



Stabilization of protein–protein interactions by small molecules

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Protein–protein interactions (PPIs) are implicated in every disease and mastering the ability to influence PPIs with small molecules would considerably enlarge the druggable genome. Whereas inhibition of PPIs has repeatedly been shown to work successfully, targeted stabilization of PPIs is underrepresented in the literature. This is all the more surprising because natural products like FK506, rapamycin, brefeldin, forskolin and fusicoccin confer their physiological activity by stabilizing specific PPIs. However, recently a number of very interesting synthetic molecules have been reported from drug discovery projects that indeed achieve their desired activities by stabilizing either homo- or hetero-oligomeric complexes of their target proteins.

Introduction

Small molecule modulation of protein–protein interactions (PPIs) has been recognized as a promising approach in drug discovery [1–3]. However, in the vast majority of cases, PPI modulation is exclusively recognized as inhibition, understandably motivated by a number of wonderful success stories that have been published in recent years. Of these, two of the most prominent examples are the nutlins developed by Roche [4,5], which inhibit the negative regulation of the tumor suppressor p53 by the ubiquitin ligase mouse double minute 2 homolog (MDM2), and navitoclax from Abbott (ABT263), which disrupts the interaction of the antiapoptotic protein Bcl-2 and apoptosis-executing proteins like Bad, Bid and Bak [6–8]. Further prominent examples are molecules that inhibit the interaction of human leukemia-derived growth factor (LDGF) with HIV integrase [9], disrupt the binding of KRas and phosphodiesterase (PDE) δ [10], transform an active tumor necrosis factor (TNF) α trimer into an inactive dimer [11] or inhibit the binding of PPI modules like the clathrin terminal domain (CTD), or bromodomains with their partners [12,13].

A number of natural products have been shown to mediate their physiological activity by stabilizing PPIs, for a review see [14]. These molecules are ample proof of the concept of small molecule PPI interface binding which results in functional stabilization of

regulatory protein complexes leading to significant physiological effects. In addition to these examples from nature itself, there are meanwhile a number of small molecules known that have been found to modulate the function of their protein target by stabilizing their homo or hetero complexes. Interestingly, many of them have been identified during screening of PPI inhibitors, for example RO2443 and phenothiazines that inhibit the function of MDMX and the protein S100A4, respectively. A few molecules have also been found in approaches directly dedicated to identifying PPI stabilizers, for example pyrrolidone1. In this review we summarize the current state of PPI stabilization by small molecules, including natural products and synthetic molecules.

Cyclosporin A

Cyclosporin A (CsA) is a cyclic undecapeptide produced by the ascomycete *Tolypocladium inflatum*, initially isolated in 1970 from a Norwegian soil sample and shown to display immunosuppressive properties [15]. Its chemical structure inclusive of *N*-methylated peptide bonds, the atypical amino acids aminobutyric acid and (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine (MeBmt) and D-alanine is biosynthesized by a nonribosomal process [16] (Table 1). The conformational, physicochemical and pharmacokinetic properties of CsA, most notably its oral absorption profile [17], continue to inspire chemists [18–20], and its effective immunosuppressant action supported FDA registration in 1983 [21]. The molecular target

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TABLE 1

Natural products that stabilize protein–protein interactions (PPIs)

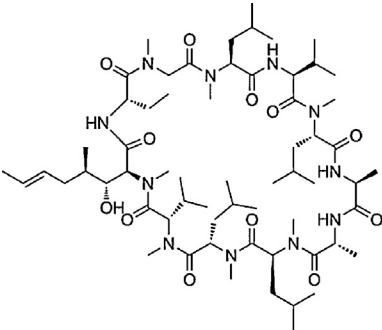
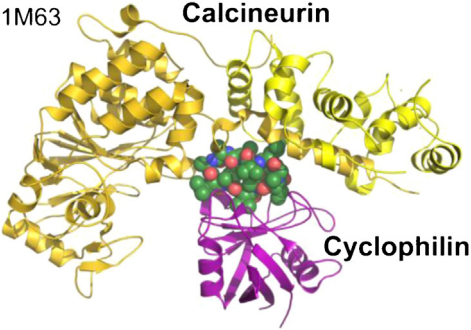
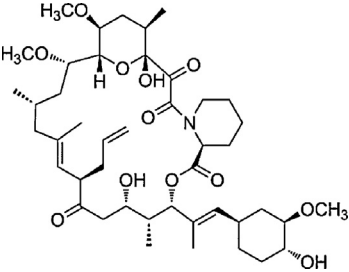
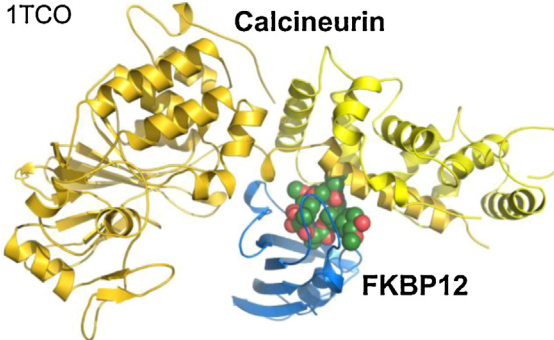
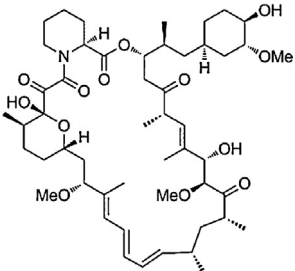
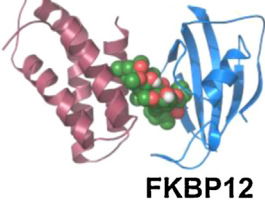
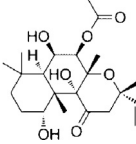
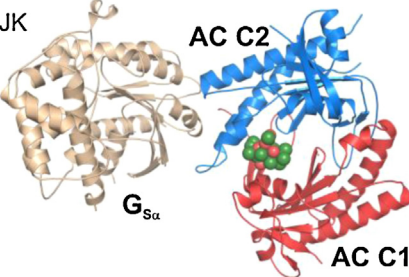
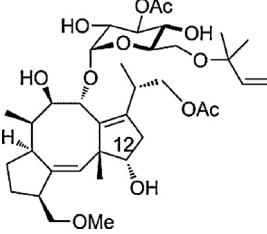
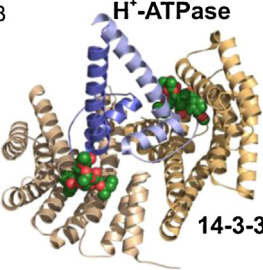
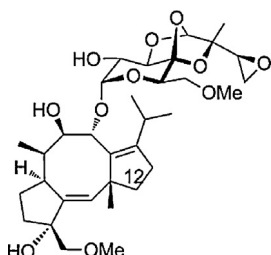
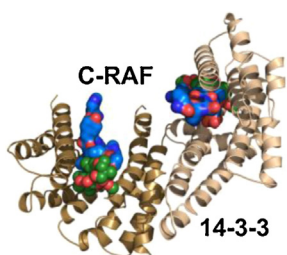
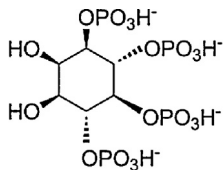
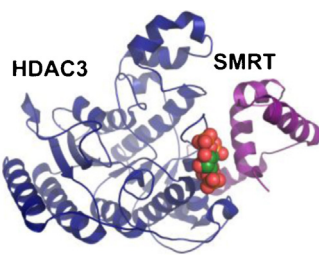
PPI stabilizer	Structure	Protein–protein complex (PDB)
Cyclosporine A		1M63 Calcineurin  Cyclophilin
FK506		1TCO Calcineurin  FKBP12
Rapamycin		1FAP mTOR  FKBP12
Forskolin		1CJK AC C2  G_{Sα} AC C1
Fusicoccin A		2O98 H⁺-ATPase  14-3-3

TABLE 1 (Continued)

PPI stabilizer	Structure	Protein-protein complex (PDB)
Cotylenin A		4IHL 
D-Myo-inositol-(1,4,5,6)-tetrakisphosphate		4A69 

of CsA was identified one year later to be cyclophilin A (CypA) [22]. Direct binding to this peptidyl-prolyl *cis-trans* isomerase leads to inhibition of its enzymatic activity explaining the usefulness of CsA derivatives for antiviral therapy [23]. However, the functionally relevant target for the immunosuppressive activity of CsA is not the individual CypA protein but rather its complex with the phosphatase calcineurin (Cn) [24]. Elucidation of the corresponding crystal structure of the ternary complex Cn-CypA-CsA showed how CsA inhibits the enzyme activity of Cn by 'gluing' CypA to the phosphatase and blocking the access to the active site [25]. Two further molecules, entirely unrelated to CsA, have also been found to display immunosuppressive activity: the macrolides FK506 and rapamycin.

FK506 (tacrolimus)

FK506 was isolated by scientists at Fujisawa Pharmaceuticals in 1984 as the active immunosuppressant component of a fermented broth from *Streptomyces tsukubaensis* [26]. FK506 is a 23-membered lactone belonging to the ascomycin macrolide class; it contains 14 asymmetric centers, nine sites of oxygenation and one amino acid, making targeted medicinal chemistry efforts a formidable challenge [27]. FK506 (tacrolimus) was approved as an oral immunosuppressant drug to be used after allogeneic transplant in 1994. The direct target for FK506 is the peptidyl-prolyl isomerase FK506 binding protein (FKBP) [28]. Very similar to CsA, also the effective physiological target of FK506 is not FKBP alone but a complex of the FKBP-bound FK506 and the phosphatase calcineurin [24]. Similar to the situation with CsA-CypA, the FK506-FKBP complex inhibits calcineurin by obstructing the active site of the phosphatase as shown in the crystal structure of the ternary complex published by scientists from Vertex [29].

Rapamycin

The third molecule that was found to have profound immunosuppressive activity and therefore helped to revolutionize transplantation medicine was rapamycin. This natural product was initially described as an antifungal metabolite from *Streptomyces hygroscopicus*, isolated from a soil sample of the island of Rapa Nui [30]. It was

found shortly afterward to have inhibitory activity toward the mammalian immune system [31]. Rapamycin is a 31-membered lactone with 15 chiral centers, an all-*trans* triene and a hemiketal masked tricarbonyl system (Table 1). Exactly like FK506, rapamycin binds to FKBP but instead of attaching to and inhibiting calcineurin the rapamycin-FKBP complex inhibits the kinase mammalian target of rapamycin (mTOR) [32] with mechanistic details revealed by the crystal structure of the ternary complex [33].

The molecular interactions established by rapamycin with FKBP12 and mTOR are in line with previously reported SAR studies including modifications at the C7 [34], C16 and C20 positions [35]. Consistent with the crystal structure of the FKBP12-mTOR-rapamycin complex, modification at the C-43 position of rapamycin is neutral to the biological activity and serves mainly to modulate physicochemical and pharmacokinetic properties of the drug, as highlighted by the marketed rapalogs [36,37]. In all three cases – CsA, FK506 and rapamycin – the resulting inhibitory complexes are established only in the presence of the small molecule, illustrating the impressing PPI-stabilizing properties of these compounds.

Forskolin

Forskolin (Fsk) was isolated in 1977 from *Coleus forskohlii* and researchers from Hoechst described it as an inotropic and blood-pressure-lowering compound [38]. Fsk achieves this activity by increasing the level of the second messenger molecule cAMP through activation of adenylyl cyclase [39]. Here, Fsk stabilizes the interaction of the C1 and C2 subunit of adenylyl cyclase, thereby enhancing the activity of this enzyme [40]. In the crystal structures of either the C2 homodimer [41] or the C1-C2 heterodimer complexes with the stimulatory α subunit of a trimeric G protein ($G\alpha$) [42], Fsk binds to a composite pocket in the rim of the dimer interface. This binding site is strongly hydrophobic and buries 90% of the solvent-accessible surface of Fsk. Stabilization of the PPI is achieved by filling this gap in the interface and eliminating the unfavorable hydration of the interior surface of the pocket. Chemically, Fsk (17- β -acetoxy-8,13-epoxy-1 α ,6- β ,9 α -tri-hydroxy-labd-14-en-11-one) is a labdane diterpene, containing

an atypical pyrane ring (Table 1). Because of its cardiovascular properties, Fsk has been the subject of several chemical optimization efforts, with special emphasis on improving its aqueous solubility [38,43–46]. Interestingly, none of the Fsk derivatives synthesized to date proved superior to the natural product with respect to *in vivo* antihypertensive and inotropic effects.

The 14-3-3 PPI stabilizers fusicoccin A and cotylenin A

The two natural products fusicoccin A and cotylenin A were first described as agents acting on plants [47,48], but were later found to show activities toward human cells [49–51]. For example, fusicoccin A shows antitumor activity [52], promotes platelet aggregation [53] and stabilizes the inhibitory binding of 14-3-3 to ER α [54]. Cotylenin A has been shown to induce differentiation in myeloid leukemia [55], display activity against breast cancer cells [56] and stabilize the inhibitory binding of 14-3-3 proteins to c-Raf [57,58]. We have furthermore shown that a semisynthetic derivative of fusicoccin – FC-THF – enhances transport of the potassium channel TASK3 to the plasma membrane by stabilizing 14-3-3 binding to the C terminus of TASK3 [59]. Taken together, the fusicoccanes are a valuable class of natural products for the targeted stabilization of the widespread 14-3-3 interactome with modifications – either by semi-synthesis [59,60] or by recombinant fermentation [61,62] potentially resulting in specific compounds. Given the fact that 14-3-3 proteins interact with hundreds of different proteins in eukaryotic cells [63], this issue of specificity is a pivotal one and stands in the focus of current efforts to develop new molecules for the modulation of the functions of this family of adapter proteins.

Inositol tetraphosphate

In humans, D-myo-inositol 1,4,5,6-tetrakisphosphate [Ins(1,4,5,6)P₄; Table 1] is produced by inositol phosphate multikinase (IMPK) that phosphorylates Ins(1,4,5)P₃ to form Ins(1,3,4,5,6)P₄ [64]. The yeast homolog of IMPK – the kinase Arg82 – has been shown to be involved in transcriptional regulation suggesting a functional role of Ins(1,3,4,5,6)P₄ in regulation of gene expression. Furthermore, Ins(1,3,4,5,6)P₄ is elevated during *Salmonella enterica* infection [65].

In 2012, Watson *et al.* [66] published the crystal structure of histone deacetylase 3 (HDAC3) in complex with the transcriptional co-repressor silencing mediator of retinoid and thyroid hormone receptors (SMRT). To the researchers' surprise they observed clear electron density of Ins(1,3,4,5,6)P₄ in the rim of the interface of the two proteins, obviously being picked up by the protein complex from its host expression system, HEK293 cells (human embryonic kidney cells). In contrast to the predominantly hydrophobic binding sites of FK506, rapamycin and forskolin, the pocket of Ins(1,3,4,5,6)P₄ displays a strong basic character. Importantly, the HDAC3–SMRT complex is not stable in the absence of the molecule, suggesting an important functional role of Ins(1,3,4,5,6)P₄ in transcriptional repression by HDAC3.

Dexrazoxane

Bisdioxopiperazines are small chemical compounds that inhibit the activity of DNA topoisomerase type II [67]. Among these remarkably simple molecules, (S)-4,4'-(1-methyl-1,2-ethanediyl)bis-2,6-piperazinedione (dexrazoxane, ICRF-187; Table 2) has been approved for use in cancer patients to prevent anthracycline-mediated

cardiotoxicity [68]. Intriguingly, dexrazoxane inhibits DNA topoisomerase II by stabilizing the closed conformation of the homodimeric enzyme [69]. Elucidation of the crystal structure for the topoisomerase II dimer complexed with dexrazoxane indicated how dexrazoxane bound to a composite, symmetric pocket in the interface of the topoisomerase II homodimer and contacts both monomers simultaneously at the exact corresponding sites in the antiparallel dimer [70].

Tafamidis

Aggregation of transthyretin (TTR) is known to be responsible for TTR amyloidoses leading to peripheral neuropathy and cardiomyopathy. The stabilization of proteins involved in misfolding processes associated to disease states was proposed to be an effective therapeutic strategy [71]. In this context, TTR amyloidosis has received widespread attention and several chemical classes of TTR kinetic stabilizers have been reported to date [72]. Among these, a benzoxazole series [73] was further optimized to 2-(3,5-dichlorophenyl)-benzoxazole-6-carboxylic acid (tafamidis; Table 1) [74], a potent TTR stabilizer that has been approved in Europe and Japan for the treatment of transthyretin-related hereditary amyloidosis. Tafamidis binds selectively to TTR variants and stabilizes the TTR dimer interface [74]. As can be seen from the crystal structure, tafamidis bound to TTR in a symmetric pocket established at the interface of the TTR. A hydrophobic subpocket is formed by the side-chains of Ala108, Leu110 and Thr112 and accommodates the 3,5-dichlorobenzene substituent, and the carboxyl group is engaged via water-mediated H-bonds with Lys15 and Glu54. Of clinical importance, tafamidis also stabilizes the most prevalently mutated TTR forms V30M and V122I.

Phenothiazines

Garrett *et al.* reported in 2008 the identification of several compounds that blocked the activity of S100A4 [75]. These inhibitors belong to the renowned phenothiazine chemical class, a family of compounds endowed with antipsychotic, antiallergic and anesthetic properties [76,77], resulting in breakthrough drugs such as chlorpromazine [78]. One of these compounds, trifluoperazine (TFP, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10H-phenothiazine; Table 1), was later shown in a crystallographic and biochemical study to inhibit S100A4 function by stabilizing an inactive pentamer [79]. In the crystallized pentamer two TFP molecules are sitting adjacent to each other in the interface contributed from each monomer, adding up to four copies of TFP that make extensive mutual contacts and thus stabilize the oligomeric assembly.

O-Methoxy-nucleozin

Influenza nucleoprotein (NP) is an essential viral factor, necessary for replication and virion assembly [80]. Hence, inhibitors of NP function could be of interest as therapeutic agents. The groups of Yuen and Wong reported the identification of nucleozin as an NP inhibitor with antiviral activity [81,82]. In 2011, researchers at Bristol Myers Squibb identified related compounds via high-throughput screening [83]. Here, expansion of the original hit nucleozin compound via substructure searches identified a series of compounds with improved properties. Among these, *ortho*-substitution of the 3-phenyl ring of the nucleozin isoxazole ring

TABLE 2

Synthetic compounds that stabilize protein–protein interactions (PPIs)

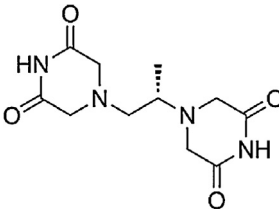
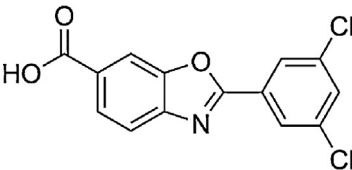
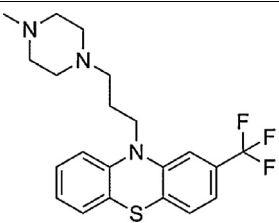
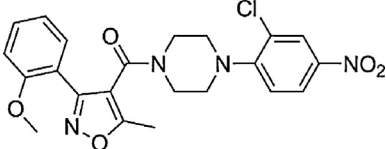
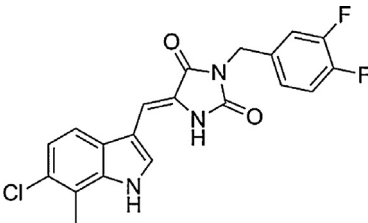
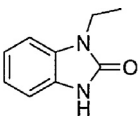
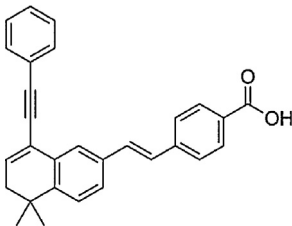
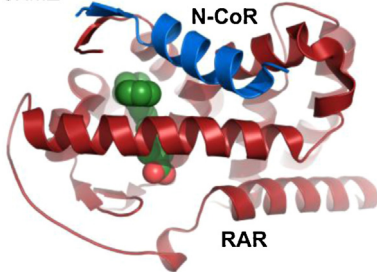
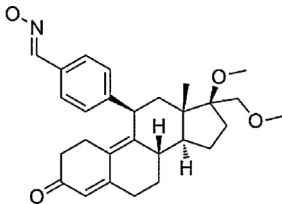
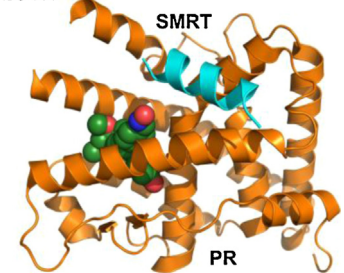
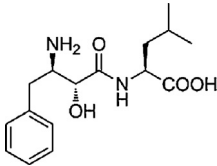
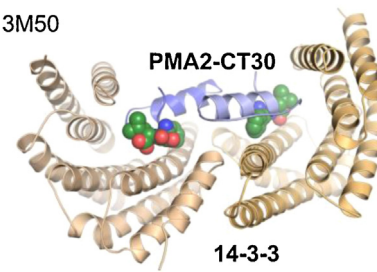
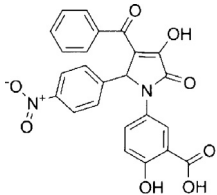
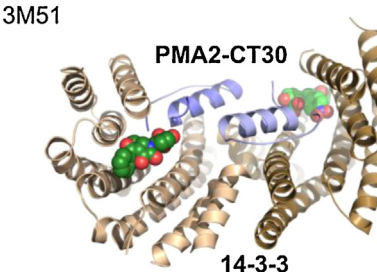
PPI stabilizer	Structure	Protein–protein complex (PDB)
Dexrazoxane, ICRF-187		IQZR Topoisomerase II Monomer A Monomer B
Tafamidis		3TCT Transthyretin Monomer A Monomer B
Phenothiazines		3KO0 S100A4 Monomer A Monomer B
O-Methoxy-nucleozin		3RO5 Nucleoprotein Monomer A Monomer B
RO2443		3U15 hDMX Monomer A Monomer B
1EBIO		4G28 Calmodulin CaMBD

TABLE 2 (Continued)

PPI stabilizer	Structure	Protein-protein complex (PDB)
BMS493		3KMZ 
Asoprisnil		2OVH 
Epibestatin		3M50 
Pyrrolidone1		3M51 

was preferred and (4-(2-chloro-4-nitrophenyl)piperazin-1-yl)(3-(2-methoxyphenyl)-5-methylisoxazol-4-yl)methanone (*O*-methoxy-nucleozin; Table 1) was the most potent inhibitor [83]. Interestingly, scaffold hopping from isoxazole to 1-methyl-1H-1,2,3-triazole was also tolerated. *O*-Methoxy-nucleozin was shown by protein crystallography to bind in the interface at the NP oligomer, bridge the dimer interface and thus stabilize the protein complex.

RO2443

p53, possibly the most important tumor suppressor in the human body, is negatively regulated by MDM2 and MDMX [84]. Whereas inhibition of binding of MDM2 to p53 has successfully been achieved by several small molecules [85], no such specific molecules have been reported for MDMX. However, to take full advantage of the strategy to stabilize p53 function, MDM2 and MDMX need to be targeted [86]. Such dual-activity inhibitors were identified by high-throughput screening by scientists at Roche and

belonged to the class of indolyl-hydantoin [87]. Surprisingly, biochemical experiments and the co-crystal structure of MDMX or MDM2 in complex with one of these compounds, (5*Z*)-5-[(6-chloro-7-methyl-1H-indol-3-yl)methylidene]-3-(3,4-difluorobenzyl)imidazolidine-2,4-dione (RO2443; Table 1), revealed that RO2443 bound to the interface of the homodimers and stabilized their interaction. This enhancement of either the MDMX or MDM2 homodimer together with a possible stabilization of the MDMX-MDM2 heterodimer includes parts of the interface with p53 and consequently excludes simultaneous binding to the tumor suppressor protein.

1EBIO

1EBIO, a surprisingly simple benzoimidazolone (Table 1), was found to stabilize calmodulin-potassium-channel interaction. Calmodulin (CaM) is a protein tethered to small-conductance potassium channels (SK) as a Ca^{2+} sensor. Binding of Ca^{2+} to

CaM leads to opening of the SK channel [88]. This opening in turn leads to reduced activation potential and vasodilatation. Abnormalities in SK channel function have been linked to schizophrenia and hypertension [89,90]. After finding that phenylurea, an additive in the crystallization solution, sat in the interface between CaM and SK, Zhang *et al.* co-crystallized the small molecule 1EBIO with CaM-SK [91]. It was shown that the known SK channel modulator 1EBIO occupies a pocket between the two proteins acting as a molecular glue. 1EBIO itself is a weak stabilizer, the structurally closely related NS309 (6,7-dichloro-3-(hydroxyimino)indolin-2-one) is 1000-times more-potent in augmenting current in whole-cell HEK293 patch clamp assays and its action could be antagonized by the SK blocker apamin [92,93].

Stabilizers of nuclear-receptor–corepressor interactions

Most nuclear receptors (NR) are ligand-activated transcription factors. In general, they can be activated by agonists or blocked by ‘passive’ antagonists [94]. In addition, antagonists can also be inverse agonists actively recruiting co-repressors. During the past decade, several interesting crystal structures of NR in complex with antagonists were solved. Two important co-repressor proteins that will be discussed below are nuclear receptor co-repressor (N-CoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT).

Asoprisnil (J867) [95] is a steroidal selective progesterone receptor (PR) modulator (SPRM) in clinical development against endometriosis. It displays mostly antagonist behavior but also weak agonist effects in fluorescence polarization and mammalian two-hybrid assays. Two crystal structures of asoprisnil in the ligand-binding pocket of the PR with SMRT or N-CoR, respectively, were solved showing that the molecule is, with its hydrophilic NOH group, in direct contact with the lipophilic side-chain of Leu2263 in the SMRT peptide (3.16 Å) [96]. Hence, changing the NOH group could lead to hydrophobic interaction between co-repressor and small molecule. Also, asoprisnil strongly recruits the co-repressor N-CoR, but only weakly recruits SRC-1.

An additional example of NR ligands facilitating co-repressor binding is the propynylbenzene bearing pan-retinoic acid receptor (RAR) inverse agonist BMS493 (Table 1). The compound strongly increases interaction of all RARs (α , β , γ) with SMRT and N-CoR. This was first shown by electrospray ionization (ESI)-MS for RAR homodimers and RAR–retinoic-x-receptor (RXR) heterodimers [97]. The dissociation constant (K_d) in a fluorescence polarization assay shifted from 3.8 μ M (agonist Am80) to 0.18 μ M for a rhodamine-labeled SMRT peptide, whereas the K_d for a fluorescein-labeled SRC-1 peptide increased from 0.47 μ M (Am80) to 12.7 (BMS493). A year later, the same group was able to solve the crystal structure of BMS493, RAR α and a N-CoR 19mer peptide [98]. The compound is accommodated in the ligand-binding pocket making new contacts with Ile396 and Leu398, which changes the α -helix 11 of RAR into a β -strand that can interact well with a β -strand in the N-CoR peptide, an occurrence that has not been observed in a NR crystal before. In addition to contacts with RAR, BMS493 also makes hydrophobic contacts with Leu2051, His2054, Ile2055 and, to a lesser extent, Ile2058 of the N-CoR peptide, hence it qualifies as a true interface binding stabilizer of PPI. BMS493 could be used as a medicinal chemistry starting point for the development of subtype selective ligands.

Epibestatin and pyrrolidone1

The phytotoxic activity of fusicoccin A could in principle qualify this compound as a promising developmental candidate for a new total herbicide. However, the complex chemical nature and high costs of production by fermentation hampers its use in plant protection. Because the binding pocket of fusicoccin in the 14-3-3–PMA2 complex has the potential to accommodate less chemically demanding molecules we screened a mid-sized (37 000) small molecule library for stabilizers of the 14-3-3–PMA2 interaction and identified the dipeptide epibestatin and a pyrrolinone (pyrrolidone1) as significantly more amenable functional substitutes for fusicoccin [99]. The corresponding crystal structures showed that the two molecules bound to different sites in the complex interface; whereas epibestatin binds to a narrow pocket between 14-3-3 and PMA2 and is literally ‘sandwiched’ in a narrow cleft between the two proteins, pyrrolidone1 binds to a relatively open pocket. This binding behavior is reflected in the dynamics of the stabilizing activity of these two compounds with pyrrolidone1 mainly enhancing the association rate of the 14-3-3–PMA2 complex and epibestatin mainly decreasing the dissociation rate [99].

Concluding remarks

PPIs are normally regarded as less tractable than other more established protein target families [e.g. kinases, G-protein-coupled receptors (GPCRs), ion channels]. Their inhibition using small molecules is considered technically challenging owing to the extended, shallow, featureless and solvent-accessible nature of the PP-binding interface [100]. Despite these limitations, PPI inhibition is receiving more and more attention in current pharmaceutical research. This is primarily because of the need to afford novel therapies in ever-complex diseases that are poorly affected by molecular regulation of standard protein classes. In the context of PPI modulation, their stabilization represents a possible alternative to impact pathophysiological conditions. The examples of PPI stabilization reviewed here would definitely support the technical feasibility of such an approach. From a medicinal chemistry perspective, it is noteworthy that a wide range of chemical structures can act as PPI stabilizers. These range from natural products (e.g. rapamycin) to typical drug-like and lead-like small molecules (e.g. tafamidis) and fragments (e.g. 1EBIO), suggesting possible opportunities for molecular intervention. These examples notwithstanding, our understanding of PPI stabilization and translatability to differentiated medicines is very much in its infancy, especially compared with established target class modulation and even PPI inhibition, where large knowledge gaps still exist. Similarly to more-established mode-of-actions, we could define a number of areas that require thorough investigation as research teams embark on PPI stabilization efforts. These can be conceptually centered on: (i) the targeted PPI(s); (ii) the link between the PPI(s) and the sought effect(s); and (iii) the link between the PPI(s) and toxicities. In the context of the targeted PPIs, screening feasibility, the relevance of the selected *in vitro* system and the stabilization interaction itself need to be considered. Accurately measuring stabilization between two partners is far more laborious than monitoring inhibition, and it often relies on biophysical methods and indirect measurements of association. Here, assay development activities need to focus on ensuring rapid turnaround of accurate data to enable chemical optimization of the PPI

stabilizers. Because of the technical difficulties associated with stabilization assays, the protein partners are very rarely studied as a whole but are generally simplified using for example molecular biology techniques. Any such manipulation should not introduce *in vitro* artifacts or limit the ability to translate biophysical and biochemical results beyond the closed system employed. Lastly, a deeper understanding of the physical forces governing specific PPI stabilization could be advantageous. As researchers move away from simplified tactics like steric hindrance and functional isosterism, better appreciation of protein conformational aspects and resulting binding pocket plasticity is required. The cooperativity, additivity and/or synergy of protein oligomerization, its induction, development and time course are also areas worth exploring, especially with respect to kinetic aspects of ligand-induced stabilization.

The link between stabilization of a given PPI and the intended physiological effect requires continuous evaluation across systems of increased complexity, from recombinant protein models *in vitro* to whole animals *in vivo*. Special emphasis should be placed on relating *in vitro* measures of protein stabilization to the observed *in vivo* effect. This can include for example relative importance and regression analyses to identify the PPI stabilization parameters most descriptive of the effect *in vivo*. Incorporation of such parameters in pharmacokinetic–pharmacodynamic (PKPD) analyses would then be crucial to assess the true significance of PPI stabilization *in vivo*. Here, given the transient nature of protein–protein interfaces and their functional inference as well as the complex dynamics of associative and stabilization processes, adequate characterization of hysteresis would be advisable.

Similarly to exerting positive pharmacological effects, stabilization of PPIs could also lead to inappropriate toxicological

response. These could be directly ascribed to the targeted PPI or could originate from concomitant PPI stabilization events. In the first case, investigation of potential effect discriminants (e.g. tissue and/or organ distribution, protein under- or over-expression, baseline state versus disease state) and accurate quantification of effect onset, intensity and duration alongside the therapeutic index are required. As discussed before, translating back this information to the PPI stabilization parameters and modality would be extremely informative to the optimization of PPI stabilizers. Because PPI networks show a certain degree of redundancy [101] and promiscuous proteins are required for network function and stability [102], it is conceivable to assume that a given PPI stabilizer could stabilize different PPIs. This raises the issue of PPI selectivity and/or promiscuity in the context of a biological and toxicological response. Efforts to investigate the potential for multiple PPI stabilization (e.g. bioinformatics and 3D structural information analyses, pathway analyses, system biology and chemogenomics approaches) and their experimental validation following the concepts highlighted before would support a more focused approach to the development of PPI stabilizers.

Finally, as in the case of other established mode-of-actions, nature has already demonstrated the potential for PPI stabilization: a feasible strategy to modulate protein function successfully that results in significant physiological responses. In addition to natural products, artificial and synthetic molecules have been found to modulate their target proteins by stabilizing inactive oligomeric states. Together with the still relatively small number of success stories of ‘intended’ PPI stabilization as mode-of-action, these examples make an important case for targeted PPI stabilization in drug discovery.

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