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Targeting Protein–Protein Interactions Perspective

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20.1 Introduction

Protein–protein interactions (PPIs) encompass a diverse range of molecular interfaces of high importance in biology. Due to their ubiquitous roles, they are often considered as possible targets for therapeutic modulation, either by inhibition or stabilization of complexes. The wide scope covered by the term PPIs means that broad statements applied to this group are an oversimplification, but they are generally considered as difficult targets from a chemical tractability (or ligandability) perspective. This chapter introduces a range of PPIs, outlines some common themes and challenges faced by their drug discovery efforts, and gives examples of progress in addressing these.

Proteins within a complex often serve to activate or inhibit each other either by conformational engagement (e.g. muscle movement, amyloid function) or by transferral or removal of a moiety (e.g. phosphorylation, methylation, ubiquitination, etc.). Going beyond simple binary PPIs, the assembling of multicomponent complexes provides biology with some of the most exquisite opportunities for integration of input signals and subsequent output. Estimates of the size of the PPI interactome are based on data derived from, for example, yeast 2 hybrid (Y2H) experiments. Early studies suggested that the *S. cerevisiae* interactome contained around 20 000 binary interactions, but this was likely an underestimate [1], and, with more careful curation and analysis, current estimates for the human interactome range from 130 000 [2] to 650 000 [3]. By contrast, the number of PPIs for which we have sufficient structural information to be able to examine the molecular details of the contact interfaces is limited to between 200 and 300 heteromultimeric complexes. An even smaller number of crystal structures are available of a PPI protein with an inhibitor bound that effectively displaces its protein partner [4]. To fill the gap of structural knowledge, a plethora of *in silico* methods have been developed to both analyze known interactions and predict likely sites for binding partners based on the structures of unbound proteins [5–10].

Despite these efforts, choosing "good" PPI targets for drug discovery remains a huge challenge, and while there is progress in understanding the significance and redundancy of any given PPI in biological pathways and the druggability of the PPI interfaces, our insights are still in their infancy, and the scale of the problem remains daunting.

20.2 Detection and Analysis of PPIs

When characterizing PPI interfaces, the type of complex must be taken into account. Broadly, PPIs are classified as either obligate or transitory, although these perhaps represent two extremes of the spectrum. Obligate PPI complexes can be formed immediately after the proteins are produced and then remain together throughout their lifetime. Transitory PPIs, by definition, have a temporary existence. Many of the methods by which PPIs are detected (e.g. library methods such as two-hybrid or phage, or physical selection methods such as co-immunoprecipitation [coIP] pull-down experiments) can lead to an overemphasis on PPIs that are bound for a considerable period of time and thus have slow off-rates [11]. Such PPIs will tend to be obligate rather than transitory. By contrast, although sometimes excluded from the PPI classification, proteases and other enzymes that have protein substrates tend to bind in a more fleeting nature; otherwise they would not be able to operate catalytically. Truly obligate PPIs will inevitably not make good drug targets as there is only a limited window of opportunity to prevent complex formation. Unfortunately, PPIs that need very brief encounters to illicit a biological response may also be difficult for other

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reasons. Firstly, they may be difficult to identify, and, secondly, it may be difficult to stop all transient interactions unless a long-acting inhibitor can be found. Fortunately, the enormous spectrum of PPI lifetimes provides many regimes for which there is potential for effective intervention.

The first review of the three-dimensional (3D) characteristics of PPIs was conducted by Cyrus Chothia and Joel Janin in 1975 based on a very limited dataset [12, 13]. Their conclusion that between 1130 and 1720 $Å^2$ of surface area is removed from contact with water when a protein is buried within a PPI has stood the test of time, although more recent studies would broaden this to 1600 ± 350 $Å^2$. Why PPIs are of this magnitude is probably a result of the basic interaction geometry of objects of the typical size of globular proteins (i.e. 60–80 Å diameter). If such an object (total surface area of 11 000–20 000 $Å^2$) is allowed to interact with another similar object to a depth of 6–8 Å (i.e. the depth of one amino acid), the surface area of contact will be c. 1/10th of the total, yielding a figure of 1100–2000 $Å^2$, which is the typical size observed. PPI surfaces have evolved to have excellent mutual surface and electrostatic complementarity. They have a higher ratio of hydrophobic-to-hydrophilic residues in the interaction area than would normally be expected for the surface of a protein [14]. This, again, is to be expected, as the coming together of two proteins to create a PPI can be considered an extension of the same driving force behind the creation of the hydrophobic core of a protein.

Jim Wells et al. pioneered the use of site-directed mutations (alanine scanning) to explore the basis of the energetics of PPIs and introduced the concept of hot spots to explain the fact that not all mutations had a deleterious effect on the binding energy [15, 16]. A hot spot is a residue that on substitution by alanine causes a significant drop (>2 kcal mol^{-1}) in the binding energy. When coupled with structural insights (particularly in relation to the presence of cavities on the surface), this can be a very powerful method for understanding whether a PPI is likely to be druggable. In fact, *in silico* analysis can be done without the need for alanine scanning, instead relying on force fields to inform on likely binding energetics. Originally pioneered by Peter Goodford with the program GRID [17], many different approaches have been developed to understand the role of surface curvature, water structure, and other parameters. These can range from simulation-based methods (e.g. WaterMap [17–19] and szmap [OpenEye Scientific Software, Inc.]) to completely empirical methods such as GRID [17] and SiteMap [20]. SiteMap characterizes the pockets of a protein according to their size, enclosure (solvent accessibility), and hydrophilicity. As these approaches all require a structural model of appropriate resolution and accuracy, their greatest utility may lie within emerging families of PPI domains where there is a high structural knowledge across the entire target class, but where the ligandability of each member remains sparsely explored. An example of this is in the epigenetic reader area discussed later in the chapter [21, 22]. In many of these families, conserved elements of binding, for example, those residues that recognize posttranslational modifications (PTM), locate the likely site of interaction and some key hot spots. However, differences in the nature (enclosure, hydrophobicity, charge) of the surrounding binding regions among family members, responsible for their biological specificity, give rise to differential ease for small-molecule PPI modulation that *in silico* methods may help predict. Over the past decade, the general role of hot spots, water structure, and the hydrophobic effect in driving the PPI have all become clearer. Hot spots have imperfect hydration, involving less stable water molecules with a shortage of hydrogen bonding partners relative to bulk water levels, and this has been referred to as the epistructure of protein surfaces [23].

While PPIs are generally considered as large, flat, and relatively featureless compared to small-molecule binding sites, their biological significance, the discovery of hot spots, and the development of a variety of surface analysis tools have increased interest in identifying those PPIs that are more tractable to small-molecule intervention. For less tractable extracellular targets, the opportunity to use therapeutic antibodies provides an increasingly exploited alternative route, especially where there is strong disease rationale but where structural insights suggest low likelihood of small-molecule success. An example is the targeting of the proprotein convertase subtilisin/kexin type 9 (PCSK9), an important enzyme in lipid metabolism. Gain-of-function mutations in this protein are linked to elevated low-density lipoprotein cholesterol (LDL-C) levels and premature coronary heart disease, whereas loss-of-function variants lead to a low LDL-C level and a reduced incidence of coronary heart disease. Given obesity trends, it is of little surprise that PCSK9 inhibition has been avidly pursued by multiple approaches, especially for patients for whom statins are not sufficiently effective [24]. An important discovery in this pursuit was that while the proteolytic enzymatic activity of PCSK9 is necessary for its intracellular maturation and subsequent secretion, the extracellular PPI between PCSK9 and the LDL receptor (LDLR) was a critical point of pharmacological intervention. The structure of the PCSK9/LDLR complex, however, revealed a large and featureless PPI surface that has proved intractable to direct small-molecule antagonism. In contrast, a monoclonal antibody (mAb) approach has enabled candidate mAbs to be progressed into clinical trials, with early results suggesting these to be effective

and well tolerated in combination with standard statin therapy [25, 26].

The role of conformational flexibility in one of the partners within a PPI has been highlighted by Tom Blundell et al. as being another important parameter in determining the small-molecule ligandability of a PPI. Proteins that undergo concerted folding of one partner upon binding tend to do this where the "receiving" protein has a more defined and rigid binding site. Proteins that bind flexible/disordered peptides tend to have relatively little induced conformational change in their own structure upon binding, and thus they represent some of the most promising targets for inhibition. Examples of this may be recognition domains such as the 14-3-3-containing proteins that recognize specific peptide sequences, especially phosphorylated motifs [27].

With such a diversity of opportunities and challenges, databases of compiled information are important resources that allow access to protein structural information. TIMBAL is a database from Tom Blundell's group that has information on compounds of MM < 1200 Da that modulate PPIs. It is automatically updated from the ChEMBL database although there is some additional manual curation. The TIMBALv2 database [28] currently covers over 50 known PPIs that are potential drug targets via inhibition or stabilization. Information includes ligands, their binding affinities, and links to the original data. By contrast the 2P2I database is a hand-curated database that covers only PPIs for which the structure of a component of a protein–protein complex with a small-molecule inhibitor or the entire complex with a stabilizer is known. This dataset has over 14 PPIs with more than 70 small-molecule inhibitors or stabilizers identified [29, 30]. These collations allow interrogation of geometrical parameters, atom and residue properties, buried accessible surface area, and other biophysical characteristics within PPI interfaces and comparisons to those from representative datasets of non-PPI-type modulators. There are also a range of tools available such as 2P2I Inspector, Score, and HUNTER [31, 32], which can be used in the analysis and design of PPI-modulating compounds.

A seminal publication by the 2P2I team based on such analyses clearly illustrates the differences in ligand efficiency metrics between drugs or potential drugs acting at PPI interfaces and those working at non-PPI interfaces [33]. The implication of this is that PPI-disrupting small molecules (where they can be found) are generally larger and more lipophilic than non-PPI-interacting compounds. This has consequences for the developability of such compounds into safe and efficacious drugs [34]. David Fry at Roche has built on these analyses and developed guidelines to assist in the design of PPI inhibitors [35]. These include the observation that while it is invariably possible to find a small peptide that binds to the proposed target, the peptidic backbone itself will ultimately not be critical for the development of non-peptide mimetics – whether these mimetics are found by high-throughput screening (HTS) of small-molecule libraries or iterative peptidomimetic work. He also observed that PPI binders possess a greater degree of three-dimensionality than a typical drug, as measured by fsp3 metrics (i.e. number of sp^3-hybridized carbons/total carbon count). It is not clear whether this is due to the need to support a larger flat interaction surface (i.e. a volume-to-surface area issue), the need for multidirectional vectors from a rigid core or that, at present, a significant proportion of PPI inhibitors have been derived from peptide and macrocycle structures that are largely sp3 in nature. Additionally, he cautioned against establishing a single set of rules or expectations for designing PPI modulators because of the wide diversity of PPIs.

One way that has emerged to address the question of which PPIs are tractable to small-molecule intervention experimentally is the use of fragment screening. Initially proposed by Phil Hajduk et al. at Abbott, tractability indices for protein targets derived from nuclear magnetic resonance (NMR)-based screening hit rates have been found to be a rapid and relatively cheap way to measure chemical tractability of target proteins by several groups [36, 37]. Fragment libraries can sample chemical space more efficiently than larger molecules. Typically this method uses ^{15}N-labeled protein and the acquisition of a ^{1}H—^{15}N NMR spectrum. In this two-dimensional spectrum, a single peak is observed for each directly bonded proton–nitrogen atom pair. The spectrum is therefore readily interpreted as a residue-resolved map, or "NMR fingerprint," that monitors the environment of the entire protein backbone. Ligand binding induces perturbation in a subset of resonances in the spectrum, which allows the binding event to be observed and may also yield the site and affinity of the interaction. While the thresholds used to classify low, medium, and high ligandability may vary between groups, hit rates of <1% are generally considered to indicate low tractability, while at the other extreme >10% considered high.

With the advance of several small-molecule PPI inhibitors into clinical trials, it is important to bear in mind that terms such as ligandability, chemical tractability, and druggability are often used interchangeably to describe only the *in vitro* potential for PPI modulation. For compounds to have utility as *in vivo* medicines, additional considerations such as selectivity, bioavailability, metabolic stability, cell permeability, and safety must also be satisfied. Given that many PPI inhibitors fall outside Lipinski's rule of five [33, 38] and novel modalities such as stabilized peptides [39] are being explored, clinically successful PPI inhibitors may expand our

understanding of the types of molecules that can be made into marketable drugs.

20.3 PPI Screening

20.3.1 Screening Technologies

There are many technologies that enable detection and screening of compounds that disrupt PPIs. Their strengths and weaknesses are well summarized elsewhere [40–42]. However, it is instructive to consider the degree of knowledge around a PPI that may determine the best choice of screening technology.

Co-immunoprecipitation of endogenous proteins is probably considered to be the gold standard assay for identification of PPIs. Traditionally the protein of interest was isolated with a specific antibody, and interaction partners that co-purified were subsequently identified by Western blotting or mass spectrometry. These immunoprecipitation experiments detect both direct and indirect interactions, and as such they do not exclude the possibility of bridging entities such as nucleic acids and other proteins. These simple experiments contain no quantitation, so information about the stoichiometry of the interactions or degree of complexation within the cell may not be elucidated. Subsequent experiments, perhaps exploiting a range of techniques and conditions, may allow some of these questions to be more thoroughly explored, but it can often prove difficult to provide all the answers, thus compounding the problems of configuring effective PPI inhibitor assays. For PPIs with good validation of cellular association, lack of specific molecular knowledge can be mitigated by use of more endogenous systems (e.g. cellular assays or use of cell lysates rather than purified recombinant proteins), enzyme fragment complementation assays [43, 44], and reporter-based readouts [45]. For example, a reporter assay based on cytoplasm-nuclear redistribution was used to find p53–MDM2 PPI inhibitors [46]. This can allow for minimum deviation from the physiological environment and can remove explicit consideration of requirements such as additional partner proteins, PTM, necessity of metabolite or cofactors, and effects of cellular compartmentalization, e.g. pH. At the other extreme for PPIs, where there are well-defined crystal structures of the complex and the molecular interactions have been elucidated to atomic resolution, there may be confidence to use simple competition assays, (e.g. ELISA-based formats, fluorescence polarization [FP], Förster resonance energy transfer [FRET], AlphaScreen), that monitor the competitive displacement of a partner protein, peptide, or small molecule from an appropriate target protein construct.

The wealth of structural data emerging from structural genomics efforts has produced another interesting category of PPI targets that combine elements from these two extremes. These targets are domains believed to have PPI function, with experimentally determined (X-ray or NMR) 3D structures, but for which a native partner and biological function have yet to be defined. Epigenetic acetyl- and methyl-lysine reader domain families are examples of this category. Faced with a poor understanding of their cellular function and the absence of known competitive ligands for assay development, biophysical direct binding methodologies [47] such as NMR, surface plasmon resonance (SPR), differential scanning fluorimetry (DSF), and isothermal titration calorimetry (ITC) have proved particularly powerful tools for this PPI category. Besides their use to find and confirm putative peptide partners, these approaches can also yield chemical probes to deconvolute the biological function of a PPI domain. In this quest, high-content techniques such as protein NMR and SPR are particularly attractive as they capture information that can be used to eliminate compounds with undesirable mode of action (MOA) at an early stage and are amenable to fragment screening.

20.3.2 Fragment-Based Screening Methods

For PPI targets where there is structural data for at least one binding partner, fragment-based methods provide an accessible way to find inhibitor starting points. Several factors probably contribute to its success for these proteins.

Firstly, the ability to sample chemical space efficiently with a relatively small compound set. As an illustration, a 1000-member set of fragments with 13 heavy atoms each is estimated to cover c. 1×10^{-2}% of the chemical space spanned by all theoretically possible molecules of that size, whereas a 1 million-member collection of compounds with 30 heavy atoms each covers just c. 1×10^{-22}% of the potential chemical space. For domain families sharing common structural features, knowledge-based fragment libraries, biased toward a known family pharmacophore, can further increase the frequency of finding hits. However, for these fragments that bind in a conserved site across multiple members of a protein family, the introduction of selectivity will be a key consideration during early optimization [48]. This is not to say that HTS cannot be successful for PPIs: for example, the first inhibitors of the p53–MDM2 [49] and BET/histone PPIs [50, 51] were first identified by diversity screening of pharma compound collections. However, the appropriate compounds to give the micromolar affinities typically required for robust detection within a traditional screening campaign may not be present for all PPI targets.

Secondly, fragment-based drug discovery often makes use of biophysical as well as biochemical methods. For proteins that lack the option of MOA confirmation by enzymatic approaches, biophysical approaches offer important orthogonal confirmation assays, as well as alternative primary hit identification opportunities, to arrive at well-validated chemical starting points for PPIs. For example, sensitive AlphaLISA assays have proved a popular discovery option for inhibitors of epigenetic reader/histone interactions [52], but experienced practitioners will usually try to validate hits using NMR, SPR, and/or ITC [53]. These techniques, especially ITC, require milligram quantities of high-quality purified protein, the production of which has normally already been established for proteins where structural studies have been successful. NMR was the first widely used fragment screening methodology, and its application led to PPI inhibitors such as those for XIAP [54, 55], B-cell lymphoma 2 (BCL2) [56, 57], and K-RAS [58, 59].

The ability to iteratively generate protein–ligand complex structures with a chronology of hit and lead molecules to guide productive chemical elaboration has also been invaluable for progress in PPI modulation. There are at least two strategies used to transform initial starting points into efficient, larger, and more potent molecules. One is to find two or more fragments binding in proximal hot spots and to find a suitable way to link them together. Another is to grow from a fragment binding at a single anchor point. The linking strategy theoretically appears more elegant but is often constrained by the limited repertoire of linkers possible and the requirement for the linkers to meet strict geometric constraints in order not to compromise the binding of the original fragments. In comparison, the fragment-growing strategy provides more freedom for development at each step and more scope for optimization. Also, it does not require the discovery of proximal hot spots, which may not exist for many targets. Therefore, while both approaches have been successful, the growing option appears to be more practical and widely adopted [60, 61].

One challenge of the fragment-based approach for PPIs is that many complexes involve more than a binary interaction between two partners. Good examples of this are the complexes involved in transcriptional and epigenetic regulation where the proteins within the chromatin and DNA binding complexes vary as genes are activated or repressed. A danger in the reductionist approach of using isolated protein truncates may therefore be lack of translation from *in vitro* activity into functional physiological effects. The ideal choice of truncate or complex is not always possible to design from the start, but extension beyond the smallest possible domain may be valuable in some instances. This is exemplified for the histone methyl reader malignant brain tumor (MBT) family, where the use of an assay protein consisting of the first three MBT domains, rather than just one domain, was critical to the development of the nanomolar UNC1215 molecule [62]. Similarly, use of the tetrameric multi-protein core MLL1 complex was necessary to understand the ability of the methyl reader WDR5/MLL1 antagonists to modulate the demethylase activity of MLL1 [63–65]. Even for the classical MDM2–p53 interaction, there is data to suggest functional antagonism can be achieved outside of direct blocking at the MDM2–p53 interaction interface [66, 67].

20.4 Examples

20.4.1 Inhibitors of MDM2–p53: A Breakthrough in PPI Targeting

The tumor suppressor protein p53 plays a critical role in cell cycle regulation, DNA repair, angiogenesis, and apoptosis. In approximately half of human cancers, p53 is inactivated by deletion or mutation of the TP53 gene that encodes the p53 protein. In the remaining half, its tumor suppressor function is inhibited by the oncoprotein murine double minute 2 (MDM2) or its homolog MDMX. Blocking the MDM2–p53 or MDMX–p53 PPI therefore provides an opportunity to return the p53 tumor suppressor activity to oncogenetic cells that retain wild-type p53.

A short 15-residue α-helical peptide of p53 is sufficient to bind the N-terminal domain of MDM2 (K_d c. 500 nM). Two helical turns place three key hydrophobic residues of p53 (Phe19, Trp23, and Leu26) into a deep hydrophobic pocket in the central region of the peptide binding groove of MDM2 (site 1, Figure 20.1a, PDB: 1YCR). In comparison, MDMX interacts with the same three key hydrophobic residues using a similar but not identical hydrophobic surface groove. While the resolution of these features allows a structure-based strategy to target the MDM2–p53 PPI, the first inhibitors of this interaction were found by HTS. Additional peptidomimetics and computational drug design approaches have followed. As a result of these efforts, several MDM2–p53 PPI inhibitors have entered clinical trials, including RG-7112 (PDB: 4PIF, Figure 20.1b).

In 2004, scientists from Hoffmann-La Roche, Inc. reported the first class of potent and specific small-molecule MDM2 inhibitors [49, 68]. The series of *cis*-imidazoline with four attached substituents were named nutlins. A co-crystal structure of MDM2 with nutlin-2 (PDB: 1RV1) showed that it is a direct orthosteric antagonist of p53 with the two phenyl rings and the ethoxyl group of nutlin-2 mimicking the three key

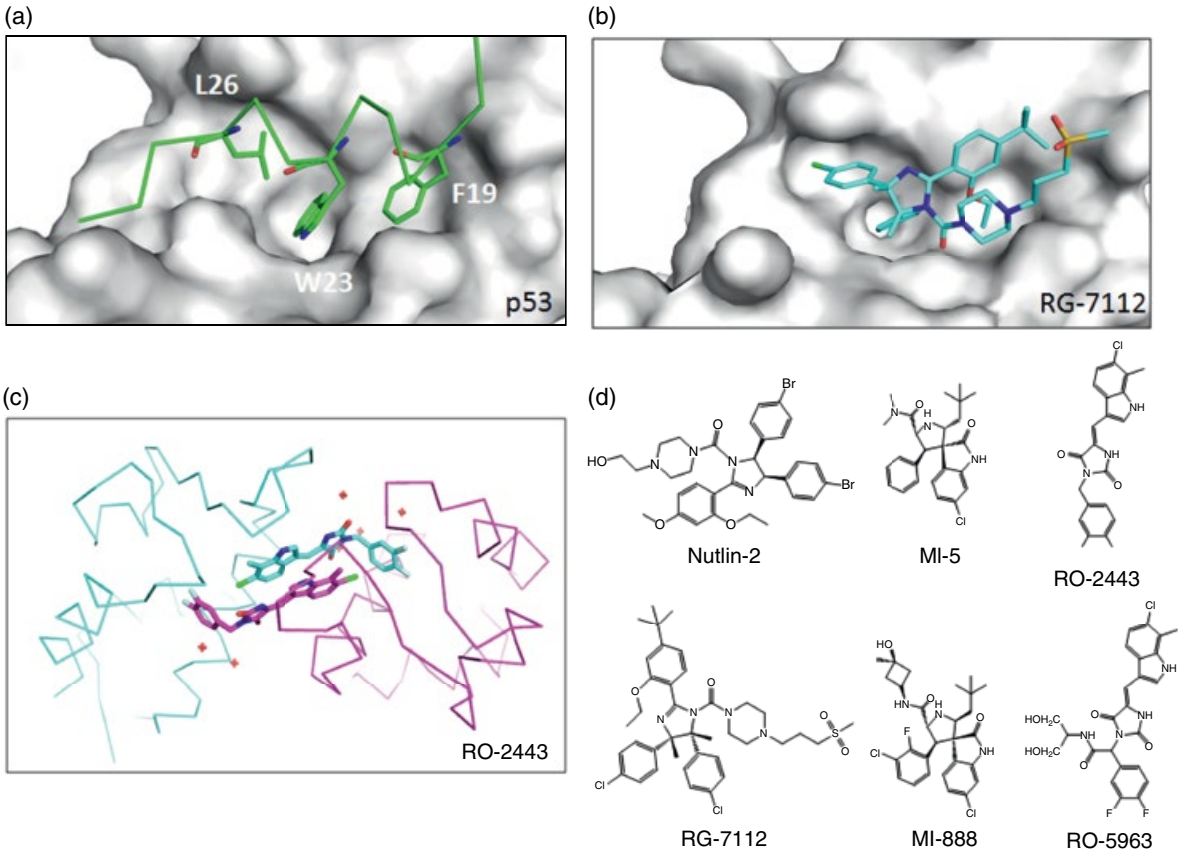

Figure 20.1 MDM2 and MDMX complex and inhibitor structures. (a) MDM2 (protein surface) with p53 peptide (stick) bound (PDB:1YCR). p53 residues F19, W23, and L26 are highlighted. (b) MDM2 (protein surface) with RG-7112 (stick) bound (PDB: 4IPF). (c) Dimer of hDMX (backbone) with RO-2443 (stick) bound (PDB: 3U15). (d) Chemical structures of RG-7112, MI-888, and RO-2443.

hydrophobic binding residues of the p53 peptide (site 1). Nutlins provided the first demonstration that MDM2 inhibitors have benefit in the treatment of cancer. Nutlin-3 was able to inhibit 90% of tumor growth in mice bearing SJSA-1 xenograft tumors with wild-type p53 and amplified MDM2, with no signs of toxicity. In 2007, an optimized nutlin, RG-7112 [69, 70], became the first of many MDM2 inhibitors to enter clinical trials for patients with advanced solid tumors [71]. The success of potent MDM2–p53 PPI inhibitors has become one of the leading examples of PPI research and has promoted the consideration of other PPIs as possible targets for small chemical compounds.

Interestingly, while nutlins occupy the same pockets as the p53 peptide, the small-molecule scaffold displays these substituents quite differently to the native peptidic backbone. For example, a phenyl ring fills the same pocket as a phenylalanine side chain but enters from a distinct direction and angle. This turns out to be a common feature of small-molecule PPI inhibitors; the greater diversity of small-molecule templates enables PPI hot spots to be accessed and sampled in ways that differ from the physiological ligand. This characteristic was exploited in the optimization of alterative inhibitors using knowledge of the crystal structure of MDM2/nutlin, yielding compounds containing diverse chemical cores, such as the benzodiazepinedione [72, 73] (TDP222669; PDB: 1T4E), chromenotriazolopyrimidine [74, 75] (AMG-232; PDB: 4OAS), and terphenyls [76]. It has also inspired the use of structure-based drug design (SBDD) for novel scaffolds such as the spirooxindole, described below.

20.4.1.1 Structure-Based Design of Spirooxindoles as MDM2 Inhibitors

In 2005, the Wang group reported the structure-based design of spirooxindole-containing small molecules as a new class of potent small-molecule inhibitors of the MDM2–p53 interaction [77]. Docking studies had led this group to identify the spirooxindole core as a novel scaffold that could direct the three key hydrophobic groups into the p53 binding pocket of MDM2. This resulted in compounds such as MI-5 (8 μM), which was designed to insert an oxindole moiety into the Trp23 pocket, a phenyl group into the Phe19 pocket, and an isopropyl group into the Leu26 pocket. Spirooxindole-containing compounds such as MI-5 have at least four

chiral centers. While it was possible to synthesize a single stereoisomer around this core, it was found that reversible ring-opening cyclization of the pyrrolidine ring in MeOH or CH_3CN/H_2O would spontaneously produce all four diastereoisomers. What could have been a purity problem turned out to be a fortuitous discovery, as one of these diastereoisomers was found to bind with much higher affinity than the intended product [78]. Its subsequent optimization led to MI-888 (Figure 20.4d), which has a Ki value of 0.44 nM, has excellent oral bioavailability in rats, and is capable of achieving complete and durable tumor regression in two animal models of human cancer upon daily oral administration. An analog of MI-888, MI-773 (undisclosed structure), has been advanced into phase I clinical development by Sanofi S.A. in 2012 [79].

Stereospecific protein binding is not unusual, but the expectation that PPI inhibitors may need to have greater "3D" character introduces a higher probability that shape complexity such as chirality will need to be a special consideration during optimization. Such compounds may also require chiral resolution at the synthesis or purification stage, and therefore insights from docking or crystal structures may be valuable to guide chemistry.

20.4.1.2 Dimer Stabilization: A Mechanism of Dual Inhibition of MDM2/X

The small-molecule MDM2 antagonists discussed so far show little activity against MDMX. Structural diversity in the p53 binding regions of these proteins, especially in the Leu subpocket, is sufficient to make the development of small-molecule dual antagonists challenging. Unfortunately, while the relative contributions of MDM2 and MDMX to regulation of p53 are not completely understood, evidence suggests that dual MDM2/X antagonists may have greater efficacy especially in tumors in which MDMX expression is high.

RO-2443 represents a novel class of indolyl hydantoins that utilize an alternative strategy to achieve dual activity versus MDM2/X ($IC_{50} < 50$ nM). Discovered by HTS of a diverse library of small molecules for suppression of p53–MDMX binding, this compound's MOA was unraveled by a number of biophysical methods including $^{1}H—^{15}N$ heteronuclear single-quantum correlation (HSQC) NMR, analytical ultracentrifugation (AUC), ITC, and ultimately X-ray crystallography (MDMX/RO-2443 complex PBD: 3U15) [66]. Unexpectedly, the inhibitor binds into and occludes the p53 pockets of MDMX and MDM2 (MDM2/RO-2443 complex PBD: 3VBG) by inducing the formation of dimeric protein complexes. A dimeric head-to-tail small-molecule core of two molecules of RO-2443 binds within a pocket formed by a homo- or heterodimer of MDM2/X protein molecules, such that a difluorophenyl group of one RO-2443 molecule occupies the Trp23 binding pocket and a chloroindole group of the other RO-2443 molecule occupies the Phe19 pocket of the same protein (PDB: 3U15, Figure 20.1c). The Leu26 pocket is left unoccupied. The X-ray structure revealed that the most accessible part of the inhibitor is the methylene bridge between the hydantoin and phenyl groups. Extension into the Leu26 pocket was therefore used to improve the physicochemical properties of the initial compounds to give RO-5963 that was suitable for cellular proof-of-concept experiments. RO-5963 demonstrated activity in cell lines expressing wild-type p53 (IC_{50} = 2–3 μM in MCF-7, HCT-116, and RKO) but not in those expressing a mutant form, showing that this novel mechanism effectively stabilized p53 and activated p53 signaling in cancer cells, leading to cell cycle arrest and apoptosis. This compound confirmed functional antagonism of the MDM2/X–p53 interaction could be achieved by sequestering MDM2/X into more stable ligand-induced dimers, thereby preventing their interaction with p53.

20.4.1.3 Other Opportunities for Inhibition

Although MDM2 and MDMX naturally form homodimers and heterodimers, these are believed to be driven by the C-terminal RING domains and dimerization of the N-terminal p53 binding domains had not been observed previously. It is therefore interesting to note that synthetic analogs of 5-deazaflavins bound to the RING finger domain of MDM2 have also been found to prevent the ubiquitination of p53 *in vitro* and *in vivo* [67]. These compounds thus provide mechanistically differentiated starting points for the development of additional p53–MDM inhibitors. Finally, a novel interaction has recently been proposed to control the binding of nutlin-like drugs and to mediate the transmission of allosteric interactions in the p53–MDM2 system. The crystal structure of MDM2 with nutlin shows two molecules of nutlin that are closely associated with the surface of MDM2 (sites 1 and 2). While one of the nutlin molecules binds to the site that has been targeted for small-molecule development strategies (site 1), hydrogen/deuterium exchange and computer simulation studies suggest that the second site (site 2) may actually be the site where nutlin first makes contact with MDM2 before tumbling into the main binding pocket and that this site of first contact may be druggable [80]. This hypothesis is strengthened by the demonstration that a mutation in this second site renders MDM2 resistant to nutlin binding [81]. If confirmed, this offers a new avenue into drug design efforts, implying that greater understanding of kinetic as well as thermodynamic signatures can lead to opportunities for PPI modulation. These observations remind us that in simplifying the PPI for biochemical and structural

convenience, we may miss unexpected interactions that could translate into cellular and *in vivo* efficacy.

20.4.2 Mimicry of Smac Peptide, IAP Antagonists

One widely used approach to rationally "design" PPI inhibitors is to use structural insights to mimic the structure of a binding partner. This includes the crystallographically determined bound conformation of short peptides such as p53, which adopts two turns of an α-helix. In the case of second mitochondrial activator of caspases (Smac), which binds to inhibitor of apoptosis proteins (IAPs), a short AVPI-NH2 tetrapeptide motif is all that is necessary to give nanomolar affinity to IAP [82]. This peptide is not long enough to adopt any regular secondary structure, but its bound conformation has inspired and informed inhibitor design.

IAPs, including cIAP1, cIAP2, and XIAP, are important regulators of cell fate and confer protection from apoptotic cell death. The IAPs contain PPI domains called BIR domains and a RING E3 ubiquitin ligase domain. Smac is an endogenous competitive inhibitor of the BIR–caspase interaction, and small-molecule mimetics of Smac represent an opportunity to stimulate apoptosis in dysregulated cancer cells. The Smac AVPI-NH2 peptide binds in an extended conformation, accessing two adjacent pockets of the BIR domains [83] (PDB: 1G73, Figure 20.2a). Many compounds exploiting these same pockets have been developed and are in clinical trials.

A combination of structure-based design and targeted compound library generation led to the identification of GDC-0152 (PDB: 3UW4, Figure 20.2b), the first peptidomimetic of Smac, to enter clinical trials in patients with locally advanced or metastatic malignancies. Other Smac mimetics, GDC-0917 [84, 85] and LCL161 [86], have followed into the clinic for a variety of oncology indications, as have non-peptide inhibitors such as SM-406/AT-406 [87]. These antagonists were designed from the AVPI peptide and all bind into the Smac site of the BIRs with IC_{50}s in the 2–60 nM range.

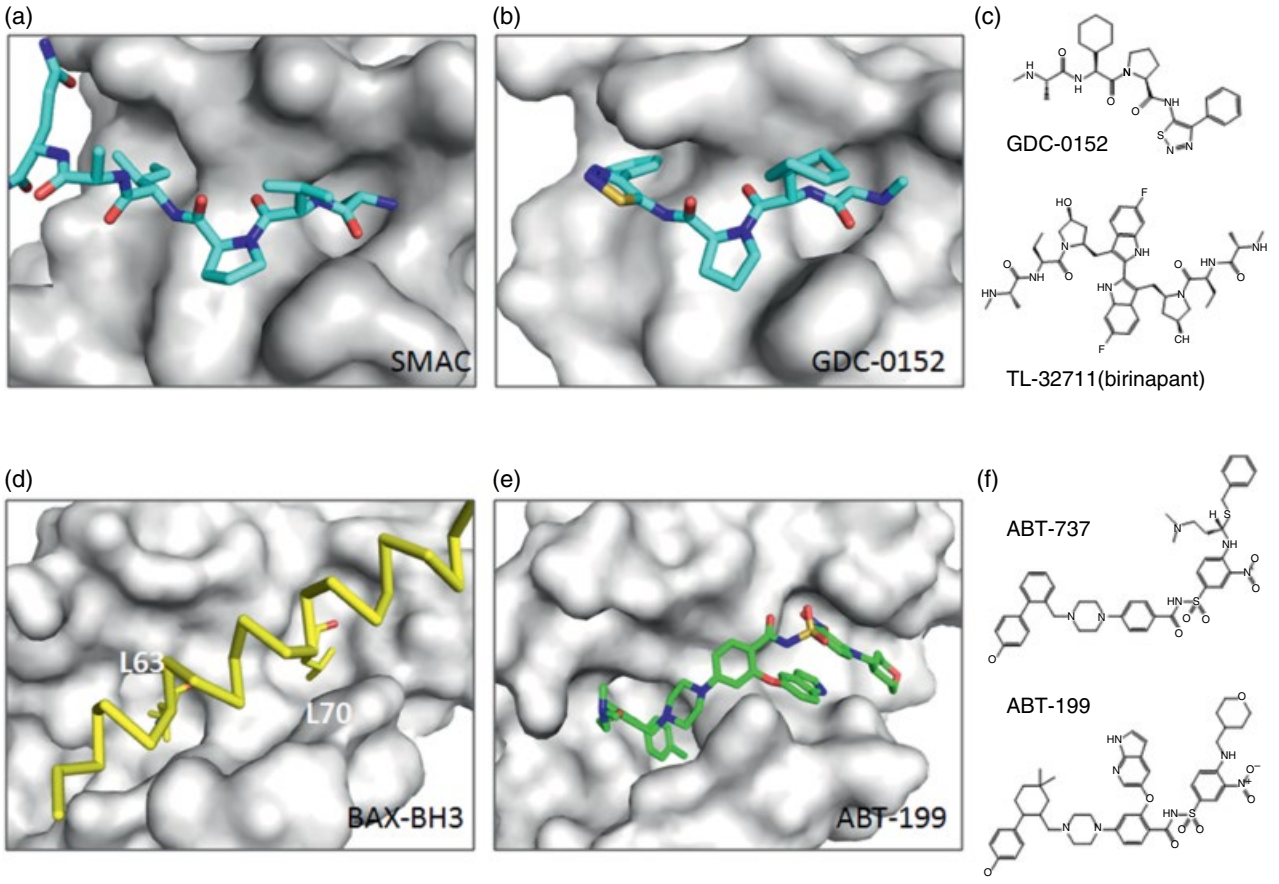

Figure 20.2 IAP and BCL complex and inhibitor structures. (a) XIAP-BIR3 (surface) with SMAC peptide (stick) bound (PDB: 1G73). (b) cIAP1-BIR3 (surface) with GDC-0152 (stick) bound (PDB: 3UW4). (c) Chemical structures of GDC-0152 and TL-32711 (birinapant). (d) BCL-2 (protein surface) with BAX BH3 peptide (ribbon) bound (PBD: 2XA0). Residues L63 and L70 are shown in stick format. (e) BCL-2 (protein surface) with ABT-199 (stick) bound (PDB: 4MAN). (f) Chemical structures of ABT-737 and ABT-199.

Another class of Smac mimetics sought to mimic not the monomer but a homodimeric form of Smac that simultaneously binds to XIAP's BIR2 and BIR3 domains physiologically [55]. Extrapolating this finding, dimeric small molecules that act as bivalent antagonists such as birinapant [82, 88] and SM-1387 [89] were made. These demonstrated better cellular potencies than their monovalent analogs but had poorer absorption properties and need to be dosed intravenously, unlike their orally active monovalent counterparts. Their utility may be to allow clinical evaluation of the relative importance of inhibition of the different IAPs since SM-406 and birinapant bind c. 50-fold more tightly to cIAP1 than XIAP.

20.4.3 Fragment-Based Approaches for BCL Antagonists

BCL2 family members BCL-xL and BCL2 inhibit apoptosis and are upregulated in many cancers, making them attractive drug targets. Their function is regulated by the binding of anti- or pro-apoptotic factors such as BAK and BAX, members of the pro-apoptotic family known as "BH3 only" proteins because they share homology with the BCL proteins only in the third homology domain. The BH3 domain forms a single α-helix that inhibits BCL function by binding into a hydrophobic groove. Mimicking the BH3 domain with small-molecule compounds has been shown to deliver significant therapeutic potential, as outlined below.

Several BH3 mimetics have been identified using NMR-based fragment screening combined with structure-based optimization. In the mid-2000s, Abbott Laboratories developed a novel inhibitor of BCL-2, BCL-xL, and BCL-w, known as ABT-737 [90]. Initially, small molecules (i.e. fragments) that bound to BCL2 were identified by mapping the NMR chemical shift that occurs upon ligand binding using $^{1}H—^{15}N$ HSQC NMR, an approach the group termed "SAR by NMR" [56]. Two compounds were identified that bound to distinct but proximal sites within the BH3 binding groove. Significantly, the two large hydrophobic pockets were identical to those occupied by key residues (such as Leu78 and Ile85 of BAK or Leu63 and Leu70 for BAX) known to be crucial for BH3 binding to BCL-xL (PDB: 2XA0, Figure 20.2d). Molecules that spanned both sites were synthesized and optimized using structural information to produce ABT-737, which has nanomolar affinity for BCL2 and BCL-xL. ABT-737 allowed the therapeutic benefit of a BCL inhibitor to be demonstrated. It showed an additive effect when used with chemotherapy and radiotherapy and enhanced the activity of several established anticancer drugs. However, its solubility and bioavailability were poor, prompting further improvements to generate the orally bioavailable ABT-263 (navitoclax) and the more soluble ABT-199 (PDB:4MAN, Figure 20.2e).

This is one of the first examples of the use of NMR to find initial low-affinity ligands for a difficult target and their optimization guided by structural insights to produce potent clinical assets. Building on this success, several other laboratories have reported the identification of additional small-molecule ligands for BCL2 and/or BCL-xL. Discovery methods include virtual screening, high-throughput diversity screening, and ligand-based design. All have been shown to inhibit binding of the BH3 peptides and to induce apoptosis in BCL gene-expressing cell lines.

20.4.4 Epigenetic Reader Proteins

PPIs involved in transcriptional regulation have become prominent drug discovery targets. There is a growing appreciation that epigenetic changes play critical roles in the onset and maintenance of many diseases. However, a detailed understanding of the molecular basis by which these changes can be modulated for therapeutic benefit is in its infancy. Discovery of selective and cell-permeable compounds that target epigenetic mechanisms may therefore help unravel the biology that underlies new therapeutic opportunities. Progress toward the discovery of small molecules that target histone reader modules and antagonize the interactions between these readers and their cognate covalent epigenetic marks on histone proteins has been rapid and accelerated by structure-based approaches.

20.4.4.1 Bromodomains

Protein lysine acetylation (AcK) is an abundant and important PTM found to occur physiologically on over 1750 proteins [91]. Acetylation of the N-terminal tails of histones is most commonly associated with gene activation, as removing the charge from the lysines weakens its association with DNA and hence allows transcription initiation factors better access to the DNA. Bromodomains (BDs) are believed to be the main "readers" of the histone acetylation mark. They are small (c.110-residue) evolutionary and structurally conserved modules present in a large number of chromatin-associated proteins and in almost all nuclear histone acetyltransferases. The human genome encodes for at least 56 BDs within 41 different proteins. Forty-three of the 56 BDs possess a conserved tyrosine and asparagine that form a key part of an acetyl-lysine binding motif.

The first 3D structure of a BD, p300/CREBBP-associated factor (PCAF), was determined by NMR in 1999 [92]. Since then 3D structures of well over half of the BD family have been determined by X-ray crystallography and/or NMR, revealing that they share a

common fold consisting of four antiparallel left-handed α-helices (αZ-αA-αB-αC). The peptide acetyl-lysine binding site sits at one end of the helical bundle, with the conserved asparagine and tyrosine residues located at the bottom of a hydrophobic pocket [93].

20.4.4.1.1 Structure-Based Approaches within BET Family

SBDD or fragment-based drug design (FBDD) approaches have been particularly successful for protein systems where there is a good structural understanding of the key elements for recognition, affinity, and selectivity. The conserved AcK binding signature within the BD protein family suggests that AcK mimetics act as hot spots for BD inhibitors. The large variations in the BC and ZA loops among the BDs that surround the AcK site offer the opportunity to gain affinity and selectivity [94].

Zhou et al. were the pioneers of BD inhibitor discovery. Following their solution of the structure of PCAF by NMR, they embarked on several NMR screening campaigns to identify small molecules that may antagonize its interaction with the HIV-Tat protein [95–97]. Rationalizing that compounds that bound in PCAF's peptide binding groove rather than within the AcK binding pocket would be more likely to have selectivity over other BDs, they targeted this site and found a class of micromolar *N*-aryl-diamines. The best of these had comparable affinity (IC_{50} of 1.6 μM) to the Tat peptide. While still of modest affinity, they were able to show that these compounds did not bind to the BDs of CREBBP and TIF1β, demonstrating that selectivity within the BD could be achieved.

Building on this success, Zhou et al. used $^{1}H—^{15}N$ HSQC screening to look for acetyl-lysine mimetic inhibitors of CREBBP [97, 98]. Changing their focus to concentrate on the AcK pocket itself, a selection of 200 compounds containing an *N*-acetyl moiety yielded an impressive hit rate of 7%, with the best compounds of modest micromolar affinity. Interestingly, despite targeting the conserved AcK site, the CREBBP hits exhibited some selectivity over the BD of PCAF. This highlighted that even within this seemingly highly conserved pocket, subtle variations allow some differentiation between members of the BD family. While encouraging, these early examples yielded molecules of relatively modest affinity, and the therapeutic potential of these BD/histone PPIs remained questionable.

The discovery of inhibitors of the BET family of BDs, such as JQ1 [99] (PDB: 3MXF) and I-BET762 [50, 100] (PDB: 5P5O) transformed the perception of BDs and epigenetic readers as druggable targets. These molecules proved that drug-like compounds are able to effectively disrupt epigenetic PPIs and offer the potential to deliver profound pharmacology *in vitro* and *in vivo*. BET inhibitors show efficacy in diverse models of acute inflammation [101], atherosclerosis [102], and hematological [103–105] and solid tumors [106, 107]; consequently multiple molecules have progressed into clinical trials [108].

All reported BET BD inhibitors occupy the AcK pocket, emphasizing its importance as an anchor for subsequent interactions. Several fragment-based approaches, supported by crystallography, illustrate the relative importance of the interactions that can be made within this site. The first study [109] used a small fragment set of a few thousand compounds chosen to contain the two features required for effective AcK mimicry: a hydrogen bonding functionality and a small alkyl substituent. The analysis of 40 crystal structures of a BET BD suggested that the H-bond interaction with the bridging water (Wb) dominates that with the asparagine, but that both influence ligand positioning (Figure 20.3a).

A diverse range of chemotypes have emerged as capable of targeting the AcK pocket of these proteins [108]: heterocyclic acetyl-lysine mimics, such as the dimethylisoxazoles [110–112] and hydroquinazolinones [113, 114] and templates that use the acetyl group itself attached to a number of scaffolds such as the tetrahydroquinolines [115].

20.4.4.1.2 Structure-Based Approaches Across Bromodomain Family

There is a wealth of 3D structural information across the BD family, but relatively little known about their biology. Vidler et al. used SiteMap to evaluate the ligandability of 24 BDs outside the BET family to identify those BDs most likely to be tractable to small-molecule ligand discovery. Of the three classifications (difficult, intermediate, and druggable), approximately half of those studied were classed as difficult [22]. The rest were almost equally divided among the druggable (e.g. PCAF) and intermediate (e.g. CREBBP) categories, suggesting that the BDs as a target class have significant potential for small-molecule inhibitor discovery. The utility of these *in silico* predictions has been difficult to judge because inhibitors of BDs in all three of the difficult, intermediate, and druggable categories have been reported, as outlined below.

GSK2801 [116] and BAZ2-ICR [117] are nanomolar inhibitors of the BDs of the BAZ2 subfamily (BAZ2A/B), classed as a "difficult" target by SiteMap. The BAZ2 proteins are characterized by a carboxy-terminal BD adjacent to a PHD finger and a WACZ motif. In addition, four other conserved motifs are found in the N-terminus of BAZ family members, namely, the LH motif (a leucine-rich helical domain), the ZB2 motif, and the BAZ 1 and BAZ 2 motifs. The biological consequence of blocking each of these domains is unknown, and chemical probes have been sought to try to address this. GSK2801

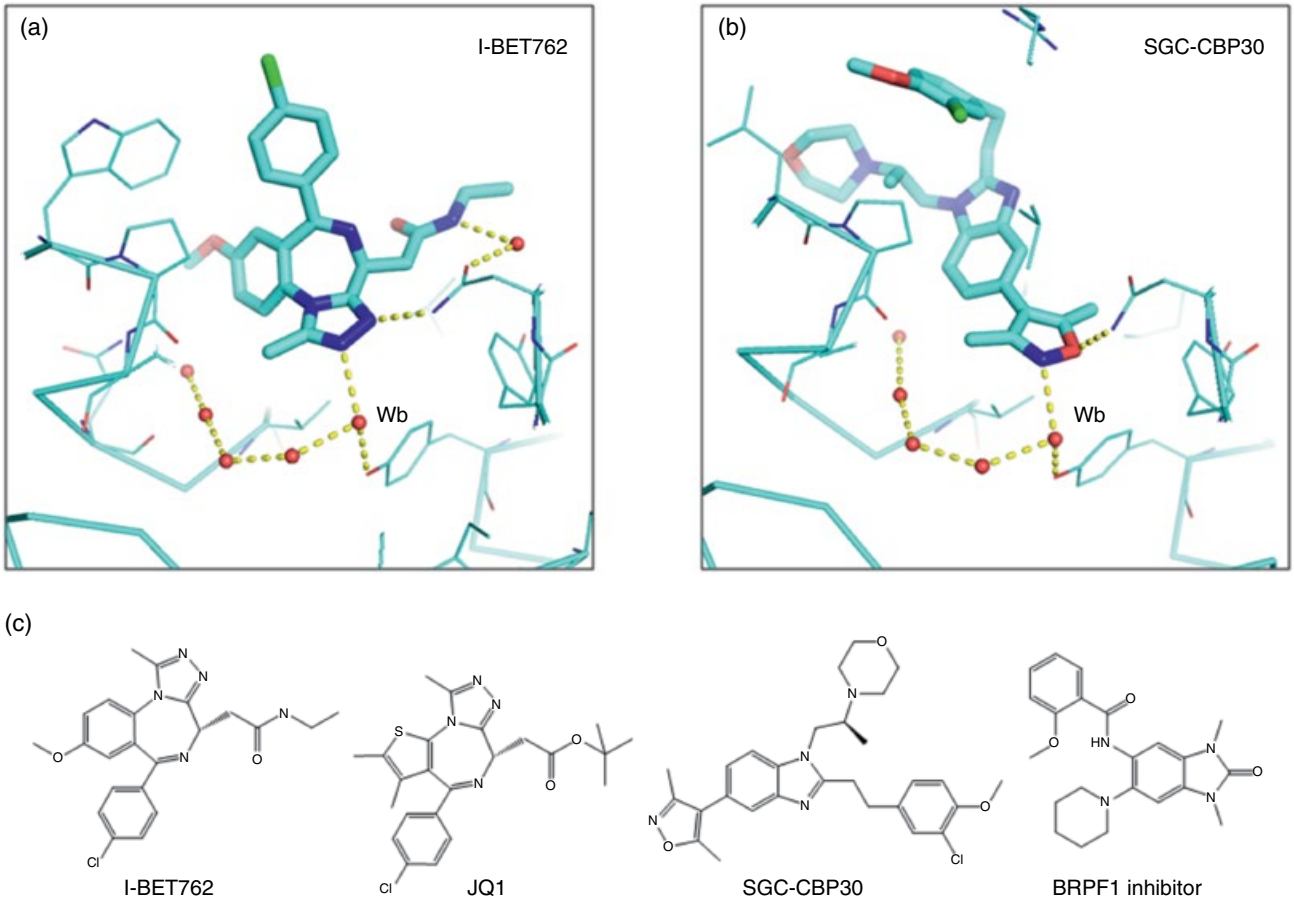

Figure 20.3 Bromodomain complex and inhibitor structures. (a) I-BET762 (stick) within acetyl-lysine site of BET bromodomain BRD4 (PDB: 3P5O). Wb is the bridging water between inhibitor and conserved tyrosine. (b) SGC-CBP30 bound into CREBBP bromodomain (PDB: 4NR7). (c) Chemical structures of bromodomain inhibitors.

originated from an indolizine fragment, initially found by cross-screening a BET inhibitor library. This showed comparable micromolar activity versus both the BET and BAZ2B proteins. Selectivity and affinity for BAZ2B were optimized by exploiting structural differences between the proteins [116]. In a similar manner, starting from a weak and nonselective fragment, a nanomolar probe compound was found and optimized for another "difficult" BD, that of ATAD2 [118, 119], a protein whose cellular levels are found to correlate with cancer patient survival rates. In this instance, X-ray structures of ligands in the BET "anti-target" were very informative in guiding the optimization as, unexpectedly, ligands could bind in differing conformations within the two proteins [119].

Focused screening of AcK pharmacophore-targeted libraries and cross-screening from BET compounds have delivered chemical probes and starting points from a number of distinct templates for another BD-containing protein, CREBBP. This target was classed as "intermediate" tractability [22]. The most potent of these compounds, I-CBP112 [120] (PDB, 5J0D) and SGC-CBP30 [121, 122] (PDB: 4NR7, Figure 20.3b), are dual CREBBP and EP300 inhibitors. Similar to other potent BD compounds, CREBBP BD inhibitors exploit regions outside the AcK pocket for selectivity and affinity. For example, the expanded lipophilic regions of CREBBP are occupied by the dimethoxyphenyl of I-CBP112 and chloromethoxyphenyl of SGC-CBP30 that confer selectivity over the BET proteins.

Nanomolar inhibitors of BRPF1 (Figure 20.3c), one of the proteins classed by SiteMap as highly druggable [22], were also optimized from benzimidazolone fragments originally found for the BET proteins [123] (PDB: 4YUE). Divergent structure–activity relationship (SAR) between BET and BRFP1 could be obtained by varying substituents off the 5- and 6-positions of this template that exploit differences in the BC and ZA loops near to the acetyl-lysine site.

In summary, the evidence so far demonstrates that finding BD inhibitors outside of the BET family is

achievable, whether *in silico* predictions deem the BDs to be difficult, intermediate, or druggable. Moreover, for each of these proteins, inhibitors with submicromolar *in vitro* potency, a reasonable profile of selectivity among the BD family, and cell permeability have also been obtained. This suggests that *in silico* methods may not always reliably forecast the ligandability of different members of this target class. However, as limited information is available about these optimization efforts, these predictions may yet prove to signify the ease with which success can be obtained.

20.4.4.2 Methyl-lysine Reader Domains

20.4.4.2.1 Introduction

Histone methylation is a very common and versatile PTM found on basic residues such as lysine and arginine. These amino acids can exist in multiple methylation states: un-methylated and monomethylated (MeK1), dimethylated (MeK2), and trimethylated (MeK3). These methyl marks are "read" by >200 methyl reader domains in the human genome. At least four distinct families of reader domains recognize methylated lysines: the zinc finger PHDs, the WD40 repeat proteins (WD40rs), the ankyrin repeats, and the large Royal superfamily, consisting of the Tudor, Agenet, chromodomain, Pro-Trp-Trp-Pro (PWWP), and MBT domains [124].

In a similar study to that discussed for BDs, Santiago et al. [21] used computational algorithms to predict the chemical tractability of 38 nonredundant methyl readers for which there are X-ray structures. The diversity of protein families and methyl reader pockets gave a highly variable picture, but some trends were seen. Folds containing deep pockets, for example, MBT and PWWP proteins, were classified as likely to be tractable, whereas those with rather open shallow sites, such as PHD domains, were not. For methyl readers of the WDR and chromo families, the situation was more mixed. Tractability was found, but located at sites outside the methyl amino acid pocket. For WDR, this was the proximal non-methylated arginine pocket, and for CBX chromoproteins, it was the groove occupied by the backbone of the histone tail. A caveat to these predictions is that they focus on isolated domains and fragments of full-length proteins. Neglecting the importance of the context of a domain and of physiological complexes has consequences, both positive and negative, for inhibitor discovery, which are highlighted in the following case histories.

20.4.4.2.2 MBT Domains

MBT domains are motifs of c.100 amino acids found in multiple repeats within proteins. To date, 9 proteins containing a total of 27 different MBT domains have been identified. Individual MBTs recognize peptides with lower methylation states, MeK1 or MeK2, via a cavity insertion mode where the methyl-lysine side chain is fully surrounded by a deep and narrow cavity. This aspect makes them relatively attractive for small-molecule inhibitor discovery in comparison to the shallow solvent-exposed MeK pockets seen within some other MeK readers. Many of the MBT proteins have been crystallized to reveal that sequential MBTs can form compact structures such as the tri-propeller of L3MBTL3 (Figure 20.4a, PDB: 4FL6). Despite the potential for multiple MBT repeats within each MBT to interact in a polyvalent manner with partner proteins, there is no evidence to suggest this is relevant to histone peptide recognition.

In contrast to this wealth of structural knowledge, the biological function of these domains is poorly understood. Attempting a target class approach, the Frye group at UNC has employed a number of screening methodologies against a panel of MBTs (and other MeK reader domains). A virtual screen identified 51 compounds that were tested in AlphaScreen assays using protein truncates of L3MBTL1/3/4 and MBTD1 [125]. Nineteen compounds from this set were found to give dose-dependent effects with IC_{50}s within the tens of micromolar range. These included direct MeK1/2 analogs as well as examples where the methylated amine was replaced by a pyrrolidine. Further optimization resulted in UNC669, which displayed a 7.9 μM affinity for L3MBTL1 and whose predicted binding mode was confirmed by its co-crystal structure in the first three MBT domains of L3MBTL1 (PDB: 3P8H). While UNC669 represented a significant advance in small-molecule inhibitors for the MBT domains, its potency was not sufficient to be useful as a chemical tool [54, 124, 126].

The group therefore designed a series of divalent compounds (e.g. UNC1021) that incorporated two MeK mimetics into the same molecule. Although there was no evidence of polyvalency as a mechanism for increasing potency against proteins containing multiple MBT domains, they were inspired by MeK binders such as 53BP1, a mammalian DNA repair factor containing tandem Tudor domains, that was known to recognize an adjacent unmodified arginine in addition to the H4K20me2 modification. Testing these bivalent compounds across a panel of methyl-lysine readers, UNC1021 showed surprisingly high affinity (IC_{50} 40 nM) for L3MBTL3 [62]. UNC1215 was made to improve the cellular activity of this starting point and displayed a similar affinity and selectivity profile to UNC1021. The co-crystal structure of UNC1215-L3MBTL3 (PDB: 4FL6) explains the basis for this compound's unexpected selectivity and affinity. It binds as a unique 2 : 2 complex, two UNC1215 molecules bridging

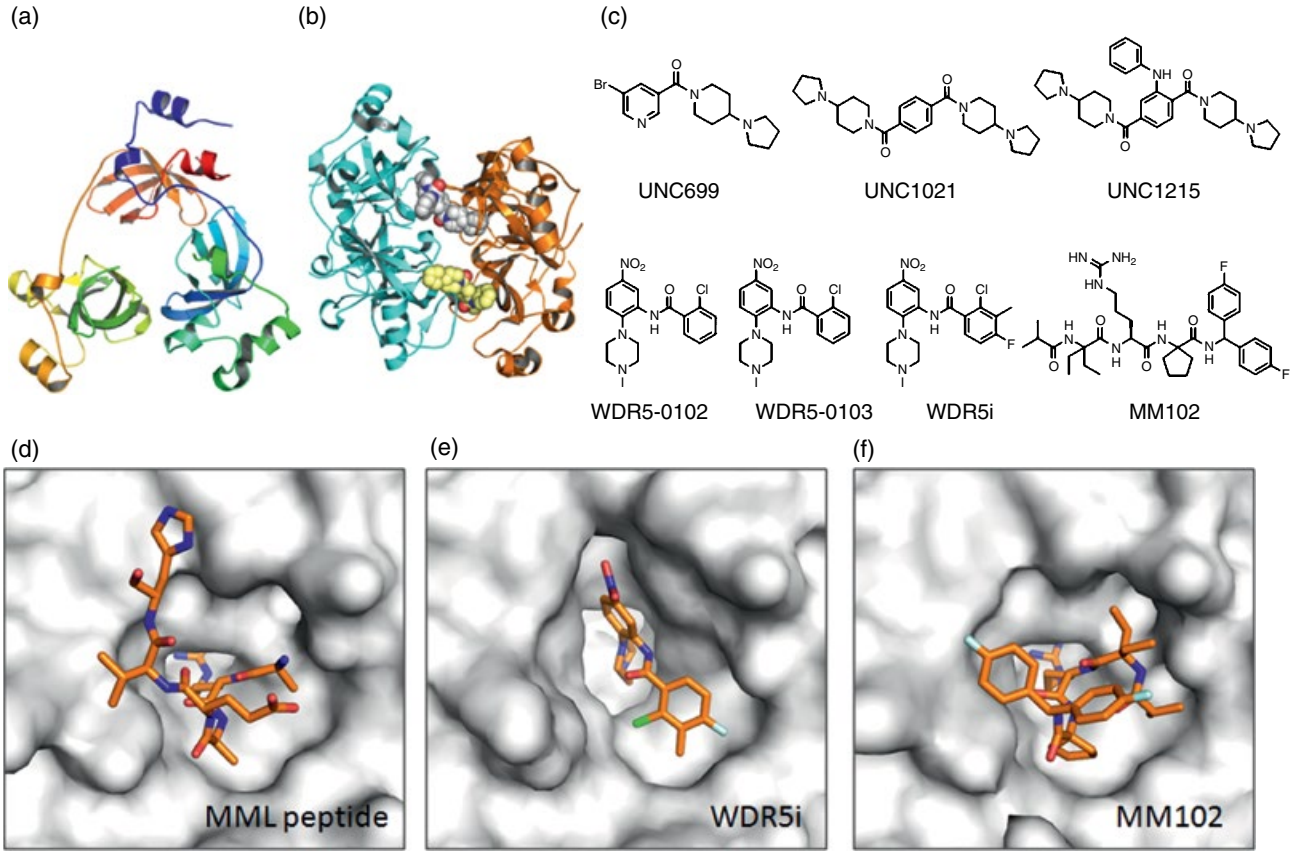

Figure 20.4 LBMBT3 and WDR5 complex and inhibitor structures. (a) Tri-propeller structure of a L3MBT3 (cartoon) monomer (PDB: 4FL6). (b) Co-crystal structure L3MBTL3 (cartoon) with dimer inducing UNC1215 (space fill) (PDB: 4FL6). (c) Chemical structures of range of methyl reader inhibitor compounds. (d) WDR5 (protein surface) with MLL peptide (stick) bound (PDB: 3EMH). (e) WDR5 (protein surface) with inhibitor WDR5i (stick) bound (PDB: 4IA9). (f) WDR5 (protein surface) with M1102 (stick) bound (PDB: 4GM8). (*See insert for color representation of the figure*.)

between two L3MBTL3 protein molecules to induce dimer formation (Figure 20.4b). In each UNC1215 molecule, one pyrrolidine interacts with the aromatic cage of domain 2 of one protein monomer, while simultaneously its other basic pyrrolidine engages the methyllysine binding site of domain 1 of a second protein monomer. Given this unusual binding mode, gel filtration experiments were conducted to confirm the ability of the compound to induce protein dimerization of the first three MBT domains of L3MBTL3 in solution. Fluorescence recovery after photobleaching (FRAP) experiments using a transiently transfected green fluorescent protein (GFP) fusion protein of the three MBT domains of L3MBTL3 showed that UNC1215, but not its negative control UNC1079, was able to alter chromatin mobility with a half-maximum effective concentration of 50–100 nM, confirming cell penetration and target engagement of this chemical probe. UNC1215 was also found to be able to disrupt the formation of nuclear foci of this truncated protein in transfected U2OS and HEK293 cells, but not when full-length L3MBTL3 was used. This led the authors to propose that other domains in the full-length protein may act cooperatively with the MBT domains to facilitate foci formation and that in their presence chemical probes such as UNC1215 have insufficient potency for efficacy due to the avidity of these increased interactions.

This example highlights both opportunities and challenges resulting from the multivalent interactions that are so prevalent within the physiological environment of histone reader proteins. By engaging MBT domains on separate proteins, UNC1215 achieves high affinity, yet its ability to inhibit these domains in a cellular context may be limited.

20.4.4.2.3 Inhibitors of WDR5 PPIs in Protein Complexes

The catalytic activity of many enzymes, including epigenetic modifying enzymes, is modulated by PPIs, providing opportunities for drug discovery. One example is the development of high-affinity peptidomimetics that antagonize the interaction of the histone methyltransferase mixed-lineage leukemia (MLL1) and its acti-

vator WDR5 from the WD40 family of methyl readers. Disruption of the MLL1–WDR5 PPI by peptidomimetics effectively decreased MLL1-fusion-mediated leukemogenesis.

Mixed-lineage leukemia (MLL) proteins are histone H3 lysine 4 (H3K4) methyltransferases (HMTs) that catalyze mono-, di-, and trimethylation through their evolutionarily conserved SET domains. The most studied of these proteins, MLL1, is often misregulated in lymphoblastic and acute myeloid leukemia (AML), and genetic abnormality of this protein accounts for 5–10% of AML in adults and 70% in infants. Wild-type MLL1 activity is tightly controlled by the formation of a core complex composed of MLL1, WDR5 (**WD**40 **r**epeat protein **5**), RbBP5 (**r**etino**b**lastoma **b**inding **p**rotein **5**), and ASH2L (**a**bsent **s**mall **h**omeotic discs-**2**-**l**ike), where all four components are required for optimal activity. The MLL1–WDR5 interaction is mediated by the **W**DR5-**in**teracting (WIN) motif of approximately 12 amino acids and is required to maintain the integrity of the MLL1 complex. MLL2, MLL3, and MLL4, whose production can be altered in other forms of cancer, participate in similar complexes.

Antagonism of the MLL–WDR5 subunit interaction has been proposed as an alternative to active site inhibition of MLL1 (PDB: 3EMH, Figure 20.4d). Two groups have pursued this goal using different approaches, both guided by structural insights [63–65, 127]. The first essentially followed a small-molecule approach from the start [124, 126]. Screening a diverse library of 16 000 small molecules in a WDR5 peptide–MLL1 fluorescence displacement assay, micromolar starting points were found and optimized to give WDR5-0102 (K_d 4 μM) and then WDR5-103 (K_d 450 nM) for which co-crystal structures (PDB: 3SMR, 3UR4) were obtained [64, 65]. These confirmed binding to the deep central cavity of the doughnut-shaped WD40 repeat fold. This site is the arginine pocket identified by the SiteMap analysis of Santiago et al. [21]. Additional exploration at three points of diversity around WDR5–102 yielded a compound, WDR5i (PDB: 4IA9, Figure 20.4c,e), with only modest improvement (K_d 300 nM). This improved compound showed no activity versus a panel of human HMTs and SET domains but, when tested in an *in vitro* MLL1 catalytic assay that requires the presence of the tetrameric core MLL complex, was found to exert inhibition in the hundreds of micromolar range. A complex multi-protein equilibrium modulates MLL cellular activity, and the effectiveness of WDR5–MLL inhibitors to change this balance will depend on the local concentration of the protein components and the nature of the complexes formed. Therefore, while these initial PPI inhibitors of the WDR5–MLL demonstrate inhibition in simple competition assays, it is likely that inhibitors of far greater potency are necessary to assess the full potential of this mode of MLL inhibition [64, 65]. This has been achieved by following an alternative peptidomimetic approach.

Co-crystal structures of WDR5 with an MLL1 peptide suggested that not all 12 amino acids of the WIN motif were critical for binding [128]. Truncations revealed that the CO–Ala–Arg–Ala–NH motif corresponding to MLL1 (3764–3766) was both necessary and sufficient to bind WDR5 and that the Ac–Ala–Arg–Ala–NH_2 tripeptide had a 120 nM affinity for WDR5 [127]. Exploration of peptidomimetics from this starting point led to MM-102 (PDB: 4GM8, Figure 20.4f). MM-102 has subnanomolar affinity in a displacement assay and the ability to inhibit MLL1 core complex methyltransferase activity in the single-digit nanomolar range [63]. Treatment of MLL1–AF9 fusion-transduced murine myeloblasts with MM-102 reduced mRNA levels of two critical MLL1 target genes required for leukemogenesis, HoxA9 and Meis-1, without altering those of housekeeping genes. This successful proof-of-concept study suggests that potent small-molecule inhibitors of the WDR5–MLL1 interaction can effectively inhibit MLL1-mediated gene transcription in cancer caused by misregulation of MLL1 [63, 127].

In this example, structural insights from protein–partner interactions inspired the WDR40 peptidomimetic that allowed this novel mechanism of MLL1 modulation to be validated, and this represents a novel therapeutic strategy for acute leukemia.

20.4.5 Competitive Antagonists: IL-2 Receptor

Interleukin-2 (IL-2) is a cytokine that binds to and activates a heterotrimeric receptor (IL-2R), which contains α, β, and γ chains, found on the surface of T cells. A hot spot for IL-2Rα on IL-2 was mapped onto an amphipathic surface by site-directed mutagenesis and NMR and confirmed by a crystal structure of IL-2/ILRα (PDB: 1Z92) [129–132]. IL-2Rα is an elbow-shaped protein consisting of two beta-sheet sushi domains, and the PPI is defined by a near parallel packing of IL-2 and IL-2Rα secondary structures, resulting in a buried area of c. 1900 $Å^2$. Each protein contributes both sequential and non-sequential residues to this binding interface, leading to a large and discontinuous contact interface. As surfaces of this type are widely acknowledged to be the most challenging for small-molecule inhibition, antibodies are a sensible alternative for extracellular PPIs. Antibodies such as basiliximab [133] (PDB: 3IU3) and daclizumab [134] (PDB: 3NFP) that block this interaction by binding the IL-2Rα have been developed and shown clinical success.

Unexpectedly perhaps, small molecules have also been found that antagonize the IL-2/ILRα interaction. One of the first examples of a small-molecule inhibitor of a cytokine–receptor interaction was Ro26-4550 (Figure 20.5a) [135]. This compound was designed as a peptidomimetic of IL-2 and therefore was expected to bind to IL-2Rα. However, ELISA and $^{1}H—^{15}N$ NMR experiments revealed the compound bound to IL-2 itself with low micromolar activity at the IL-2Rα binding site [131]. X-ray crystal structures of unliganded IL-2 (PDB: 1M47) and Ro26-4550-bound IL-2 (PDB: 1M48) show few differences in main-chain conformation between the free and bound forms, but a previously largely flat surface (corresponding to the IL-Rα hot spot) adapts by rearrangement of surface side chains to bind Ro26-4550 in an emergent groove [136]. NMR studies and X-ray structures suggest this part of the protein is inherently flexible, with two loops adjacent to this region somewhat disordered in solution and variable conformations observed in several X-ray crystal structures. This is supported by thermodynamic measurements of the Ro26-4550–IL-2 interactions, which suggest that binding "captures" a low-energy conformation rather than "inducing" a high-energy one. The high degree of adaptability in the hydrophobic portion of the binding site allows for the creation of pockets and grooves into which small molecules can bind.

Although Ro26-4550 is not an ideal starting compound, it inspired attempts to develop small-molecule compounds with greater affinity, the most potent of which is SP-4206 (Figure 20.5a), which showed 60–100 nM activity in both a protein inhibition assay and an IL-2 binding assay (SPR). The X-ray crystal structure of

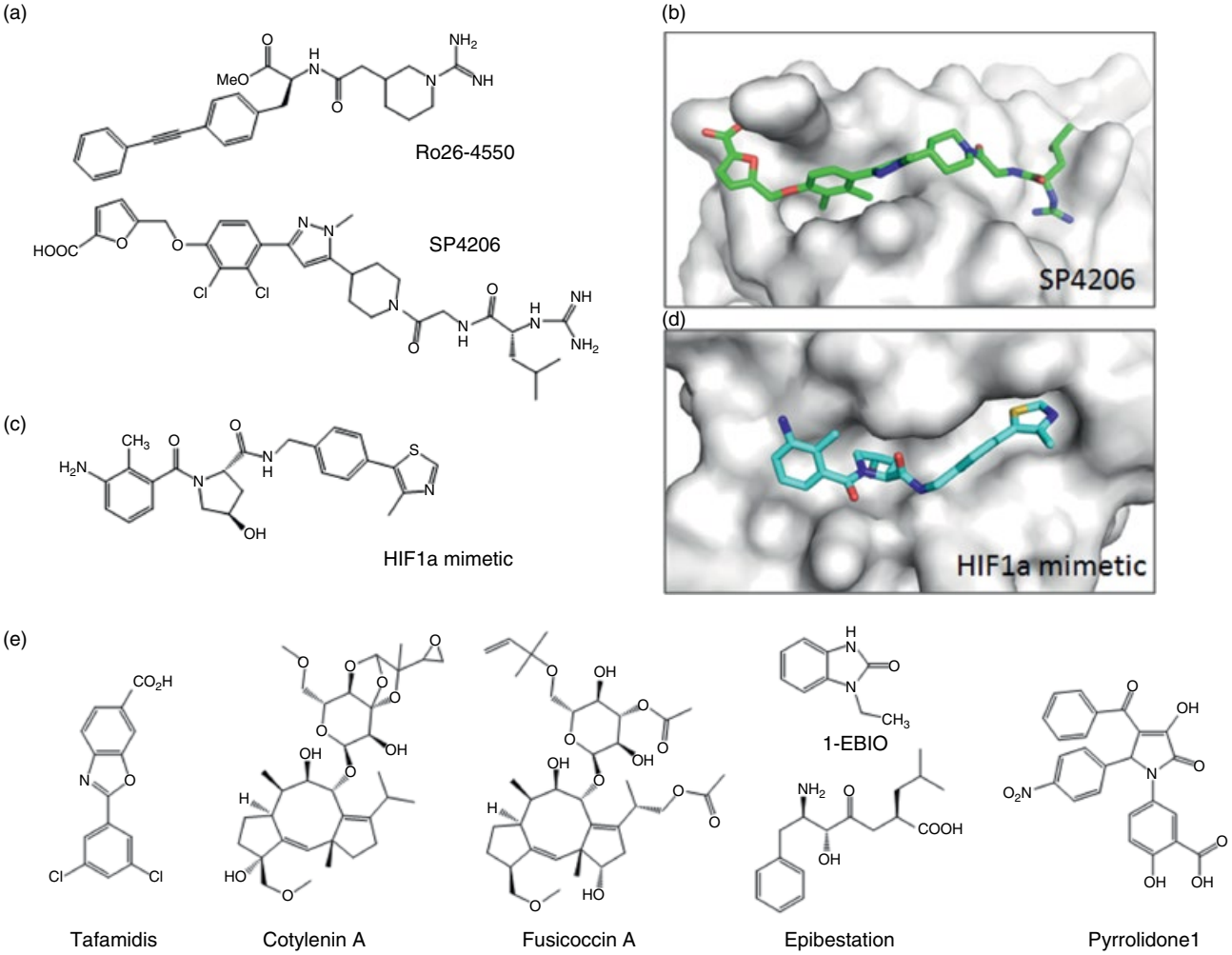

Figure 20.5 IL-2 complex and inhibitor structures. (a) Chemical structures of Ro26-4550 and SP4206. (b) IL-2 (protein surface) with SP4206 (stick) bound (PDB: 1PY2). (c) Chemical structure of Von Hippel–Lindau (VHL) HIF1a mimetic from PDB: 4B9K. (d) VHL protein (white surface) with bound inhibitor compound (stick; PDB: 4B9K). The central hydroxyproline mimics the binding of a peptide derived from HIF1a. (e) Chemical structures of protein stabilizers.

SP-4206 complexed with IL-2 (PDB: 1PY2, Figure 20.5b) shows that it binds to the hydrophobic pocket and basic groove of IL-2, with both these regions adapting through loop movements and side-chain rotations to give optimal compound–protein complementarity.

A key feature of reports of hit discovery and optimization for IL-2 binders is the extensive use of a spectrum of biophysical and structural techniques (NMR, SPR, AUC, X-ray) from an early stage. This validated even very weak compounds binding at the protein–protein interface and ensured chemical optimization began on the right track. This was particularly important given the highly adaptive nature of the IL-2R binding site, where it would have been difficult to predict changes in compound binding site and mode. It should also be noted that Ro26-4550 inspired the exploration of fragment-assembling and tethering approaches that may have utility for detection of fragment binding at especially difficult interfaces where particularly weak binding may be expected [136, 137].

20.5 Stapled Peptides

Notwithstanding the success that can be achieved with small molecules, modulation of some PPIs remains a challenge, especially those that lack a well-defined concave pocket. Another avenue makes use of peptidic modalities [138]. These present the advantages that their innate modularity and flexibility yield naturally complementary and adaptable biological interfaces. Unfortunately, native peptides do not make good conventional drugs as they are, typically, metabolically labile, do not generally cross membranes, and tend to have poor oral bioavailability. Additionally, the conformational freedom of short peptides can result in large entropic penalties upon binding, which may lead to weak affinities. Constrained peptides are one way of addressing some of these issues. For example, hydrocarbon-stapled peptides have been used as tools to understand the biology of protein complexes and also as a potentially new class of therapeutic agents. Most research has concentrated on stabilizing α-helical conformations.

Using ring-closing metathesis (RCM) developed by Blackwell and Grubbs [139], the Verdine group has optimized α-helix stabilization by introducing synthetic α,α-disubstituted nonnatural amino acids of different lengths and stereochemistries and demonstrated increased stability against proteolysis. The group's first validation of this strategy involved stapling a peptide from the BH3 domain of BID. This disrupted binding of BH3 to BCL-2 *in vitro* and demonstrated the *in vivo* biological activity consistent with this disruption [140]. Other targets addressed with stapled peptides by the same group include antagonists of MDM2–4–p53 [141] and beta-catenin [142], as well as binding to the NOTCH1/CSL complex to prevent the formation of the ternary NOTCH1/CSL/MAML1 complex necessary for NOTCH signaling [143]. Encouraged by these advances, Aileron Therapeutics was founded to advance and commercialize the stapled-peptide technology for drug discovery.

20.6 Alternatives to Small-Molecule Orthosteric Inhibition

Allosteric modulation can be an alternative to orthosteric binding. The example of RO-2443, which stabilizes MDM dimers to give functional antagonism, has been discussed above. Another novel approach involves bivalent molecules that conscript the cell's proteosomal pathways to degrade the target protein rather than to inhibit its interactions.

20.6.1 Protein Degradation

PPIs control not only signaling pathways but also degradation pathways. The ubiquitin–proteasome system (UPS) is the principal conduit for protein turnover in all eukaryotic cells. Ubiquitin-dependent proteolysis requires the attachment of polyubiquitin chains to a substrate via the actions of three enzymes, E1, E2, and E3, which marks the substrate for degradation by the 26S proteasome. An innovative alternative strategy may be to degrade one partner protein or even the entire complex using a PROTAC approach. **Pro**teolysis-**ta**rgeting **c**himeric molecules, or PROTACs, are bifunctional molecules consisting of three covalently linked components: an E3 ligase binder, a linker, and a target protein binder. This triggers the recruitment of an E3 ubiquitin ligase to a specific targeted protein of interest, resulting in the tagging of that protein with ubiquitin and initiating its elimination by the UPS.

This unlocks new ways of drugging targets, as the PROTAC may exploit the chemically tractable parts of the target, rather than confining itself to only sites with native functional relevance that may be traditionally "undruggable." Degradation may also offer advantages in pharmacodynamics arising from the timescale of *de novo* protein synthesis. Additionally, there is the potential for lower doses where the PROTAC mechanism allows the degrading molecules to be recycled, thus acting catalytically in contrast to conventional stoichiometric small-molecule approaches.

The initial demonstration of this approach was made by Sakamoto et al. [144] using Protac-1 to degrade

methionine aminopeptidase-2 (MetAP-2) via the Skp1-Cullin-F box (SCF) ubiquitin ligase complex. Protac-1 consisted of an IκBα phosphopeptide that is recognized by the F-box ubiquitin ligase β-TRCP linked to the angiogenesis inhibitor ovalicin that covalently binds to MetAP-2. They demonstrated that MetAP-2 can be tethered to SCFβ-TRCP, ubiquitinated, and degraded in a Protac-1-dependent manner.

Subsequent PROTAC molecules involved E3 warheads that were essentially minimal peptide motifs that could target the molecules to the E3 substrate site. For example, the first estrogen receptor (ER) PROTAC published by Crews et al. consisted of a 17-O-linked estradiol motif linked to the HIF-1α hydroxyproline-containing pentapeptide that binds the E3 ligase Von Hippel–Lindau (VHL). Unfortunately the pentapeptide VHL binder renders the molecule poorly cell permeable and thus is of limited therapeutic use. A second generation [145] (PDB: 4B9K, Figure 20.5c,d) had better physicochemical properties [146, 147] and could be further simplified using structural insights to demonstrate the catalytic properties of PROTACs and their effect in an *in vivo* setting [148].

The field has been further fueled by the discovery of another category of E3 ligase ligands – thalidomide and related immunomodulatory drugs (IMiDs) – that bind to the E3 ligase cereblon (CRBN) [149]. Like the simplified VHL warheads, these IMiDs have good affinity, specificity, and a known binding pose. Chimeric PROTACs for an increasing number of degradation targets have been successfully designed using the new generation of VHL and CRBN warheads. These chemical protein knockdown tools have been effective in a variety of settings, suggesting PROTACs may have wide utility in dissecting biological pathways and tackling less tractable targets such as PPIs [150].

20.6.2 PPI Stabilizers

Many protein complexes are dynamic, and pharmacological modulation can be achieved not only by PPI inhibition but also through their stabilization. Ottmann et al. [151] suggested this was a common mechanism of regulation within nature but an underexploited opportunity in drug discovery. They classified stabilizers into allosteric, where stabilization of the protein complex is achieved by binding at a distal site, and direct or interfacial, where the small molecule stabilizes the complex by binding directly at the interface, bridging the components. Interestingly, an analysis by the Klebe group concluded that cavities formed at interfaces of transient protein–protein complexes had similar properties to those of enzymes, leading them to conclude that these interfacial pockets formed were as druggable as classical enzyme pockets [152]. This was followed by work by Greene et al., which successfully used a computational approach to select small molecules that bind to a trypsin inhibitor binary complex that were demonstrated to stabilize this complex by ITC [153].

A number of natural products such as FK506, rapamycin, brefeldin, forskolin, cotylenin, and fusicoccin function by this stabilization MOA, and a wide range of other chemical structures have also been demonstrated to act by this mechanism. This includes lead-like small molecules such as tafamidis (Figure 20.5e) that inhibits the aggregation of transthyretin (TTR) responsible for TTR amyloidosis that leads to peripheral neuropathy and cardiomyopathy (PDB: 3TCT) [154] and fragments such as 1-ethyl-2-benzimidazolinone (1-EBIO) that has been found to stabilize the calmodulin–potassium channel (CaM-SK) interaction [151, 155].

Small- and intermediate-conductance Ca^{2+}-activated potassium (SK) channels, activated by Ca^{2+}-bound calmodulin (CaM), have an important role in regulating membrane excitability. Abnormalities in SK channel function have been linked to schizophrenia and hypertension. Development of small molecules targeting these channels has often been hampered by difficulty in achieving potency and selectivity, perhaps due to the absence of structural and mechanistic insights into their MOA. The structure of CaM-SK revealed that the crystallization additive phenylurea (PBD: 4G27) bound in the interface between CaM and SK. This prompted Zhang et al. [155] to explore interfacial binding compounds of the 1-EBIO class and to study how the molecular nature of the binding pocket contributes to the potency and selectivity of these compounds. While 1-EBIO is a weak stabilizer, the structurally related NS309 (6,7-dichloro-3-(hydroxyimino)indolin-2-one, PDB: 4J9Z) is 1000 times more potent in augmenting current in whole-cell HEK293 patch clamp assays.

A generic platform to exploit complex stabilization has been proposed by targeting conserved PPI structural motifs such as that of the 14-3-3 family of proteins [156]. These are small eukaryotic proteins with highly conserved sequences that form a family of ubiquitous adaptor modules. They act as docking hubs for diverse classes of phosphoproteins and peptides and play important roles within many PPI networks. Both stabilization and inhibition of the interactions of 14-3-3 with binding partners have been demonstrated. The first protein crystal structures showed these proteins to have 9 or 10 α-helices, and they usually form homo- and/or heterodimer interactions along their amino-terminal helices. Commonly 14-3-3 proteins bind phosphorylated serine or threonine residues in the context of peptides such as R[SFYW]XpSXP or RX[SYFWTQAD]Xp(S/T)X[PLM] (where an "X" can be several, but not any of the

20 amino acids and the lower case "p"| indicates the site of phosphorylation). However binding of non-phosphorylated ligands is known. This interaction occurs along the amphipathic binding groove. To date, the crystal structures of at least six classes of these proteins have been resolved [27, 156].

The first reported 14-3-3 stabilizers were the plant natural products cotylenin A and the fungal fusicoccin A. Originally described by their ability to bind to the plant analog of 14-3-3, they were also found to have activities in human cells. Cotylenin A has been shown to induce differentiation in myeloid leukemia, displays activity against breast cancer cells, and stabilizes the inhibitory binding of 14-3-3 proteins to c-Raf (PDB: 4IEA, 4IHL). The fusicoccin A toxin that induces wilting in plants by binding the 14-3-3-PMA2 plant complex also binds to 14-3-3/Raf1 complexes (PDB: 3IQV) as well as stabilizing the inhibitory binding of 14-3-3 to proteins such as ERα. Importantly, less complex small molecules have been found to stabilize 14-3-3 complexes.

The dipeptide epibestatin and a pyrrolinone (pyrrolidone1) occupy different parts of the fusicoccin binding site in the 14-3-3–PMA2 interface; epibestatin binds to a narrow pocket sandwiched between the two proteins (PDB: 3 M50), whereas pyrrolidone1 (PDB: 3 M51) binds to a relatively open pocket [157]. These binding differences are reflected in the dynamics of the stabilizing activity of these two compounds. Pyrrolidone1 mainly enhances the association rate of the 14-3-3–PMA2 complex, whereas epibestatin mainly decreases the dissociation rate [157].

These examples demonstrate that PPI stabilization can be achieved by small molecules but also highlight that our understanding of PPI stabilization and its translation to medicines is very much in its infancy, compared with established target class modulation and even PPI inhibition. Considerations include how to identify appropriate PPI complexes to target and questions of "selectivity" especially for proteins such as 14-3-3 that have an abundance of partner proteins. In the context of screening feasibility, in addition to the normal consideration of the relevance of the selected *in vitro* system, accurate measurement of stabilization between two partners may be more difficult than monitoring inhibition. Often, biophysical methods and indirect measurements of association are employed, but not all of these are amenable to screening of large compound libraries or lend themselves to the routine assays required to support iterative chemistry. Finally, given the transient nature of protein–protein interfaces and potentially the stepwise nature of their assembly and disassembly, special attention needs to be given to not only the equilibrium modulation of the PPI but also the association and dissociation processes involved in the stabilization of the complex.

20.7 Conclusion and Perspectives

PPIs play a critical and ubiquitous role in biology. They are important drug targets for which there are now several success stories. Their diverse nature has required a diverse range of approaches to achieve success. Use of antibodies, HTS, fragment-based hit identification, SBDD, and novel modalities such as stapled peptides have all played a role in this achievement. Although the challenges for many PPIs remain daunting, their high value will continue to prompt exploration of innovative approaches to deliver therapeutic opportunity, for example, using protein degradation and stabilization. This has been demonstrated in two recent examples of inhibitors that stabilize multimer complexes of therapeutic interest. The inactive p97 [158] and active eiF2B [159, 160] ligand-bound structures, captured by cryo-electron microscopy (cryo-EM) at almost atomic resolution, hail the increasing power of this imaging technique to deliver critical insights within drug discovery programs [161]. All these innovations are likely to yield benefit for drug discovery as a whole and increase the breadth of our druggable portfolio of targets.

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