discovery of PPI stabilizers, and could represent an unbiased assay system for the *post hoc* identification of PPI stabilizers

14–3-3 proteins could represent a useful platform to aid in this effort, since there are many examples of 14–3-3 PPIs that have been shown to be amenable to stabilization by small molecules, typically of the natural-product type. This provides a modular platform to study the order of binding events, kinetic and thermodynamic properties and the scope for selectivity. Additionally, 14–3-3 PPIs are known to generate a rather well-defined pocket, which has been successfully exploited by nature in the form of fusicoccanes. The availability of a PPI-stabilizer class such as the fusicoccanes allows us to use these PPIs as platforms for the development of new screening methods that are capable of identifying novel PPI-stabilizer chemical starting points. Additionally, further development of computational techniques, fragment-based approaches or peptide tethering approaches where fragments are added to an existing PPI interface [155] would represent advances in the understanding of PPI stabilization and would truly allow us to explore its potential in drug discovery.

One area where targeted stabilization of PPIs could be transformational in drug discovery is in the modulation of transcription factor activity. Transcription factors are implicated in the development of many diseases [156] and inhibition of their transcriptional activity (for example either by various knockdown methods or with antibodies) often leads to a desirable phenotype. Transcription factors are typically considered to be undruggable with small molecules since they generally exert their cellular effects as part of multicomponent protein complexes with large, relatively featureless interaction surfaces. Transcriptional activity only occurs when the factors are in the nucleus thus promotion of nuclear export is one method to inhibit their transcriptional activity. Given that the nuclear localization (import/export) and subsequent proteosomal degradation of many transcription factors is regulated by phosphorylation and subsequent binding to 14–3-3 proteins, stabilization of 14–3-3/transcription factor complexes could in principle represent a way of 'directly' inhibiting transcription factor activity. In the case of such 14–3-3/transcription factor complexes, both components of the PPI complex are known, increasing the likelihood of *in vitro* biophysical stabilization of the PPI translating to a relevant functional outcome. Inhibition of 14–3-3 proteins has been shown (using a reporter gene assay) to increase the transcriptional activity of a transcription factor, which is negatively regulated by 14–3-3 proteins (FoxO3a [157]). The opposite strategy of stabilization of a 14–3-3/transcription factor interaction has been shown to be viable for ER [158]. The ready availability of 14–3-3/phosphopeptide crystal structures could also enable structure-based design to be applied to transcription factor inhibitors, something which is currently impossible for the vast majority of transcription factors due to their often inherently disordered structure. The challenge in this case is achieving selectivity toward 14–3-3 complexes with other off-target proteins. There are some promising data that suggest that selectivity is possible and the ability to perform structure-based drug design on the complexes should enable further selectivity to be achieved [159].

The potential for high selectivity, the ability to gain access to previously undruggable targets and the list of impressive compounds reviewed here, in our opinion, advocates for an increased effort toward developing a better understanding of PPI stabilizers, along with the techniques required to identify and develop lead structures. The stabilization of PPIs is becoming an approach that can be of tremendous value to the medicinal chemistry community.

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Declaration of interest

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