1
Protein–Protein Interactions: An Overview
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1.1
Introduction

Protein–protein interactions (PPIs) are implicated in almost all biological processes for any given protein engaged in complexes with other proteins for the majority of its lifetime. In this regard, proteins function not merely as single, isolated entities, but display their roles by interacting with other cellular components. The different interaction patterns are at least as important as the intrinsic biochemical activity status (e.g., of a protein kinase) of the protein itself. Therefore, to understand the biological role of a protein it is of the utmost importance to know the underlying PPI network. This holds especially true in the case of diseases where, for example, mutations in oncogene or tumor suppressor proteins are recognized as the cause for malignancies. An impressive recent example for the relevance of the PPI interplay is the finding that active-site inhibitors targeting the oncogenic kinase B-Raf can under certain circumstances activate the underlying signal transduction pathway (mitogen-activated protein kinase (MAPK) pathway) instead of inhibiting it [1–3]. This finding is a strong reminder that nature in the majority of cases ultimately relies on regulating protein function by PPIs. In addition to taking into account this important concept for the drug development process, targeting PPIs significantly enlarges the "druggable genome" that was initially estimated to comprise around 1500 single protein targets [4]. While this number is still several times higher than the 266 human protein targets actually addressed by currently approved drugs [5], there are diseases that lack a good "conventional" target like an enzyme, receptor, or ion channel. By adding the number of PPIs occurring in the human body, the so-called protein–protein "interactome," this situation will definitely be improved. As the size of the interactome has been estimated to lie between 130 000 [6] and 650 000 [7], successfully addressing PPIs will vastly expand our opportunities for pharmacological intervention.

1.2
Role of PPIs in Human Physiology

Direct physical interactions of proteins are intricately implicated in the majority of processes in living organisms (Figure 1.1). For example, reception and propagation of growth signals can start with the binding of a proteinaceous signaling molecule like the epidermal growth factor (EGF) to its cell surface receptor (EGFR). This binding triggers the intracellular assembly and activation of signaling complexes comprised, for example, of adapter proteins like Grb2 and Sos and small G-proteins like Ras that – again by physically interacting – activate protein kinases like Raf. Activated Raf then stimulates a phosphorylation cascade via the kinases MEK (mitogen-activated protein kinase/extracellular signal-related kinase) and ERK (extracellular signal-related kinase) that ultimately leads to gene activation via transcription factors like Sp1 and Elk [8]. As each of these steps necessitates direct binding of the components of this signal transduction chain, small molecules inhibiting these interactions could disrupt this proproliferative signaling. Furthermore, stabilization of the inhibitory binding of regulatory proteins like the Raf kinase inhibitory protein (RKIP) [9] and 14-3-3 to components of the pathway (e.g., Raf) might also produce a therapeutic benefit.

Many cellular functions like motility are related to functional changes in the cytoskeleton. For example, dynamic assembly and disassembly of actin filaments are based on the interaction of actin with itself and with protein partners like ADF/cofilin and profilin [10]. Biological (surface) recognition, like in the immune system, is also mediated by PPIs as in the case of binding of lymphocyte function associated antigen (LFA)-1 presented on the surface of immune cells to intracellular adhesion molecule (ICAM)-1 found on the surface of endothelial cells [11]. This interaction enables immune cells to attach to the walls of blood vessels and to migrate into neighboring tissue to initiate inflammation.
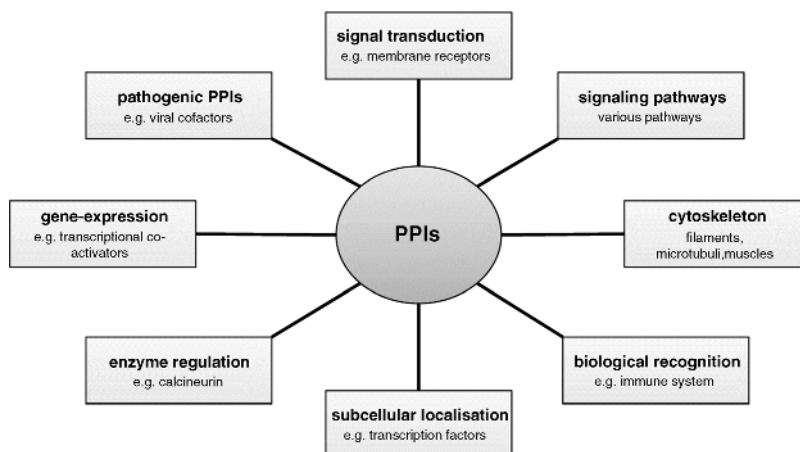
Figure 1.1 Examples of the role of PPIs in human physiology.

The control of subcellular localization is another important aspect of protein regulation performed by PPIs. For example, the transcription factor NFκB is prevented from nuclear import upon complexation with its negative regulator IκB [12]. The 14-3-3 adapter proteins play a similar role in the case of the FoxO transcription factor family [13]. Also, direct regulation of biochemical activity by PPIs is performed many times by PPIs. The phosphatase calcineurin is activated upon complexation with Ca^{2+}-activated calmodulin and repressed upon binding to cabin (calcineurin binding protein) or calcipressin [14]. Another important process involving PPIs is the functional constitution of transcriptional complexes. While transcription factors of the Tcf (T cell factor) LEF (lymphoid enhancer factor) family can directly bind to DNA, transcription starts only when coactivators like β-catenin additionally interact with Tcf/LEF [15]. Many proteins of disease-causing organisms need host proteins as cofactors for their pathogenic activity. For example, exoenzyme S from *Pseudomonas aeruginosa*, an opportunistic, pneumonia-causing bacterium, has to interact with host 14-3-3 proteins to be able to transfer an ADP-ribose moiety from NAD^+ to small G-proteins like Ras [16], thereby inhibiting its target proteins [17].

1.3
Regulation of PPIs

Given the importance and number of PPIs in the living cell it is no surprise that they have to be tightly orchestrated at any moment in time. The occurrence and perseverance of PPIs is governed by the two principal variables local concentration and intrinsic binding energy of the binary interaction [18]. The first is regulated by transcriptional and translational mechanisms, subcellular (co-)localization, degradation rates, and temporary storage. The second can be influenced by covalent modifications like phosphorylation, and by changes in pH, ionic strength, and temperature (Figure 1.2). Furthermore, additional PPIs can modulate binary interactions. They can be inhibitory when, for example, the interaction interface of one partner is masked by binding to the same interface or by simple sterical obstruction. They can also be stabilized, for example, when the third interacting protein binds simultaneously to both protein partners. Such a "bridging" or "assembly platform" function has been described for the A-kinase anchoring proteins (AKAPs) [19] and the kinase suppressor of Ras (KSR) [20]. It is now clear that the local architecture of such signaling complexes is one of the keys to understand regulation and specificity of signaling events.

1.4
Structural Features of PPI Interfaces

PPIs can be established between identical and nonidentical protomers leading to homo- or heterodimeric complexes, respectively. In the following, a number of examples are discussed in more detail. Small-molecule inhibitors have been identified for these PPIs (Table 1.1), strongly validating the general approach to pharmacologically interfere with the interaction of proteins.
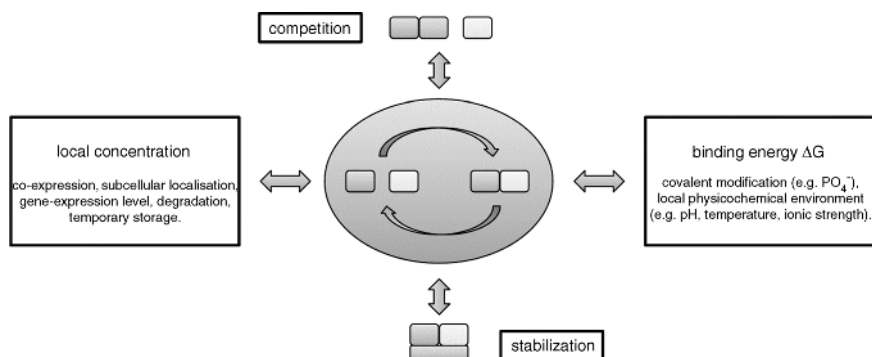
Figure 1.2 Factors governing the occurrence and perseverance of PPIs. Important control mechanisms for the oligomerization state of interacting proteins. The association–dissociation equilibrium between monomeric and multimeric states is regulated by the partners' local concentration and their mutual binding affinity. Additional cellular or pharmaceutical factors can compete for one partner or stabilize the dimeric complex. (Adapted from Nooren and Thornton [18].)

Table 1.1 Small-molecule PPI inhibitors from the pharmaceutical industry.

Target	Compound	Identification	Affinity (µM)	Reference	Company
Bcl-2	ABT-737	SAR by NMR	$K_i = 0.001$	[21]	Abbott
HDM2	Nutlin-2	HTS (biochemical)	$IC_{50} = 0.14$	[22]	Roche
TNF-α	SP307	combinatorial fragment assembly	$IC_{50} = 22$	[23]	Sunesis
RSV F$_1$	BMS-433771	HTS (cellular)	$EC_{50} = 0.012$	[24]	BMS
RSV F$_1$	JNJ 2408068	HTS (cellular)	$EC_{50} = 0.00016$	[25]	Johnson & Johnson
iNOS	PPA250	HTS	$IC_{50} = 0.082$	[26]	SSP
iNOS	compound **21b**	HTS	$K_d = 0.00029$	[27]	Berlex Biosciences
iNOS	compound **6**	HTS	$IC_{50} = 0.012$	[28]	Adolor
B7.1	compound **2**	HTS	$IC_{50} = 0.030$	[29]	Wyeth
HPV E1/E2	compound **10**	HTS	$IC_{50} = 0.35$	[30]	Boehringer Ingelheim
ZipA	pyridylpyrimidine 1	HTS	$K_i = 12$	[31]	Wyeth
IL-2	SP4206	tethering	$K_d = 0.07$	[32]	Sunesis
HDM2	TDP665759	HTS	$IC_{50} = 0.7$	[33]	Johnson & Johnson
LFA-1	LFA878	HTS	$IC_{50} = 0.05$	[34]	Novartis
LFA-1	compound **4**	epitope transfer	$IC_{50} = 0.0014$	[35]	Genentech/ Roche
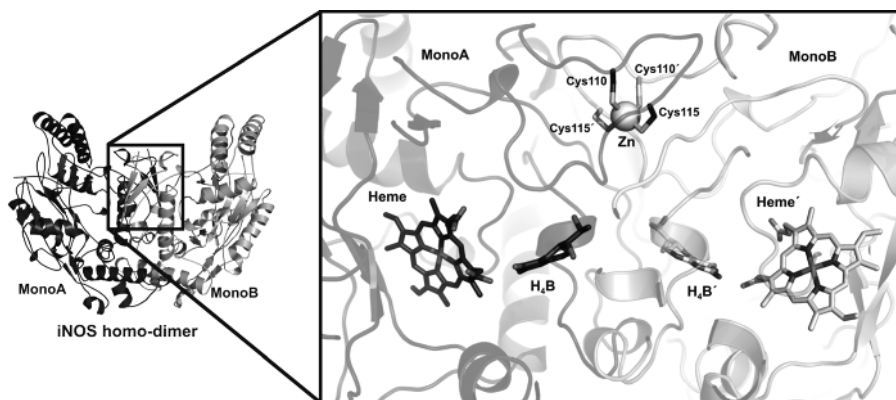
Figure 1.3 Structure of the iNOS homodimer. General topology of the iNOS dimer, and expanded, detailed view of the dimer interface with the cysteine-coordinated zinc ion and the cofactors H_4B and heme.

1.4.1
iNOS Homodimer

An example for a homodimeric protein complex is the inducible nitric oxide synthase (iNOS) that produces the signaling molecule NO from L-arginine [36–38]. To perform its catalytic activity NOS depends on the tightly bound cofactors tetrahydopterin (H_4B), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and iron protoporphyrin IX (heme). This enzyme is only active as a homodimer, and the crystal structures of the (dimeric) oxygenase domain [38] explained this fact by showing that the dimerization interface shapes the functional binding sites for the cofactors H_4B and heme (Figure 1.3). It also displays a large intersubunit cavity of about 750 Å^3 that is separated from the surrounding bulk solvent when a zinc ion is coordinated by two cysteines from protomer A and two cysteines from protomer B. With 69% nonpolar and 31% polar amino acids, the interface of the iNOS dimer shows a distribution that is typical for the majority of known homodimers. The contact surface of roughly 2900 Å^2 is rather flat. Nonetheless, mainly due to the special situation characterized by interface-bound cofactors, inhibitors of dimer formation could be identified successfully.

1.4.2
β-Catenin/Tcf4 Complex

The Wnt pathway found to be constitutively activated in many colorectal cancers is dependent on the interaction of β-catenin with transcription factors of the Tcf/LEF family. Normally, the transcriptional coactivator β-catenin can be sequestered in the cytoplasm and the Tcf transcription factor is inhibited by complexation with negative regulators of the Groucho family [39]. Upon Wnt activation, β-catenin is translocated into the nucleus and binds to Tcf to constitute the active transcriptional complex [40].
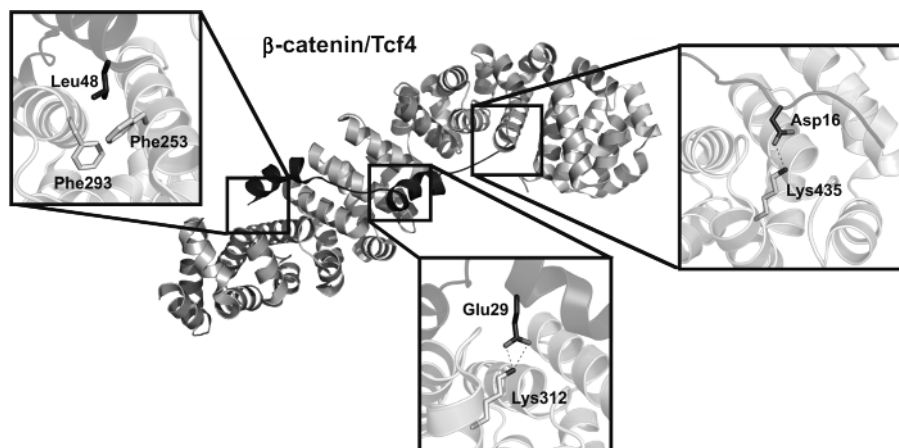
Figure 1.4 Complex of Tcf4 (black ribbon) bound to β-catenin (gray ribbon). Three hotspots of the interaction are presented in structural detail with key residues of Tcf4 (black sticks) and β-catenin (light gray sticks) labeled. Polar contacts are shown as black dotted lines.

The crystal structure of the human β-catenin/Tcf4 complex [41,42] revealed the multisite binding nature of the interaction with three regions of Tcf4 to be important for binding to β-catenin (Figure 1.4): (i) an extended N-terminal sequence, (ii) a kinked α-helix, and (iii) a second extended segment followed by the C-terminal α-helix. The binding module of Tcf4 wraps around the 12-membered armadillo-repeat region of β-catenin. Three essential interaction "hotspots" have been identified in the β-catenin/Tcf4 interface; a salt bridge between Tcf4 Asp16 and β-catenin Lys435, a hydrophobic contact of Tcf4 Leu48 to Phe253 and Phe293 of β-catenin, and a second salt bridge between Glu29 of Tcf4 and β-catenin Lys312. Disruption of one (or several) of these contacts by a small-molecule PPI inhibitor may successfully abolish binding of Tcf4 to β-catenin.

1.4.3
LEDGF/HIV-IN Complex

For a productive infection HIV depends on the viral integrase (IN) that integrates the genetic material of the virus into the host cell's DNA [43]. The human transcriptional coactivator LEDGF (lens epithelium-derived growth factor) is an essential host protein as cofactor for the function of IN that, among others, locates IN to the nucleus [44]. The interaction is mediated between the catalytic core domain (CCD) of IN and the IN-binding domain (IBD) of LEDGF [45]. The IN CCD/LEDGF IBD complex crystallized as an IN CCD dimer with two LEDGF IBD copies attached at opposing sites (Figure 1.5) [46]. An interhelical loop of IBD binds to a pocket at the IN dimer interface burying approximately 1300 Å^2 of protein surface. Binding is driven by the hydrophobic contact of LEDGF residue Ile365 to a pocket concomitantly established by IN residues Leu102, Ala128, Ala129, and Trp132 from one chain of the IN dimer (chain B), and Thr174 and Met178 from the other chain of the
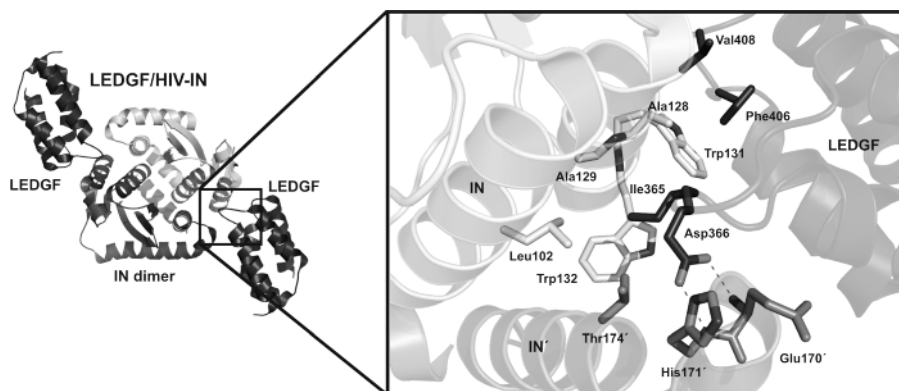
Figure 1.5 Crystal structure of the LEDGF/IN complex. Residues important for the interaction are depicted as black (LEDGF) or light gray (IN) sticks and polar interactions are represented by dotted black lines.

dimer (chain A). A second hydrophobic interaction is formed by Phe406 and Val408 of LEDGF that contact Trp31 of chain B of the IN dimer. Furthermore, LEDGF Asp366 makes a bidentate hydrogen bond to the main chain amides of Glu170 and His171 from chain A of IN.

Since mutational studies had shown that Ile365Ala, Asp366Ala, and Phe406Ala substitutions in LEDGF completely abrogate the LEDGF/IN interaction these sites identified in the crystal structure represent promising hotspots for PPI inhibition by small molecules.

1.4.4
HPV E1/E2 Complex

Another PPI that is essential for the pathogenicity of a viral infection is the E1/E2 complex of human papillomavirus (HPV). For successful replication a so-called prereplication complex must be formed that consists in the case of HPV of only two proteins, E1 and E2. E1 is the viral initiator protein that recognizes the viral origin and converts into the functional helicase [47]. For its full function, E1 needs to bind to E2 that helps to target E1 monomers to viral origins and assists in the assembly of the active helicase [48,49]. The overall topology of the HPV E1/E2 complex resembles a "C" with the top and the site formed by the E2 activation domain and the bottom by E1 burying $940\,\text{Å}^2$ of surface area per protomer [50]. Several essential contacts especially in the loop region between helices 2 and 3 in E1 have been identified, for example Arg454 that forms a salt bridge with Glu43 of E2. A hydrophobic hotspot is the interaction between Ile461 of E1 and Tyr23 as well as Leu98 of E2 (Figure 1.6).

While the interaction surface of the globular E1 is rather flat, the corresponding contact surface of E2 displays some pocket-like features that would allow binding of

HPV E1/E2
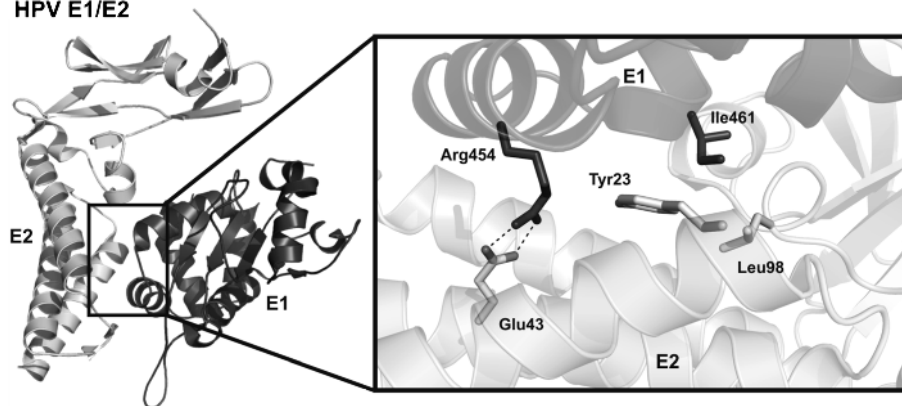
Figure 1.6 Structure of the HPV E1/E2 complex. Hotspot residues are shown as black (E1) or light gray (E2) sticks and polar contacts are depicted as black dotted lines.

small molecules. Therefore, it is no surprise that a successfully identified PPI inhibitor of the E1/E2 interaction was found to target E2 rather than E1 [51].

1.4.5
IFN-α/IFNAR Complex

Interferons (IFNs) are important signaling molecules that were discovered in the late 1950s as agents that interfere with the replication of the influenza virus [52], prompting their use as effective antiviral treatments. Due to their recognized role in enhancing immune responses and the modulation of normal and tumor cell survival, IFNs are also used in some cancer and multiple sclerosis therapies [53]. However, in certain pathophysiological conditions such as type I diabetes, IFN signaling can have deleterious effects, leading, for example, to inflammation that results in apoptosis of insulin-producing pancreatic β-cells [54]. Therefore, also the identification of pharmacological agents that attenuate IFN action by inhibiting binding of IFN-α to its receptor (IFNAR) is of therapeutic interest. In this regard, elucidation of the structural basis of IFN-α interactions with IFNAR was considered important. The structure of the IFN-α2/IFNAR2 complex was reported in 2011 (Figure 1.7) [55].

Examination of the interaction interface reveals that the single most important amino acid of IFN-α2 for binding to IFNAR2 is Arg33, forming an extensive hydrogen-bond network with the side-chain of Thr44 and the main-chain carbonyl oxygen atoms of Ile45 and Glu50 of the receptor. Mutating Arg33 to alanine reduces the affinity of the interaction by a factor of 4×10^5, literally abrogating the binding of IFN-α2 to IFNAR2 [55]. Another important polar contact is a salt bridge between Arg149 of IFN-α2 and Glu77 of IFNAR2, whose disruption by the mutation Arg149Ala reduces the affinity of the complex by two orders of magnitude. With regard to hydrophobic interaction clusters, two can be found in the IFN-α2/IFNAR2
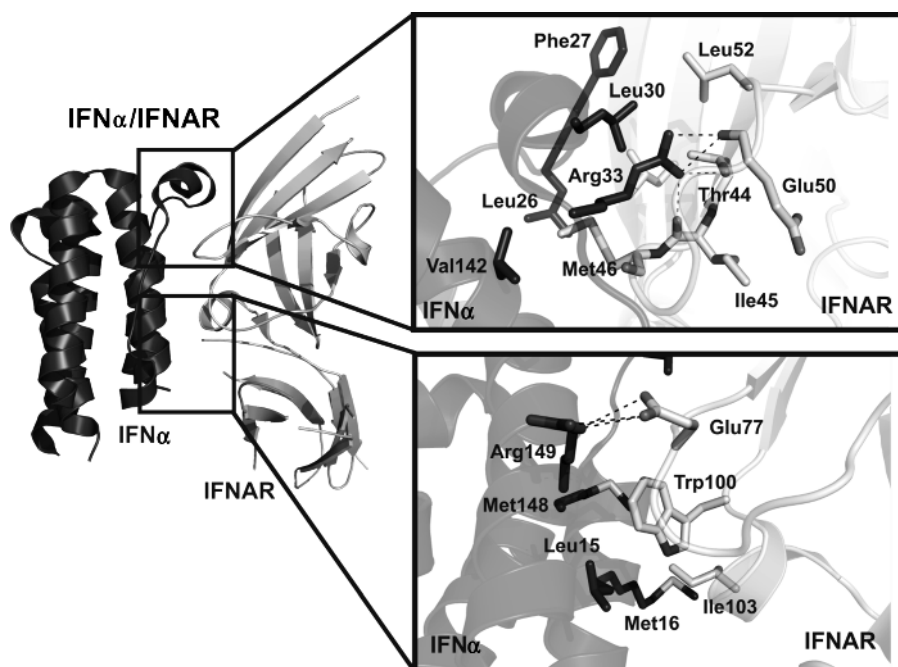
Figure 1.7 Structure of the IFN-α/IFNAR2 complex. Two hotspot regions have been identified in the extensive interface. Residues from IFN-α that contribute to essential contacts are shown as black sticks and those of IFNAR2 are shown as light gray sticks.

interface. The first is formed between Leu15 and Met16 of IFN-α2 and Trp100 and Ile103 of IFNAR2. The second involves a hydrophobic patch comprised of Leu26, Phe27, Leu30, and Val142 of IFN-α2 that contacts a corresponding patch in IFNAR2 build from Thr44, Met46, and Leu52. The substitutions of Met148Ala in IFN-α2 or Ile103Ala in IFNAR2 reduces binding 10- to 30-fold.

1.4.6
TNF-α Trimer

A further important protein hormone molecule is the tumor necrosis factor (TNF)-α that is produced predominantly by activated macrophages and lympho-cytes, and plays a central role in inflammation processes [56]. TNF-α's name is derived from its activity to induce hemorrhagic necrosis of certain transplantable tumors in mice and its cytotoxicity towards a variety of tumor cells in culture [57,58]. The physiological functions of the molecule are conferred by binding to surface-expressed receptors [59]. Therapeutic antibodies that directly target TNF-α like etanercept (Enbrel™; Amgen Incorporated, Thousand Oaks, CA/Wyeth Pharmaceuticals/Pfizer, Collegeville, PA), infliximab (Remicade™; Centocor, Horsham, PA/Schering-Plough/MSD, Kenilworth, NJ), and adalimumab (Humira™; Abbott Laboratories, Abbott Park, IL) have produced significant advances in the
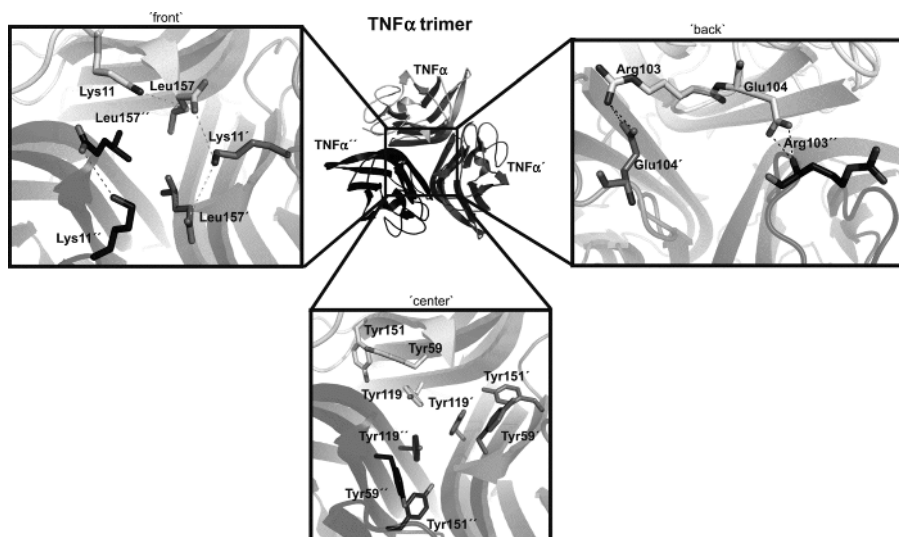
Figure 1.8 Structure of the TNF-α trimer. Three areas important for formation of the trimer have been identified, one each at the "front" (top) and the "back" (bottom) of the trimer and one in the center of the molecule.

treatment of rheumatoid arthritis and corroborated the feasibility of addressing this signaling protein. Active TNF-α has been shown to be a trimer in solution [60]. The crystal structure of the TNF-α trimer (Figure 1.8) revealed an interface that buries 2200 Å² of each subunit involving some 40 residues [61]. Eighteen of these are glycine, alanine, valine, leucine, isoleucine, or proline, five are tyrosine or phenylalanine, another eight are uncharged polar, and the remaining nine residues are charged. The latter are responsible for polar intersubunit interactions that are predominant at the top of the dimer where an intrasubunit disulfide bride is also located. Salt bridges can be found between Glu104 of one and Arg103 of the adjacent subunit, and between Lys11 and the terminal carboxylate at Leu157. A hotspot of hydrophobic interactions is formed by a cluster of three tyrosines (Tyr59, Tyr119, and Tyr151). Notably, this is the region that binds a small molecule that has been identified to disrupt the functional TNF-α trimer [23].

1.5
Identification of PPI Inhibitors

In the past years numerous PPI interactions have been addressed successfully with small-molecule inhibitors, adding up to several hundred molecules targeting more than 40 protein complexes [62]. In addition to many reports from academic institutions, the pharmaceutical and biotech industry plays an important role (Table 1.1). A wide variety of methodological approaches and techniques have been used for

the primary identification of PPI inhibitors. An encoded combinatorial chemistry library was screened in a whole-cell assay for inhibitors of NO production identifying pyrimidinimidazoles that inhibit iNOS activity by disrupting homodimer formation [63]. These molecules were further optimized to yield compound **21b** (Table 1.1 and Figure 1.9) that inhibited NO production in A172 cells with IC_{50}s in the subnanomolar range [27]. Small-molecule PPI inhibitors of the β-catenin/Tcf interaction, like ZTM00990 (Figure 1.9), were identified from a library of 7000 purified natural products [64]. The group of Debyser reported the *in silico* identification of a lead compound disrupting the LEDGF/p75 interaction with HIV IN [65]. To this end,
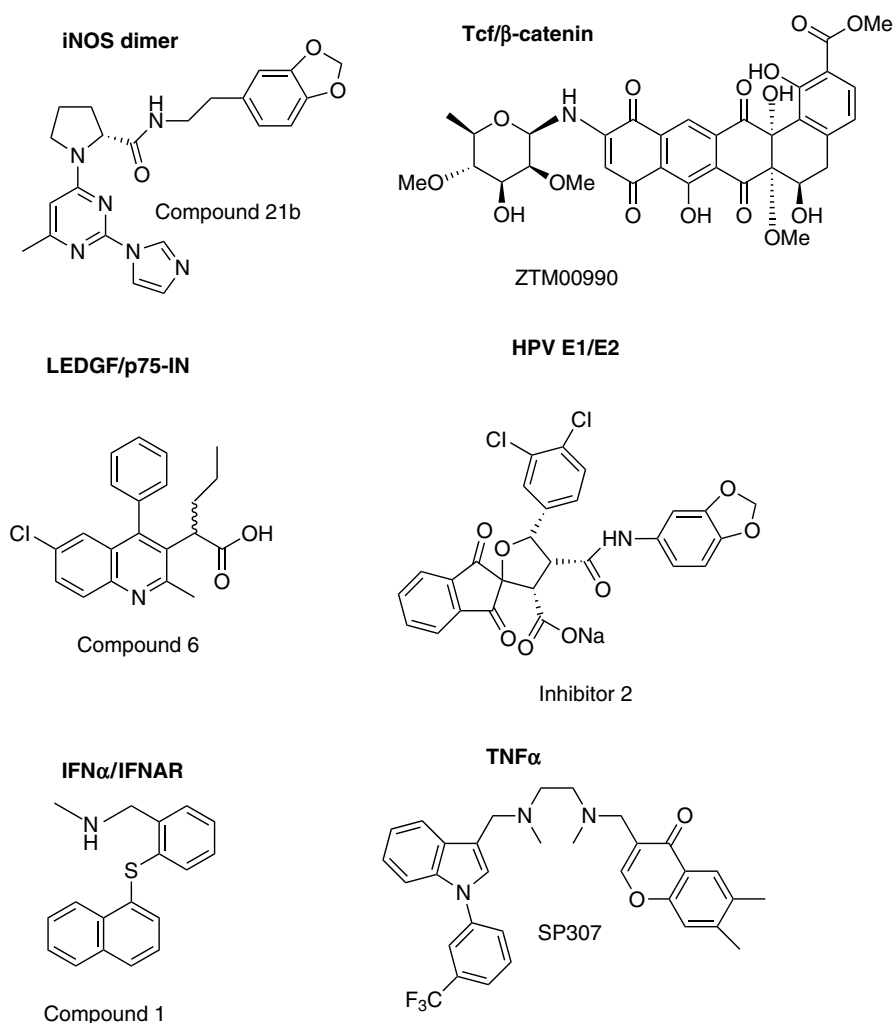

Figure 1.9 Small-molecule inhibitors of PPIs.

they started their investigations with a 200 000-compound virtual library that was scanned for suitable small molecules. Remarkably, the algorithm employed was so powerful that only 25 compounds had to be tested in a biochemical assay to identify and validate the hit molecule compound **6** (Figure 1.9). A screen for HPV E1/E2 interaction inhibitors with a 140 000-compound library produced one lead structure for further development [30]. A derivative thereof (inhibitor **2**, Figure 1.9) was later cocrystalized with E2 revealing the compound bound to the pocket that lies in the contact surface with E1 [51].

Recently, Schneider *et al.* reported the first PPI inhibitor of the IFN-α/IFNAR interaction [66]. Starting from the nuclear magnetic resonance (NMR) structure of unbound IFNAR (Protein Data Bank ID: 1TIF) they identified druggable sites on the protein interaction surface. These were used for the generation of a pharmacophore that was screened against a 556 763-virtual-compound library identifying one lead compound (compound **1**, Figure 1.9). To inhibit TNF action, a 285-membered initial library was used as a starting point for a combinatorial fragment assembly strategy that led to the identification of 15 fragments whose possible combinations were subsequently tested. These investigations revealed a molecule (SP307, Figure 1.9) that potently disrupted the TNF-α trimer, thereby abrogating the binding to its receptor [23].

In addition to the examples presented here in more structural detail, there are some "classical" success stories of PPI inhibition with small molecules. Among them, disruption of binding of the ubiquitin ligase MDM2 to the tumor suppressor protein p53 by Nutlin-2 (Figure 1.10) identified by scientists from Roche [22], the benzodiazepinediones (Figure 1.10, TDP665759) from Johnson & Johnson [33], and PB11 from the Dömling group [67] are well-known examples. Furthermore, "SAR (structure–activity relationships) by NMR" was used to identify the precursor fragments of the Bcl-2/Bak inhibitor ABT-737 (Figure 1.10) [21], and "tethering" was employed to identify small molecules that bind to interleukin (IL)-2 and disrupt the interaction with its receptor, IL-2R (SP4206, Figure 1.10) [32]. From a 250 000-compound library, 19 molecules were identified that inhibited the ZipA/FtsZ interaction, such as pyridylpyrimidine 1 (Figure 1.10) [31]. A smaller library was successfully employed in high-throughput screening (HTS) campaigns as in the case of the search for inhibitors of the PICK1 PDZ domain where 44 000 compounds were screened in a fluorescence polarization format, resulting in the identification of FSC231 (Figure 1.10) [68]. In addition, a screen for eIF-4E/eIF-4G interaction inhibitors with only 16 000 compounds yielded successful hits like 4EGI-1 (Figure 1.10) [69]. Recently, a lead structure (pitstop 1, Figure 1.10) was identified by screening 17 000 compounds in an enzyme-linked immunosorbent assay-based assay and were subsequently developed into potent PPI inhibitors of clathrin-mediated endocytosis [70]. This compound binds to the terminal domain of clathrin which disrupts the interaction with clathrin-binding accessory proteins like amphiphysin, AP180, and synaptojanin. The group of Botta reported the identification of a small-molecule inhibitor of the c-Abl/14-3-3 interaction by employing structure-based pharmacophore modeling, virtual screening and molecular docking
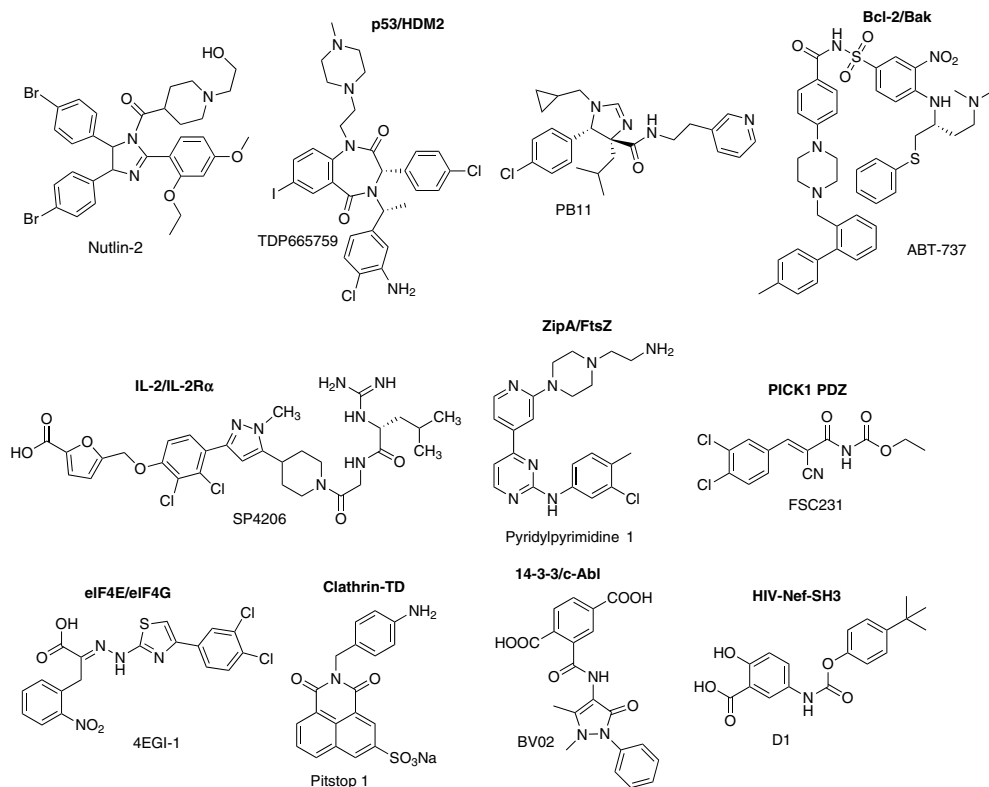
Figure 1.10 Further inhibitors of PPIs.

simulations. They also started with roughly 200 000 compounds, of which finally 14 compounds were tested in cellular and biochemical assays, resulting in the identification of one lead structure (BV02, Figure 1.10) [71]. Furthermore, a low-micromolar-active inhibitor of the HIV Nef–Src homology 3 (SH3) interaction (D1, Figure 1.10) was found by docking a 1990 compound virtual library into a pocket in the Nef–SH3 interface [72].

1.6
Conclusions and Outlook

The examples of successful inhibition of PPIs illustrate the principal feasibility of this approach in drug development. With an estimated number between 130 000 and 650 000 PPIs in the human body, it is in principle plausible to identify a "druggable" PPI for every disease or (patho)physiological condition. Since nature regulates protein function mainly by interaction with other proteins, the strategy to

modulate PPIs with small molecules is an ideal concept to complement more classical approaches of pharmacological intervention. One can, for example, envision that simultaneously targeting a prosurvival pathway with active-site inhibitors and PPI modulators might produce a maximum benefit in cancer therapy. As the examples of the LEDGF/HIV IN, HPV E1/E2, or the ZipA/FtsZ PPI inhibitors show, new active agents against viral or bacterial infections might be also developed based on "hitting" essential (and unique) PPIs in these organisms. Over the last 10 years our knowledge about how to target PPIs with small molecules has dramatically increased, holding great promise for future clinical applications of this kind of compounds.

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