Small molecules, big targets: drug discovery faces the protein-protein interaction challenge.

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Abstract

Protein-protein interactions (PPIs) are of pivotal importance in the regulation of biological systems and consequently are implicated in the development of disease states. Recent work has begun to show that, with the right tools, certain classes of PPI can yield to the efforts of medicinal chemists, and the first PPI inhibitors have reached clinical development. In this review we describe the research leading to these breakthroughs and highlight that within the PPI target-class there exist groups of structurally-related PPIs. For each of these groups we use examples of successful discovery efforts to highlight the research strategies that have proved most useful.

Introduction

The importance of the complex network of direct interactions between proteins, known as the interactome, to both biological systems and to the development of disease-states is widely recognized. Despite this, inhibitors that function by directly disrupting the interaction between two proteins remain an under-represented target class in drug discovery and PPIs are viewed as challenging, and in some cases essentially 'undruggable'. However, work in recent years has begun to show that certain classes of PPI are amenable to small molecule inhibition; typically these inhibitors disrupt the interaction between a globular protein and a single peptide chain on the partner protein and do so by binding into pockets on the surface of the globular protein. PPIs can be classified into groups based on common structural elements in both the globular protein and the peptide chain. Most notably, the presence of secondary structural features within the peptide-chain such as α-helices and β-strands has important ramifications for the design of inhibitors functioning by mimicking and displacing these peptides. In this review we explore emerging evidence that within a given structural class certain drug discovery strategies may be more applicable than others – thus providing guidance to researchers entering the field. We focus specifically on small molecules that inhibit PPIs by interacting directly with the binding interface of one protein partner rather than through an allosteric mechanism or binding at a catalytic site.

Structural biology and classification of PPIs

Structural biology often plays a central role in PPI research. Generally, PPI projects are supported by X-ray crystallography, although some groups have had great success with protein-based NMR spectroscopy[1]. Whilst it might be preferable to have access to a structure of the complete PPI to inform a drug-discovery effort, in practice one or both of the proteins is often truncated or modified to facilitate the structural biology. Typically, on-going structural information is obtained during the hit identification and optimisation process from X-ray or NMR structures of key compounds bound to the target. Further information can be gleaned from *apo* structures of globular protein but partners that can reveal significant conformational changes that may occur on binding. High-resolution protein X-ray structures can also reveal the location of water molecules at the protein surfaces. Analysis of water networks combined with

computational approaches can allow assessment of whether such waters should be displaced or treated as effectively part of the protein surface[2].

A critical development in the understanding of PPIs was the realisation that the interactions driving the affinity of a pair of proteins are not distributed evenly across their surfaces. Rather, certain residues or regions, termed hot-spots, are largely responsible for driving binding. This can be explored experimentally by a process known as alanine-scanning or hot-spot analysis[3, 4]. Thus the impact of sequentially mutating residues to alanine (or alanine residues to glycine) on the affinity of a pair of proteins can be measured. Within the literature a degree of ambiguity exists as to whether a hot-spot must be a specific residue or whether it can also refer to a cluster of residues such as those giving rise to a pocket – sometimes called a hot-region. For clarity, we have chosen to use the terms 'hot-spot residues' and 'hot-spot pockets' to clarify the nature of the hot-spot to which were are referring.

The buried surface area for protein-protein interfaces for which inhibitors have been discovered, varies between 1000-6000 Å^2 [5]. Under 2000 Å^2 the total interaction surface is usually limited to a single patch whereas larger interactions are typically composed of several patches, separated by solvent-exposed residues[6]. There is no simple correlation between the total surface area buried upon complexation and binding affinity; hot-spots contribute disproportionately more binding energy[4]. Analysis of alanine scanning data indicates a preference for Trp, Tyr and Arg as hot-spot residues. To a lesser extent the polar residues Asp, and His are also enriched[4]. It has been proposed that hot-spots interact cooperatively and tend not to be distributed evenly over the interface but group within tightly packed local clusters[7]. Unsurprisingly, hot-spot residues identified from alanine scanning studies are structurally conserved[7]. On exposed protein surfaces, the conservation of a Trp residue, and to a lesser extent Phe or Met, correlates with a PPI binding site[8]. Others have reported an increased frequency of Trp and Tyr at PPI interfaces as compared to solvent-exposed protein surfaces[9]. An analysis by Hu *et al.* found a similar pattern but also highlighted the propensity for polar amino acids such as Arg, Asp and His in PPI interfaces[10]. The importance of polar interactions in PPIs has also been recognised and discussed[11].

Methods for experimentally detecting PPIs have been recently reviewed[12] and will not be commented on further here. Bioinformatic approaches have also been useful[12], for example in identifying putative interactions through sequence similarity with known human PPIs or phylogenetic analysis of PPIs from lower-order eukaryotic species[13]. A combined analytic-prediction approach has recently estimated the entire interactome to involve 300,000 binary interactions[14]. Several databases have been created to aid study of PPIs.[15] STRING[16] is a database of predicted and known PPIs, TIMBAL[17] provides small molecule inhibition data, 2P2IDB[18, 19] PICCOLO[20], and others[15] provide structural information and analysis.

PPIs can be homo- or heteromeric and classified as either obligate - strong and long-lived or non-obligate - weaker and transient[21]. Looking across a broad set of PPIs, the range of affinities spans nearly one million-fold, from the picomolar to high micromolar[5]. It has been proposed that interfaces are often predominantly hydrophobic with a surrounding ring of polar residues[22] or a mixture of hydrophobic patches interspersed with polar interactions and water molecules scattered across the interface[23]. Obligate PPI complexes often resemble a larger globular structure with the interface similar to the internal structure of each globular protein.[24] On the other hand, non-obligate PPI interfaces are typically smaller and not as clearly dominated by hydrophobic interactions, this likely reflects the unfavourable energetics of exposing a hydrophobic patch to the solvent on dissociation[11, 21, 24]. Wells *et al*. have recently discussed classifying interfaces based upon the complexity of the binding epitopes.[25]

It is helpful to divide PPIs into a series of structural classes (figure 1)[26].

1. Pairs of globular proteins that interact through a discontinuous epitope with no significant structural changes on binding.

2. Interactions between a pair of globular proteins in which one or both proteins undergoes significant conformational change on binding.

3. PPIs involving a globular protein interacting with a single peptide chain.

4. PPIs between two peptide chains.

The latter two classes can be further differentiated, depending on whether the peptides undergo significant conformational changes on binding. In some cases the peptide is an intrinsically disordered peptide or region of a protein which folds into a specific conformation on binding[24], whilst in other cases a pre-folded region of a protein interacts with the partner protein – although in practice a continuum exists between these two extremes. In the pre-folded scenario the remainder of the protein is effectively acting as a scaffold to stabilise and present the peptide in a suitable conformation for binding. The significance of disordered peptides in PPIs has been reviewed[24].

Building on this analysis, in table 1 we highlight here a range of structural classes, some of which have been previously proposed[27] (table 1). In the latter half of this review we take examples of the different classes, expand on their characteristics and provide examples of successful small molecule discovery efforts.

PPIs between a pair of globular proteins, exemplified by the interaction between IL-2 and IL-2R[28] (see below) remain formidable targets for drug discovery. The interaction surfaces of such proteins have been found to be flatter on average and thus potentially less suitable for the binding of a small molecule ligand[29]. The interaction between two peptidic regions presents a very different challenge owing to the lack of a defined binding site, and the potential of one or both peptides to be intrinsically disordered when uncomplexed. For example, considerable effort has been put into developing inhibitors of the interaction between c-Myc and Max[30]. The c-Myc–Max structure consists of a four-helix bundle in which each monomer forms two α-helices separated by a loop. However, when uncomplexed both of these proteins are intrinsically disordered, therefore structural biology of this system is very difficult and the nature of any potential binding site is hard to define. In general the reported inhibitors have potency only in the micromolar range and their mechanisms of inhibition are not fully understood. It should be noted that some of the reported chemotypes contain structural elements identified as so-called PAINs[31] with the potential to interfere with biological assays. Moreover, there is only limited structural evidence showing that these inhibitors operate as orthosteric inhibitors of the c-Myc–Max interaction. However, Metallo and co-workers have taken a rational approach to validating the reported inhibitors. Truncated and mutated proteins were screened to identify the

ligand-binding regions, confirming this binding with NMR spectroscopy and circular dichroism.[32, 33] Despite this work, clearly this is an exceptionally challenging PPI from a drug discovery perspective and much work is required to further characterise these inhibitors and to develop molecules with the potential to become drugs.

On the other hand, interactions where one partner interacts through a single peptidic region and the other is globular have been proposed to be the more druggable[26]. When bound, the peptide typically adopts one or more secondary structural elements, which may or may not be present in the unbound peptide, and which then interact with the surface of the globular protein. These structural elements differentiate a series of structural groups for which aspects such as strategy and likely success-rate may vary quite significantly. In addition, common structural motifs may occur in the globular protein. Thus we argue that *PPIs with similar secondary structural elements are often best tackled using similar strategies*.

In the second half of this review we highlight examples with different structural elements and outline the strategies used to tackle them. In the interaction of the BAD/BAK peptides with BCl-2, the peptide presents an α-helix into a defined groove on the globular protein. Other peptides display a continuous epitope on a β-sheet or strand motif, as exemplified by the XIAP–Caspase 9 and Keap1–Nrf2. In some cases a single residue on the peptide acts as an anchor group dominating the interaction as is seen with the interaction of bromodomains with histones containing acetylated lysines. Table 1 provides further examples of PPIs in each of these categories.

Structural examination generally reveals direct interactions between the hotspots on partner proteins. However there is an asymmetry in the nature of the hot-spot interactions between globular proteins and peptides: hot-spot residues on the peptide typically access hot-spot pockets on the globular protein[24]. Lead discovery efforts targeting PPIs have perhaps inevitably led to the discovery of inhibitors binding to the globular partner and thus displacing the peptide: the small molecules typically mimic the interactions made by the peptide and place groups into the hot-spot pockets on the globular protein.

Some PPIs, for example RAD51–BRCA2[34] and HIV integrase–LEDGF[27] consist of multiple peptide binding elements separated by regions providing little interaction

energy. In one sense these can be thought of as being an intermediate case between a globular–peptide interaction and a full globular–globular interaction. However, it seems that often a single peptide region is dominant, and may provide much of the binding energy. In practice, small molecule inhibitors are typically found to bind to this single high-affinity region[35] and in doing so they disrupt the PPI[36]. We argue that such cases are best classified structurally using their high affinity sites only.

Identification of hits and leads against PPIs

Developing a molecule capable of accessing a series of hot-spot pockets on the globular protein surface, whilst keeping molecular properties within conventional limits can be challenging. Compared to binding pockets on classical targets, those accessed by PPI inhibitors have been found to be smaller in volume and PPI inhibitors tend to bind into a higher number of pockets[37]. The relative clustering of binding hot-spots has ramifications in the design of small molecule inhibitors, PPI intervention with a small molecule is more likely to succeed when interaction hot-spots are tightly clustered in space, such as in a short α-helix binding cleft as opposed to being distributed over an extended interface[38]. This picture is further complicated if there are significant changes in the globular-protein's structure between the *apo*, peptide- and inhibitor-bound complexes[39]. A wide variety of strategies have been employed to identify hits and leads against PPIs.

Screening strategies

Lead molecule screening

Conventional high-throughput screening (HTS) presents a number of challenges when targeting PPIs, namely low hit rates, weakly potent hits, and difficulties in removing false positives - as discussed by Wendt[27]. It is difficult to know to what extent low hit rates reflect the inherent limitations of the HTS approach, the relative difficulty of binding to a protein surface or the biases of many compound collections towards classical target classes. Nevertheless, HTS has been successfully applied, particularly to the identification of molecules mimicking a non-continuous epitope consisting of side-chains on an α-helix, such as successful screens against MDM2–p53[40-43], ZipA–FtsZ[44] and HPV E2–E1[45]. It is not clear whether this reflects a potential inherent ease

of finding hits against these targets or the frequency that they have been screened against.

Screening strategies have also been successfully applied to find natural product inhibitors.[46] Whilst this approach has led to a wide range of active chemotypes against a variety of PPIs, structurally these hits may prove challenging to optimise.

Fragment screening and optimisation

Over the past decade fragment-based drug discovery (FBDD) has become a standard part of the medicinal chemists' arsenal[47-49]. FBDD has been shown to be particularly effective against targets such as PPIs, generally viewed as 'undruggable'[15, 50, 51] as fragments are not likely to be inherently biased towards one target class over another[47]. It has been shown that there is a good correlation between hot-spot regions of a protein and fragment binding sites[51, 52].

Using FBDD to tackle PPIs presents challenges: firstly, a low affinity fragment hit is unlikely to be able to disrupt a PPI. The most common approach is therefore to use a screening cascade of biophysical screening techniques to screen for binding to the globular protein partner. There is often a trade-off between the throughput of a screening technique, the extent of false positives and negatives and also the degree of structural information. For this reason many groups use a suite of techniques to identify well-characterised hits with defined binding sites[53]. The cascade may include *inter alia* thermal shift (TS)[54], surface plasmon resonance (SPR)[55], ligand- or protein-based NMR[56], X-ray crystallography[57] and isothermal titration calorimetry (ITC)[58]. Once a hit has been identified, structural techniques such as protein NMR or X-ray crystallography can be used to establish whether the fragment binds at the PPI interface or alternatively displacement or blocking experiments with a tool ligand (often a peptide derived from the partner protein) can be used to make this assessment. This approach has a number of potential weaknesses. Firstly, it may prove necessary to mutate the protein target in order for it to be stable in the absence of its binding partner[53]. Moreover, the energy required to drive conformational

changes at the binding-site may render a fragment unable to bind with detectable affinity. This latter problem has been circumvented using tethered fragments[59].

The potential benefits of tailoring a fragment library to the PPI target-class have been discussed[60-62]. Recent data suggests PPI fragment hits to be more likely to be charged, somewhat larger and more lipophilic, but no more three-dimensional than those against non-PPI targets[51]. A number of reports have appeared in which potent inhibitors discovered by means other than FBDD have been retrospectively fragmented to generate compounds that would be unlikely to have been found through a fragment-based approach.[61, 63, 64] The energetic costs associated with water displacement and conformational mobility have additionally been proposed as potential challenges in targeting PPIs with fragments[61].

There are relatively few reports of fragment optimisation to generate improved-potency leads targeting PPIs, with success against the Bcl-2 family[51], XIAP–Caspase-9[51], HIV-IN–LEDGF[65], RAD51–BRCA-2[35] and bromodomains[51] being notable exceptions[51]. This may reflect the inherent challenge of accessing two or more hot-spot pockets separated by regions not offering the potential for increased affinity. In our experience it can prove difficult to realise iterative improvements in potency as a fragment is gradually grown from one hot-spot pocket towards another[66]. Whilst, it may seem more attractive to try to link two fragments, each binding to a different hot-spot pocket; in practice such fragment-linking can be very challenging[67].

Rational-design: peptides and peptidomimetics

The rational design of PPI inhibitors has focussed on efforts to mimic peptides using a range of different strategies (figure 2)[68]. Such mimetics, whether peptide- or non-peptide-based, have a tendency to fall well outside conventional drug-like space and present very different challenges to conventional small molecule drug design.[69]

Much design work has been based on the rational design of general scaffolds, substituted with the relevant amino acid side-chains, mimicking the 3 major recognition motifs found to modulate PPIs: α-helices, β-strands and reverse-turns[68].

In α-helix interactions, the binding energy is largely derived from the hydrophobic interactions of the side-chain residues present on one face of the helix with the partner protein (figure 2, A). One design strategy is to seek to modify a helical peptide in order to maximise its helicity and possibly also improve its properties[70]. Most notable in this respect is the rapidly expanding field of peptide stapling[71] (figure 2, Aiii). Typically metathesis chemistry is used to covalently link non-natural amino acid side-chains, leading to peptides with improved affinity and cellular penetration[72], examples of which are being investigated in the clinic[73]. A related approach seeks to replace the hydrogen bonds in a helix with covalent linkers[74]. Also of note are β-peptides - formed from β-amino acids - which can adopt a number of secondary structural elements, notably helices, and may show good pharmacokinetics[75, 76].

An alternative approach is to use non-amino acid building blocks (figure 2, Ai). For example, Hamilton has developed a ter-phenyl scaffold with 3 points of attachment to represent 3 consecutive amino acids on one face of a helix (i, i+3/4 and i+7 residues)[77]. This approach has been successfully applied to the inhibition of PPIs such as Bcl-xL–Bak and MDM2–p53[78, 79]. Boger designed a polyamide scaffold that represents the i, i+4, i+7 residues; improved synthetic ease allowed facile creation of an 8,000-member library of mimetics for *in vitro* screening[80]. Despite the high affinity of these mimetics, their structural simplicity can result in poor selectivity across closely related PPI families. Seeking to address this, other groups have enhanced mimetic complexity[81].

Developing mimetics of continuous epitopes such as β-strands and turns and of short peptide sequences with no clear secondary structure presents a very different challenge. These generally present an epitope in which hydrophobic side-chain interactions and hydrogen bonding interactions with the more exposed peptide backbone functionality are important (figure 2, B). The generation of reverse-turn mimetics, most notably β-turns, has been applied developing molecules which bind to integrins[68], SH2 domains such as that of Grb2[82, 83] and inhibitors of the CD2–C58[68] interaction amongst other targets[68]. Mimics of β-strands are less prevalent[84], likely due to these secondary structure motifs making few hydrophobic interactions; typically using hydrophilic amide backbone hydrogen bonding interactions to stretch across flat featureless protein surfaces to link residues benefiting from hydrophobic

hot-spots. However, designing a mimetic simpler than a sequence of amino acids is demanding as such structures have high conformational flexibility and poor metabolic stability, consequently efforts have focused on increasing rigidity via conformational restraint[84].

Computational approaches

Computational techniques are commonly used to complement both screening and design approaches and to identify focussed screening sets from commercially available compound collections[85]. Given the structure of a PPI it is possible to use computational tools to assess druggability and identify possible small molecule binding pockets. Dr PIAS[86] is a software tool that identifies similarities in pocket structure from a database of known PPI inhibitors and targets. The presence of conserved surface residues at interfaces has been exploited to predict where small-molecules might bind[87]. Meireless *et al.* have suggested the importance of the burial of Solvent Accessible Surface Area (SASA) and have introduced the ANCHOR system for identifying 'anchoring' residues that are deeply buried upon binding[88].

There have been many reports of computational approaches to PPI inhibitor development[89, 90] including the use of docking and pharmacophore-based searching[91]. The importance of accurately modelling water molecules has also been highlighted[2]. The flexibility and solvent-exposed nature of PPI binding sites makes the application of these approaches to static protein structures challenging. The frequency with which protein conformational changes are observed in crystal structures upon binding a protein partner or small molecule ligand[39] suggests that virtual screening is unlikely to be straightforward. MD simulations of unbound proteins have shown that druggable PPI sites have a higher propensity to form surface pockets than non-PPI sites and that these predicted pockets match those found in inhibitor-bound structures[92]. Brown and Hadjuk have implemented an empirically validated 'druggability algorithm'[93] for snapshots of MD simulations, suggesting an explanation for the dynamics and druggability observed for Bcl-xL[94]. MD simulations with halogenated benzene as a probe have been reported as an approach to identify both exposed and cryptic pockets from ensembles of protein conformations[95]. Conformational mobility can also be revealed experimentally, for example through analysis of crystal binding interactions that may reveal cryptic pockets opened by

crystal contacts[96]. Consideration of conformational ensembles and their relative energetics should prove to be a useful tool for targets where large conformational changes are expected. The prediction of the ability of some PPI interfaces to accommodate small molecules by dynamic pocket formation could well aid in target selection.

Lead to Candidate optimisation

Once the binding site of a ligand moves beyond established hot-spots it can be difficult to derive significant potency from additional interactions, which can make optimisation to development compounds highly demanding. The problem is most pronounced for cases where the binding surface is relatively flat and featureless. There is a tendency for MW and other properties to be outside conventional 'drug-like' chemical space making the process of identifying a drug with good physicochemical and pharmacokinetic properties more difficult[19]. Thus the drive for improved potency as MW increases must always be balanced against a subsequent potential decline in developability properties and selectivity. Unlike kinases, for example, where ATP-competitive inhibitors must compete with high cellular concentrations of ATP, PPI inhibitors may not suffer from the same potency attrition in moving from biochemical screens into cell studies, depending upon the exact cellular concentration of the partnering proteins. It remains to be seen whether PPI inhibitors in general can afford to be less potent, at the advantage of maintaining favourable physicochemical properties. Table 2 shows the examples of small-molecule development candidates identified against PPIs known to the authors and that have entries on www.clinicaltrial.gov.

Examples of the different classes of PPI

Globular–helical peptide – discontinuous epitope

Bcl-2 family–BH3 domain

The Bcl-2 family of proteins are pivotal in the regulation of cell death through control of the integrity of the outer mitochondrial membrane. Pro-apoptotic Bcl-2 family

proteins such as Bak and Bax play a role in causing apoptosis. The effect of these proteins is blocked when they are sequestered by anti-apoptotic binding partners such as Bcl-2 and Bcl-xL. Small molecules which disrupt this interaction, by binding to the anti-apoptotic Bcl-2 family proteins, have been designed to induce apoptosis of cancer cells[97].

Structurally, the pro-apoptotic proteins possess a number of BH3 domains containing an α-helical motif that drives binding to the anti-apoptotic family members through predominantly hydrophobic interactions. In each case a critical 4-turn α-helical portion of the BH3 domain on the pro-apoptotic protein binds in an extended hydrophobic groove, of length around 20 Å, on the surface of its binding partner (figure 3, A, B). Led by Abbott laboratories, development of small molecule inhibitors over the last two decades has resulted in three molecules progressing to the clinic[98]. These inhibitors bind to the globular Bcl-2 family proteins and mimic the α-helical BH3 domain.

NMR solution structures and X-ray crystal structures of Bcl-xL with many peptide and small molecule ligands have been described[99-105]. Analysis of these structures has revealed significant Bcl-xL backbone flexibility, dependent on the binding partner. Early virtual-screening based approaches, with no consideration of protein surface flexibility, yielded flat, rigid compounds of limited affinity[106-108]. A more refined approach resulted in identification of moderately potent small molecules that could provide interesting starting points for future elaboration although no progression has been published[109].

The elongated, discontinuous α-helical nature of the BH3 peptide partners has inspired the design of secondary structure mimetics[110] such as terephthalamide-based structures[111, 112]. Chemically "stapling" BH3 peptides has resulted in highly specific, biologically-active inhibitors of both the Bcl-xL and Mcl-1 interactions[73, 113, 114]. Despite potential hydrolytic liabilities, following *iv* dosing, a stapled BID BH3 peptide has exhibited activity in xenograft studies[113].

In common with other globular protein–α-helix PPIs, a number of high-throughput screens have been reported, including cases leading to promising hits with MW <500 and low μM IC_{50} values *in vitro*[115, 116]. A group at BMS obtained co-crystal structures

of their leads and subsequent optimisation resulted in low nM inhibitors of Bcl-2–Bim and Bcl-xL–Bim in biochemical assays; although their relatively high MW resulted in low aqueous solubility and limited cellular potency[115].

The Wang group (University of Michigan, Ann Arbor) and others have primarily used structure-based virtual screening to identify small molecule potent Bcl-2 family inhibitors: YC137[117], TW-37[118] and compound 21[119]. Meanwhile Fesik (Abbott laboratories) has used ^{15}N HSQC protein-NMR to drive a fragment screening and linking strategy, followed by extensive optimisation guided by further NMR work. This led to the orally-bioavailable inhibitor **1** (**navitoclax**, **ABT-263**) that is currently in a number of phase 2 clinical trials for the treatment of a variety of cancers (figure 3, C and D).[120]

Subsequently, "pan-inhibition" of the Bcl-2 family proteins was shown to result in mechanism-specific toxicity (thrombocytopenia); specifically attributed to binding to Bcl-xL. To circumvent this toxicity, re-optimisation based on compound **1** (**ABT-263)** produced sub-nanomolar Bcl-2 binder **2** (**venetoclax**, **ABT-199**) with 3 orders of magnitude selectivity over Bcl-xL and Bcl-w[98]. Selectivity was achieved by introducing a new polar interaction based on the observation of a crystallisation artefact in which the tryptophan side chain of a Bcl-2 crystal-packing mate formed a hydrogen bond with Asp103 of an adjacent Bcl-2, which is a Glu residue in Bcl-xL. Thus an indole moiety was incorporated into the scaffold of **1** (**ABT-263**) (Figure 3, D), recapitulating the interaction with Asp103 and leading to selectivity over Bcl-xL. Bcl-2-selective **2** (**venetoclax**, **ABT-199**) has now also progressed to the clinic as a combination therapy and is currently under evaluation in phase 3 for chronic lymphocytic leukemia (www.clinicaltrials.gov). Since the Abbott work, further examples of structure-guided discovery leading to potent Bcl-2 family inhibitors by merging newly identified groups with elements of the Abbott structures have been reported[121, 122].

Discussion

The Bcl-2 protein is flexible and has three hot-spot pockets, leading to high molecular weight inhibitors and potentially poor developability properties. This is a good example of how identification of additional binding sites can be achieved by

conducting a second fragment screen in the presence of hit fragments from an earlier screen. Notably, in this case selectivity was required over a number of structurally-similar PPIs, despite similarities of the α-helix binding grooves of the Bcl-2 family members, it has proved possible to engineer selectivity for Bcl-2 over Bcl-xL.

Looking across the broader set of globular–helical peptide PPIs (table 1) a wide range of approaches for discovering leads have been used, with fragment-, lead- and natural product-screening, and peptidomimetic strategies all bearing fruit. Notably, the much studied MDM2–p53 interaction has proved less amenable to fragment screening than the Bcl-2 family, but HTS and peptidomimetic approaches have been successful[123]. Structurally, the peptides and peptidomimetics have been based on amino acid and non-amino acid oligomers as well as stapled peptides (figure 2, Ai and iii). In addition to these 'oligomeric' structures, there has been considerable success using non-oligomeric scaffolds mimicking the orientation of the side-chains present on a helix. These may either be linear scaffolds (figure 2, Aii), as in the Bcl2 binders described above, or compact cores (figure 2, Aiv), as for the MDM2–p53 inhibitors[124].

Globular–peptide - continuous epitope

XIAP-SMAC

Caspase-9 is a cysteine-dependent protease that plays a vital role in apoptosis. Binding of the BIR3 domain of XIAP (X-linked Inhibitor of Apoptosis Protein) to Caspase-9 causes inhibition of Caspase-9 activity, preventing apoptosis and hence allowing tumour cell survival. Similarly, Caspases 3 and 7 are inhibited through binding to the BIR2 domain of XIAP. A small molecule that inhibits this interaction is of interest in promoting apoptosis in cancer cells. Caspase-9 binds XIAP with an *N*-terminal continuous-epitope tetrapeptide motif (ATPF) that accesses a hydrophobic groove on the BIR3 domain of XIAP (figure 4, A, B). Interestingly, an endogenous protein inhibitor of the XIAP–Caspase-9 interaction exists in the form of SMAC (Second Mitochondria-Derived Activator of Caspase, also known as DIABLO), which operates by binding and sequestering XIAP, via a similar binding motif, tetrapeptide AVPI. This utilises the same binding groove as Caspase-9[125]. It is

thought that the BIR2 domain of XIAP also interacts with another molecule of SMAC to form a 1:2 complex via the same tetrapeptide motif.[126] In addition, XIAP is member of a family of Inhibitor of Apoptosis Proteins (IAPs), including c-IAP1, c-IAP2, Livin and Survinin, which also interact with SMAC through highly conserved BIR domains. Therefore small molecules that bind to the BIR3 domain of XIAP at the SMAC binding site usually bind to other IAPs as well as XIAP.

The tetra-peptide AVPI derived from SMAC, binds XIAP with a K_D of 0.58 μM[127] and so provides a potential starting point for small molecule development, leading to optimised inhibitors such as compound **3** (figure 4C and D). Impressively, single digit nanomolar affinity was achieved for some of the compounds and cytotoxicity against several human cell lines with concomitant increase in Caspase activity was demonstrated, as well as inhibition of tumour growth in a mouse xenograft model[128]. Various strategies have been employed to impose a conformational constraint on the peptide by building a bicyclic ring system, fusing the proline with the analogous position of the neighbouring valine[129, 130]. A representative molecule **4** (**SMAC037**) is shown (figure 4, D). In general, conformational constraint of a peptide has the potential benefits of increasing potency through reducing the entropic barriers to binding and interaction of the constraining elements with the protein itself.

Dömling and co-workers have reported a novel strategy to identify inhibitors of the XIAP–SMAC interaction[131]. The approach consists of identifying an 'anchoring' residue from the SMAC interaction motif, in this case the alanine of AVPI. Virtual libraries were then constructed incorporating the alanine, and docking was performed. The compounds that scored best were then synthesised and tested for the ability to abrogate the protein interaction. Although the compounds were only modestly potent (IC_{50} values 10-100 μM) as compared to peptide mimetics, the strategy represents an interesting methodology to generate novel scaffolds.

Pellechia *et al*. have applied fragment-based methods to XIAP in an effort to generate compounds from the peptide with improved drug-like properties[132]. Similar to Dömling's 'anchoring' approach, libraries based upon alanine were screened virtually and selected compounds were tested for binding. In this case, 2D NMR spectroscopy was employed to identify small, weakly binding fragments. Through inspection of

the structure with AVPI and further library synthesis, a hit fragment was elaborated to give a compound with a K_D of 2.5 uM, with superior human plasma stability and permeability compared to the AVPI peptide.

The observation that SMAC must bind to both the BIR3 and the BIR2 domain of XIAP to effectively disrupt XIAP-mediated caspase inhibition has more recently led to the development of bivalent ligands, designed to bind BIR3 and BIR2 by linking together two identical monovalent peptide mimetics. In some cases, such as SM-164, the bivalent analogue of monovalent ligand SM-122, an approximately 1,000-fold improvement in effect on inducing apoptosis in tumour cells is observed.[133] Birinapant (TL32711) from Tetralogics Pharma is another bivalent ligand, but dispenses with a linker, with two monovalent ligands directly attached to each other through an indole. Birinapant is currently in phase 2 trials, as is LCL-161 (a monovalent ligand) from Novartis. Several other monovalent ligands are in phase 1 such as AT-406 and GDC-0152 (Table 2).

The majority of XIAP inhibitors are not selective for XIAP over other members of the IAP family, in particular c-IAP1 and c-IAP2. In addition, within the domains of XIAP itself, inhibitors tend to bind to both the BIR2 and BIR3 domain. Recently however, inhibition of the BIR domains of c-IAP1 has been linked with the undesirable production of TNFα[134], prompting efforts to design selective inhibitors which particularly target the BIR2 domain of XIAP and not the BIR domains of c-IAP1 and c-IAP2.[135, 136] Such inhibitors are anticipated to have different pharmacodynamics profiles to the less selective compounds.

Keap1–Nrf2

Nrf2 (Nuclear factor-erythroid 2-related factor 2) is a transcription factor that plays a key role in protecting cells against the damage caused by carcinogens, oxidants and other toxic chemicals through the induction of detoxifying enzymes. Keap1 (Kelch-like ECH associating protein 1) inhibits the activity of Nrf2 by facilitating protein degradation through ubiquitination. Oxidative stress is detected by Keap1 through modification of reactive cysteine residues leading to the release of Nrf2, reduction of ubiquitination and thus to up-regulation of key cytoprotective proteins[137]. Inhibition of this regulatory process has been proposed

to have potential therapeutic benefit in a number of diseases including cancer, diabetes, atherosclerosis, Alzheimer's disease and arthritis.

Nrf2 possesses two binding motifs that drive its interaction with Keap1: the high affinity ETGE (DxETGE) unit and the more weakly-interacting DLG (LxxQDxDLG) unit. It has been proposed that a 2:1 complex in which a Keap1 homodimer binds to a single molecule of Nrf2 controls the ubiquitination of Nrf2 and thus increases Nrf2 levels at times of oxidative stress. The suggested mechanism for this regulation is that the high affinity ETGE binding unit acts as a hinge whilst the low affinity DLG behaves as a latch: at times of oxidative stress conformational changes in Keap1 cause dissociation of the DLG 'latch' leading to conformational changes in the Nrf2 protein that reduce the extent of Nrf2 ubiquitination thus increasing overall protein levels[138].

The X-ray crystal structure of the murine Keap1 DGR-CRT domain (mKeap1-DC) revealed a striking six-bladed beta-propeller structure, with each blade composed of four anti-parallel β-sheets[137]. Further structures showing peptides derived from Nrf2, containing the high affinity ETGE motif, bound to mKeap1-DC have been reported[137, 139]. A second crystal structure of the DLG motif revealed a very similar binding mode [138].

A number of efforts to identify inhibitors of the Keap1-Nrf2 PPI have been reported. It has been shown that the shortest ETGE containing peptide to give similar affinity to Keap1 is an acetylated 9-mer with a K_D of 23 nM[140]. The SAR of a series of heptapeptides has been explored with the most potent showing sub-micromolar IC_{50} values for disruption of the PPI[141, 142]. Two high throughput screens for inhibitors of the Keap1-Nrf2 PPI have been reported[143, 144]. Symmetrical inhibitor **5** was reported to have an IC_{50} of 2.7 μM and to show evidence of target engagement in cells. A crystal structure of **5** bound to Kelch-DC central domain of Keap1 showed that it accesses the same binding site as the ETGE and DLG motifs (figure 5, C, D). Exploring this structure, Jiang *et al*. noted that **5** lacks the polar interactions made by the glutamic acid residues in the ETGE motif. Based on this observation they designed the diacid **6** that was found to have a much-improved K_D of 9.9 nM as determined by biolayer interferometry, and activity of 29 nM in an FP assay (figure 5, D). This compound was also shown to

have activity in a number of cell-based assays[145]. Other small molecule leads have been identified by a virtual screening approaches utilising both a pharmacophore-based search and docking[146, 147].

Discussion

Many inhibitors of PPIs with a short continuous epitope can be traced back to peptides and peptidomimetic leads based on the binding unit (figure 2, B, v). There is a fundamental difference in the nature of peptidomimetics in this case and the helix mimetics discussed in the previous sections.

As with the XIAP−caspase 9 interaction, commonly, a linear peptide makes a sequence of hydrogen bonds with the protein surface via the peptide backbone, and also positions hydrophobic groups that interact in surface pockets. There may be no net energetic benefit in displacing surface waters from hydrated interfaces to replace them with interactions with the epitope backbone. However, if these surface waters are displaced by a small molecule and the hydrogen bond is not re-made, it is likely an enthalpic penalty will result. The challenge is to design a small molecule that bridges hydrophobic hot-spots, with a linker that will satisfy these hydrogen bonds. It can be difficult to design a more efficient linker to achieve this than the peptide unit. α-Helical peptides cannot interact in this manner with a protein surface due to the internal back hydrogen bonding of helices, and in general non-peptide small molecules have not achieved the same levels of potency as peptidic compounds. Sensible strategies to increase potency include constraint of the peptide by introduction of cyclic amino acids and bridges between residues. The nature of the residues in a short peptide sequence will have significant bearing on the ease of developing potent inhibitors and endowing these with good ADMET properties.

The PDZ domain is a conserved peptide binding motif which has been identified in >140 proteins. Typically this globular motif binds to a short *C*-terminal peptide in which the terminal four amino acids are of particular importance. Ligands are typically negatively-charged, forming an interaction with a Lys or Arg residue in the PDZ domain. Extensive studies with peptides have been reported and also some initial results using virtual screening, lead-like molecule and fragment screening[148]. The integrins are another important class of PPI featuring a continuous epitope, in this

case the need to mimic a zwitterion has made the development of compounds with good developability properties challenging[149].

In the case of Keap1–Nrf2 the β-propeller structure makes this structurally very different from the above interactions; the presence of concave binding surface with a number of charged residues may be responsible for the notable successes screening against this target. Notably, WDR5–MLL represents another example of a peptide binding into the hole in a β-propeller[150-152], it remains to be seen whether other related PPIs may be discovered.

Globular-globular discontinuous epitope

IL-2–IL-2R

The cytokine IL-2 (interleukin-2) and its associated cell surface receptors, IL-2Rα (p55), IL-2Rβ (p75) and IL-2Rγ (p64, γc) play a critical role in the immune response. IL-2 and IL-2Rα are expressed by activated T-cells, extracellular IL-2 binds to IL-2Rα and the resultant complex then joins with the β- and γ-subunits triggering an immune response. Antibodies that act as antagonists of the interaction between the receptor IL-2Rα, and IL-2 have been shown to be clinically effective as immunosuppressive agents, providing target validation for this as a therapeutic approach and supporting efforts to develop a small molecule inhibitor[28, 153].

Compared to most other PPIs targeted in drug discovery, this interaction is arguably a true globular-globular interaction. Analysis of the binding surface of IL-2 reveals hydrophobic patches surrounded by polar groups[28]. It is proposed that desolvation of these patches drives binding of the proteins.

A number of small molecule inhibitors have been reported, these bind to the cytokine IL-2 and thus mimic residues from IL-2Rα, which are derived from various non-continuous structural elements in contrast to the previous examples in which the inhibitors mimicked a single structural element. A group at Roche attempted to design a small molecule capable of acting as an antagonist of IL-2Rα leading to the discovery of compound **7** that inhibited IL-2–IL-2Rα binding with an IC_{50} of 3 μM

(figure 6, D). Subsequent ^{15}N HSQC protein NMR studies led to the surprising discovery that **7** binds to IL-2 rather than, as expected, the corresponding receptor[154]. Wells and co-workers at Sunesis solved X-ray structures of **7** bound to IL-2 and of the *apo* cytokine[155]. Comparison of these two structures revealed that the binding site for **7** has a rigid region into which the polar head of **7** binds and an adaptive region forming a recessed channel used for binding the lipophilic biarylacetylene unit.

Sunesis have reported a number of IL-2 ligands. A conventional structure-based design approach led them to a series of guanidines, however they found that potency reached a low micromolar plateau[156, 157]. On the other hand, a fragment-tethering strategy proved more fruitful. A set of IL-2 mutants in which residues at the perimeter of the IL-2 "hot-spot" were converted to cysteine was screened against a library of 7,000 disulfide-containing fragments. This identified a region, accessible from two mutant cysteines, with a preference for binding small aromatic carboxylic acids[156] that were subsequently shown by crystallography to bind at the end of the hydrophobic channel[155]. Overlaying the crystal structure of their lead with the modeled fragments led to the design of hybrid molecules - the most potent of which **8** (**SP4206**) has a K_D for IL-2 of 100 nM as determined by SPR, and an IC_{50} of 60 nM for inhibition of the IL-2–IL-2Rα interaction[156]. In further tethering studies, Sunesis have explored the impact of simultaneous binding of the Roche compound **7** and tethered fragments, finding examples of both cooperativity and competitive binding associated with the complex behaviour of the flexible regions of the IL-2 protein, moreover this behavior was found to be associated with binding into a further cryptic pocket in the adaptive region[158].

Discussion

In the case of globular–globular PPIs, the interaction does not naturally divide into a target-protein to which inhibitors will bind and a displaced peptide, as demonstrated by the ambiguity regarding the target in the initial Roche work. The lack of a continuous epitope precludes rational peptidomimetic type approaches and makes screening and optimisation particularly formidable. Roche benefited from serendipity and Sunesis from the tight binding afforded by the tethering approach. There have

been relatively few successful examples of hit identification, and the development of hits into potential drugs seems particularly challenging. At this stage it is not clear whether any general strategies towards developing inhibitors of these interactions exist.

PPIs with an anchoring residue

Bromodomains and other epigenetic readers.

Epigenetics, the study of heritable changes not caused by alterations to the underlying DNA sequence, is currently an area of intense research[159]. A key facet of epigenetic regulation is the acetylation of lysine residues or methylation of lysines and arginines. Such residues can be 'read' by using a structurally-distinct form of PPI. These 'readers' feature classes of structurally-related protein domains such as the bromodomains, which bind to acetylated lysine residues. Related reading domains that recognize other sequences include tudor domains, chromodomains, MBT domains, PWWP domains and PHD domains[159].

These PPIs represent a very different kind of target and a number of recent excellent reviews have documented this rapidly emerging area[159, 160]. We limit ourselves to a number of comments. Firstly, the anchor residue provides a very specific focus for drug discovery efforts. Secondly, the domains bind to their modified histone partners with varying degrees of sequence specificity and often very weakly - 10-100 μM is not uncommon in the case of bromodomains. Finally, multiple related domains have been identified, for example there are proposed to be 61 bromodomains[161]. Thus drug discovery in this area bears some similarity to the development of kinase inhibitors in that the protein targets have a common binding motif surrounded by sub-pockets that may confer the potential for selectivity. In a study by Filippakopoulos *et al.,* a systematic screen of 33 bromodomains against an array of singly acetylated lysines in histone peptides revealed a variety of specificities.[161] Whilst some acetylation marks were almost ubiquitously recognized by all the bromodomains studied, other lysine acetylations were bound specifically by only a small number of bromodomains,

indicating a recognition event influenced by the sequence of nearby histone-peptide residues. In particular, the effect of neighbouring trimethylated lysine, acetylated lysine, phospho-threonine and phospho-serine was also studied which highlighted the importance of post-translational modification of adjacent residues on the anchor-residue recognition process. Similarly, some bromodomains were found to bind selectively to only a small number of acetyl lysines whilst others are more promiscuous. This clearly has consequences for inhibitor design and reported compounds have varying selectivity; inhibitors can show reasonable selectivity between families but reduced selectivity between closely related bromodomains.[162] A number of bromodomain inhibitors have entered the clinic (table 2), exemplified by compound **9 (I-BET762, GSK525762**) (Figure 7).[163]

Discussion

Epigenetic reader domains are not unique in employing a modified anchor residue, (table 1) for example the interaction between farnesylated KRAS and PDEδ has recently been the subject of successful small molecule inhibitor development[164]. SH2 domains bind to short peptide units containing a phosphorylated tyrosine. Efforts at discovery of small molecules blocking the interaction of SH2 domains with their client proteins have had some success but in this case the strong negative charge of the anchoring group and the peptidic nature of the scaffolds has resulted in small molecule mimics with undesirable physicochemical properties[27]. Overall, two main strategies have been employed to identify hits against PPIs featuring anchor residues: fragment-based drug discovery and peptide mimetics. FBDD seems ideally suited to identifying fragment hits binding into the 'anchor-pocket' – this has proved particularly useful against the bromodomains. Where the anchoring residue forms part of a short continuous epitope, for example in the SH2 domains, leads with obvious peptidic elements are common.

Outlook

Over the past 20 years there has been a clear increase in research efforts towards the development of PPI inhibitors. This has led to some notable successes using an array of different strategies and to the first PPI inhibitors entering the clinic. It is clear from this work that careful structural analysis of a PPI allows an assessment of both druggability and of the most appropriate screening and design approaches. The success stories to date are characterised by a willingness to adopt new approaches such as fragment-based discovery, tethering, NMR and biophysical screening and novel design strategies. It is notable that relatively few targets have progressed as far as the clinic and that a number of the clinical candidates fall outside normal drug-like space suggesting that the demands of optimising initial leads to development compounds are not trivial.

Over the coming decade, PPIs have the potential to enter into the drug discovery mainstream. A greater engagement from groups in large pharmaceutical organisations with the capacity to drive the later stages of lead-optimisation for these demanding targets should help to bring a wider range of targets to the clinic. This will require a shift away from a 'one-size fits all' R&D mind-set that has the potential to reject targets that do not fall into a narrow definition of tractable. The recent move within industry to smaller, more innovative, nimble research units should encourage the risk taking and innovation required to make this happen. Moreover, the increasing focus that academia is placing on drug discovery research should allow a broader range of drug discovery targets to be explored.

In both academia and industry an increased focus on PPI targets will require a strong commitment to structural biology and biophysics. From a medicinal chemistry perspective, whilst we recognise that ideally the properties of development compounds should meet Lipinski-like criteria this will likely prove incompatible with developing sufficiently potent inhibitors for some PPIs. In these cases improving our ability to discover developable compounds outside rule of 5 chemical space is a key challenge for medicinal and computational chemists.

Thus far, in common with many cutting edge areas of research it has been oncology that has benefited most from pioneering research efforts. In the future

we expect to see an increased focus on other important disease areas, although the challenges associated with some therapeutic areas – most notably CNS diseases, given the likely physicochemical properties of PPI inhibitors - are likely to be formidable.

Overall, if the drug discovery community is able to step up to the challenge posed by PPIs there are tremendous opportunities to develop new approaches to the treatment of disease in this diverse and fascinating target class.

Acknowledgements

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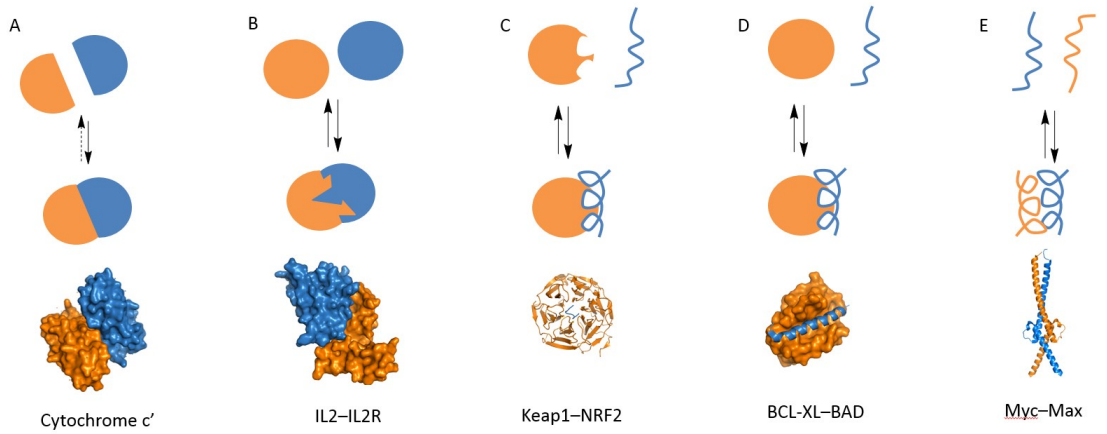
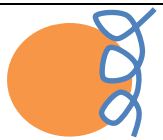
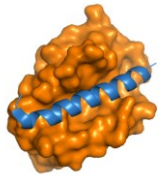
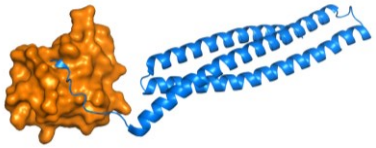


Figure 1. Structural classification of PPIs with cartoons representing the protein partners and examples in which globular peptides are represented by surfaces or cartoons and peptides by cartoons: (A) Interaction between two globular proteins with preformed surfaces. (B) Interaction between two globular proteins with an induced binding surface. (C) Interaction of a rigid globular protein with a peptide. (D) Interaction of a flexible globular protein with a peptide. (E) Interaction of two peptides.

Table 1. Further classification of globular-peptide and peptide-peptide PPIs with examples highlighted in bold and illustrated in surface and cartoon form.

PPI Class	Description		Examples (target–displaced)	Example Structure
Globular–helical peptide, discontinuous epitope.	Helix with a discontinuous epitope binding into a groove.		MDM2–p53 **BCL-XL–BAD/BAK** ZipA–FtsZ S100B–p53 β-catenin–Tcf3–Tcf4 McL–BH3 Sur-2–ESX	
Globular–Peptide, continuous epitope	Continuous epitope on β-sheet/strand and loops binding into surface with pockets.		**XIAP–SMAC** HIV integrase–LEDGF Integrins RAD51–BRCA2 PDZ domains NRP1–VEGF-A Menin–MLL	

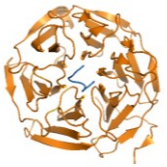
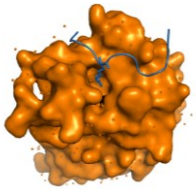
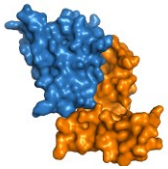
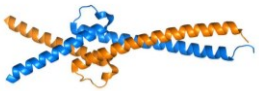
	Binding into pocket in a β-propeller		**Keap1–Nrf2** WDR5–MLL	
Globular-peptide, anchor residue.	Peptide with anchor residue due to post-translational modification binding into a pocket.		**Bromodomains** PDEδ–KRAS SH2 domains PLK1 PBD–peptide VHL–HIF1α	
Globular–Globular, discontinuous epitope	Two proteins both presenting discontinuous epitopes		**IL2–IL2R** TNFα–TNFα E2–E1	
Peptide-Peptide	A pair of helices with an elongated binding interaction.		**Myc–Max** NEMO–IKK Annexin II–P11 (S100A10)	

Table 2 – PPI inhibitors known to the authors to have reached clinical development.

Name[a]	Developer[b]	Target	Therapeutic Area	Phase[c]
Navitoclax/ABT-263 (**1**)	AbbVie	BCl2 family	Cancer	Phase 2
Venetoclax/ABT-199 (**2**)	AbbVie	BCl2 family	Cancer	Phase 3
Obatoclax (CEP-41601, GX-015-070)	Teva (Cephalon)	BCl2 family	Cancer	Phase 3
RG7112, RO5045337	Roche	MDM2-p53	Cancer	Phase 1
RO5503781	Roche	MDM2-p53	Cancer	Phase 1
AMG 232	Amgen	MDM2-p53	Cancer	Phase 2
CGM097	Novartis	MDM2-p53	Cancer	Phase 1
DS3032b	Daiichi Sankyo	MDM2-p53	Cancer	Phase 1
SAR405838	Sanofi/University Michigan	MDM2-p53	Cancer	Phase 1
MK-8242	Merck	MDM2-p53	Cancer	Phase 1
JNJ-26854165	J&J	MDM2-p53	Cancer	Phase 1
Lifitegrast (SAR1118)	Shire / SARcode Bioscience	LFA-1/ICAM-1	Dry Eye	Phase 3
Tirofiban	Merck	αIIbβ3	Cardiovascular	Approved
Orbofiban	Searle	αIIbβ3	Cardiovascular	Phase 3[149]
Xemilofiban	Searle	αIIbβ3	Cardiovascular	Phase 3[149]
Sibrafiban	Roche	αIIbβ3	Cardiovascular	Phase 3[149]
BMS-587101	Bristol-Myers-Squibb	αLβ2	Psoriasis	Phase 2
BIRT 2584	Boehringer Ingelheim	αLβ2	Psoriasis	Phase 2
Valategrast	Roche	α4β1	Asthma	Phase 2[149]
IVL745	Sanofi-Aventis	α4β1	Asthma	Phase 2[149]
Firategrast (SB-683699)	GlaxoSmithKline	α4β1	Multiple Sclerosis	Phase 2
AJM300	Ajinomoto	α4β1	Ulcerative colitis	Phase 2[149]
JSM6427	Jerini	α5β1	Macular degeneration	Phase 1
AT-406	Ascenta Therapeutics	IAP	Cancer	Phase 1
GDC-0152	Roche / Genentech	IAP	Cancer	Phase 1
Birinapant (TL32711)	Tetralogics Pharma	IAP	Cancer	Phase 2
LCL-161	Novartis	IAP	Cancer	Phase 2
AEG40826/HGS1029	Aegera/HGS	IAP	Cancer	Phase 1
GDC-0917	Roche / Genentech	IAP	Cancer	Phase 1
GSK525762	GSK	Bromodomain family	Cancer	Phase 1[d]
CPI-0610	Constellation Pharmaceuticals	Bromodomain family	Cancer	Phase 1
Ten-010	Tensha Therapeutics	Bromodomain family	Cancer	Phase 1
OTX015	Oncoethix	Bromodomain family	Cancer	Phase 1

RVX-208	Resverlogix	Bromodomain family	Cardiovascular	Phase 2

a. Names or registration numbers taken from www.clinicaltrials.gov.

b. Key organisations involved in the development listed.

c. Latest phase corresponds to that registered on www.clinicaltrials.gov unless alternative reference is provided. In some cases the phase may correspond to a trial which has not yet started or may have been withdrawn prior to dosing.4

d. www.gsk.com

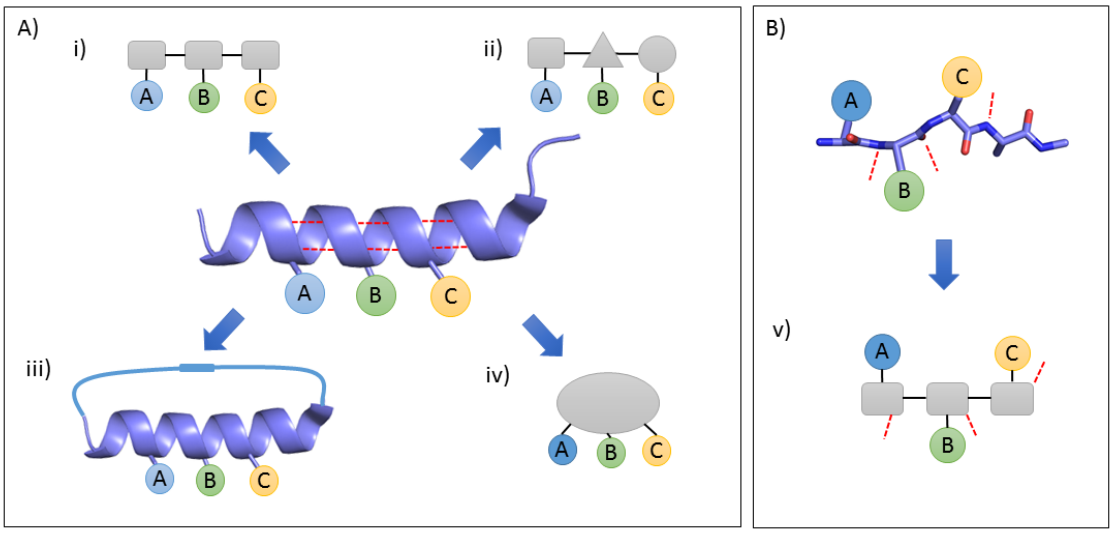


Figure 2: Strategies for developing peptidomimetics. (A) Mimicry of three hotspot residues of an α-helix can be achieved with (i) a linear oligomer with identical repeating units (ii) a linear molecule assembled from different units (iii) a "stapled" peptide and (iv) attachment of groups to a central scaffold. Peptide backbone hydrogen bonds are indicated by red dashed lines. (B) Mimicking the residues on a continuous epitope can be achieved with (v) a linear oligomer, which commonly must also make hydrogen bonds to the surface (red dashed lines).

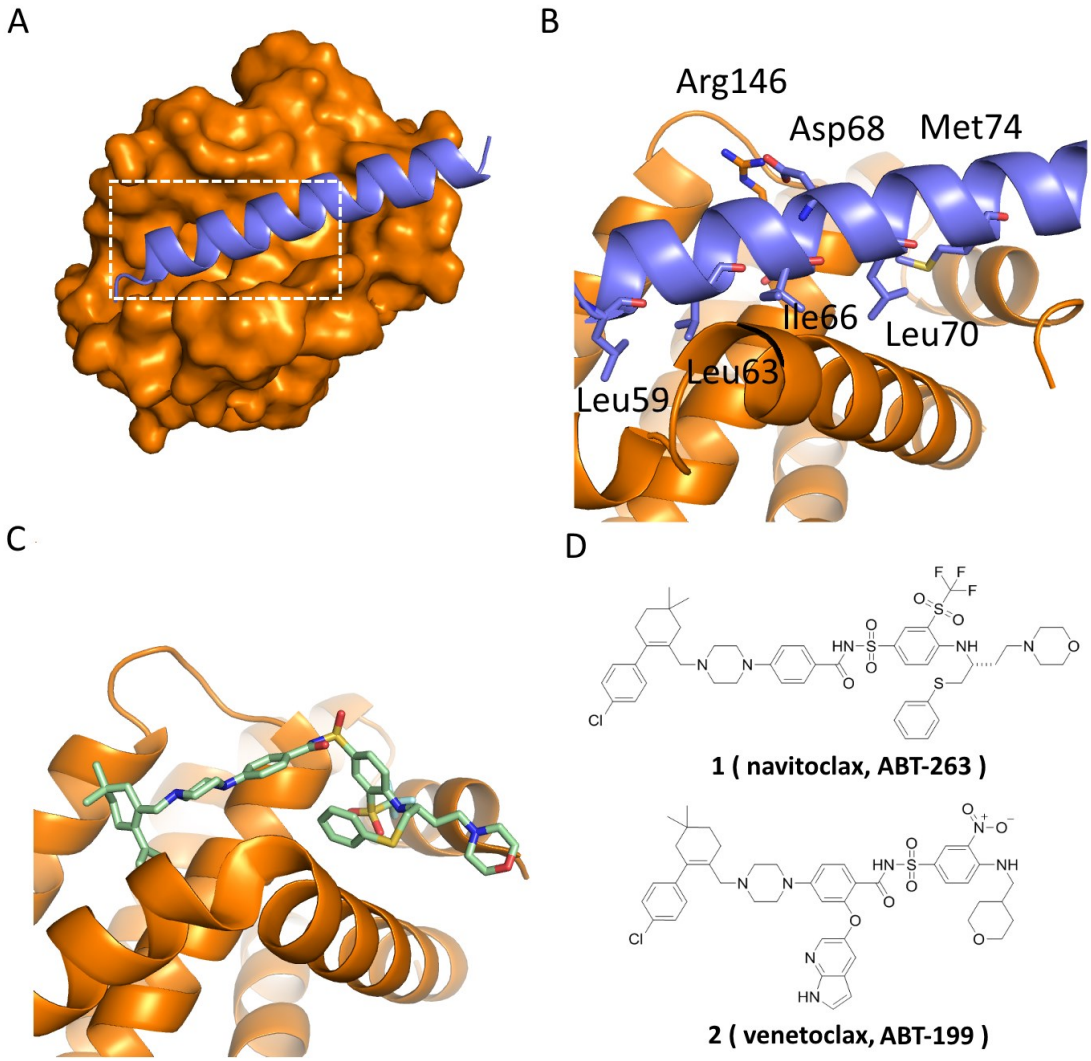


Figure 3: (A) X-ray crystal structure of Bax BH3 peptide (blue cartoon) bound to Bcl-2 (orange surface) pdb code:2xa0. (B) Detail of this interaction with Bcl-2 shown as orange cartoon and key residues as sticks. Structural investigation and binding analysis of pro-apoptotic BH3 domains has revealed the importance of 4 conserved hydrophobic residues (Leu59, Leu63, Ile66, Leu70) in binding to their protein partners [105, 165]. In addition the interaction of Bax with Bcl-2 displays a significant hydrophobic interaction with Met74[165]. Significant electrostatic interactions are also observed, with Asp68 from Bax forming a salt bridge with Arg146 from Bcl-2[165]. (C) X-ray crystal structure of **1** (**navitoclax**, **ABT-263**) bound to Bcl-2 viewed from same orientation as B (pdb code:4lvt). Initial screening identified biaryl fragments that were found to bind in the location of the dominant hot-spot in the centre of the hydrophobic groove of Bcl-xL, occupied by Leu78 of Bak in the PPI. A second fragment screen was carried out in the presence of a hit biaryl-fragment leading to identification of fragments binding at a proximal site occupied by Ile85 of Bak in the PPI.

These two fragments were linked[1] and NMR solution structures were then utilised to guide optimisation of the resultant compounds. In *apo* structures the groove is not well defined, but binding the small molecules induces structural changes that create large hydrophobic pockets. These structural changes were not observed in complex with fragments but were induced when fragments were linked and elaborated.[166] (D) Chemical structure of compounds **1** (**navitoclax**, **ABT-263**) and **2** (**venetoclax**, **ABT-199**).

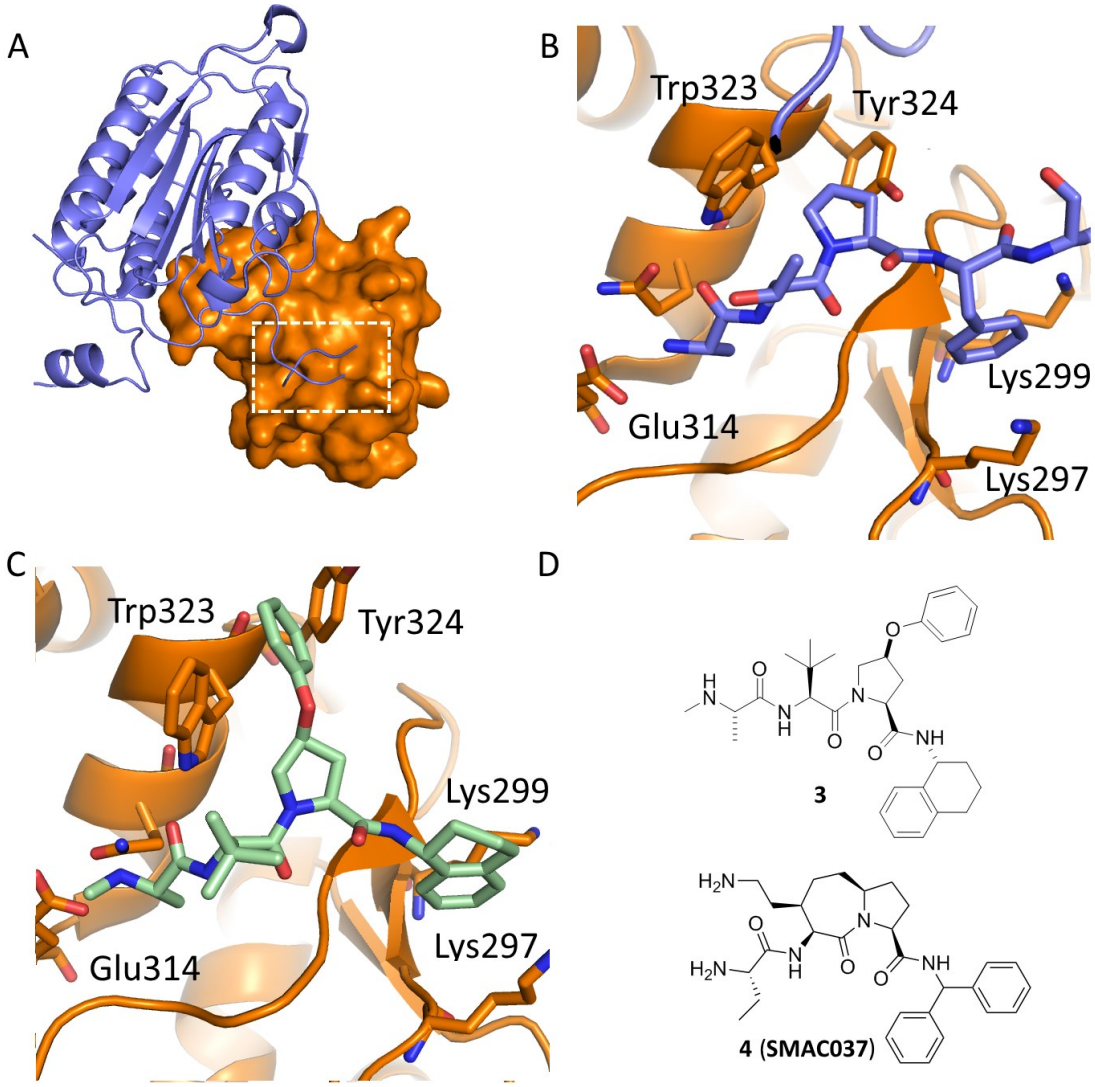


Figure 4: (A) Crystal structure of Caspase-9 (blue cartoon) bound to XIAP (orange surface) pdb code: 1nw9. (B) Detail of this interaction with XIAP is shown as orange cartoon and key residues on XIAP and Caspase-9 as sticks. The proline ring of Caspase-9 hydrophobically packs against the side chain of Trp323, whilst the phenylalanine is buried in a surface pocket of XIAP formed from the hydrophobic sidechains of Leu292, Lys297 and Lys299. The *N*-terminal alanine of Caspase-9 interacts with the sidechain of Trp310. Hydrophilic interactions are important too; the N-terminus forms a salt-bridge with Glu314, and the backbone of Caspase-9 forms hydrogen-bonding interactions with backbone and sidechains of XIAP. (C) X-ray structure of small molecule **3** bound to XIAP viewed from same orientation as B (pdb code: 1tft) [128] Fesik *et al.* have reported a series of compounds based upon the AVPFY penta-peptide, which binds XIAP with a K_D of 60 nM[128]. Through an iterative process of library screening and design, a series of capped tripeptide analogues were synthesised. The NMR structure of the BIR3 domain of XIAP bound to the AVPI peptide identified two

hydrophobic areas on the protein surface to grow towards; Trp323 and Tyr324 from the 4-position of the proline ring and from the *C*-terminus onto the hydrophobic side-chains of Lys297 and Lys299. Although there is some contact between the native peptides and these regions, they are more fully exploited with the small molecules, such as compound **3** (D) Chemical-structure of inhibitors **3**[128] and **4** (**SMAC037**).[130]

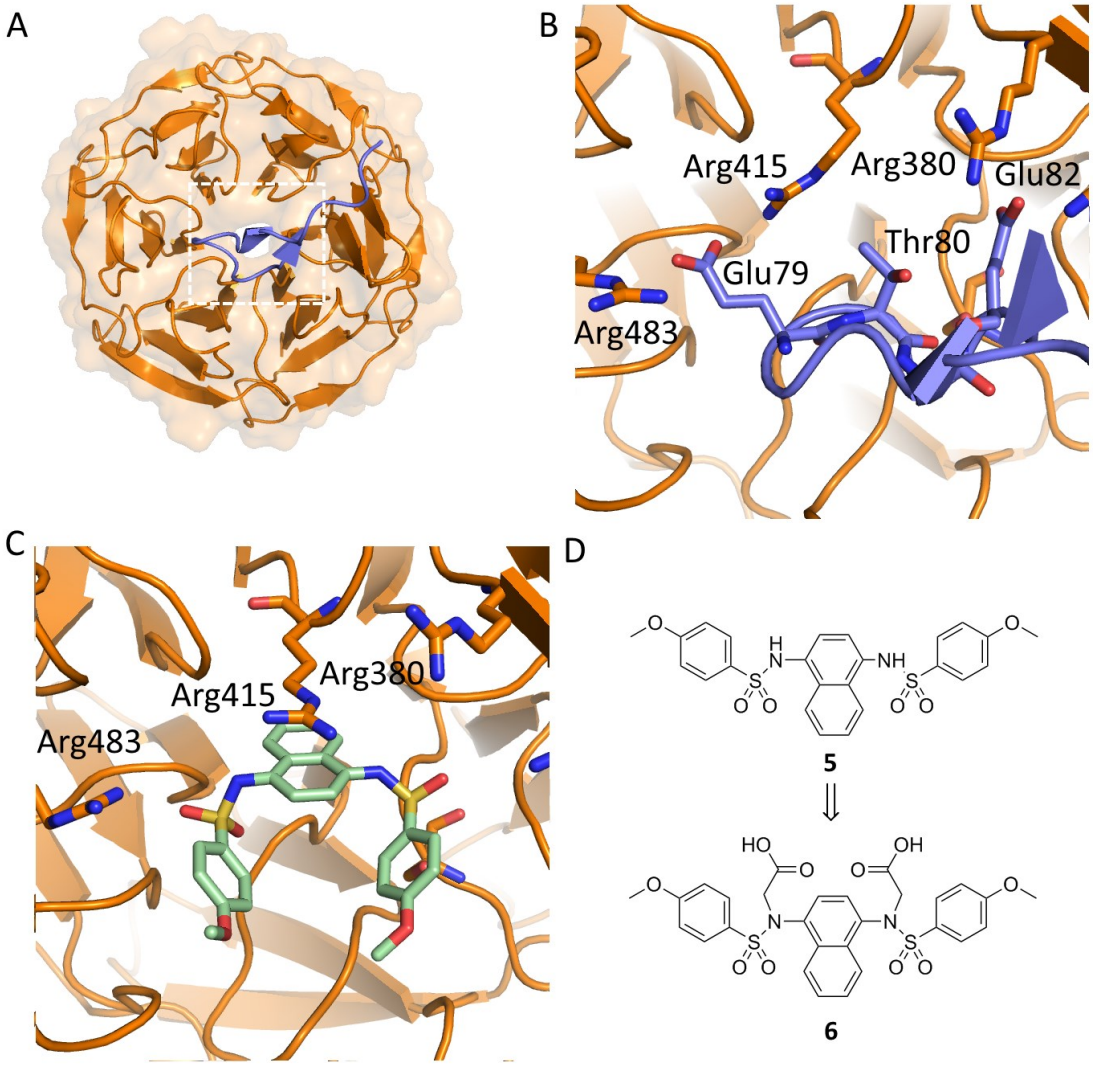


Figure 5: (A) Crystal structure of Nrf2 high affinity "ETGE" peptide (blue cartoon) bound to Keap1 (orange surface and cartoon) pdb code: 2flu. The propeller has a hydrophobic inner cavity containing a well-ordered water network and possessing a number of basic Arg residues. The peptide adopts a tight 4-residue β-hairpin, binding into the hole generated at the centre of the propeller; (B) Detail of same interaction in A with Keap1 shown as orange cartoon and important residues on Keap1 and Nrf2 as sticks, of particular note are key interactions between the two glutamic acid residues, at either end of the ETGE unit, and the Arg residues in the Keap1 binding site. The structure is consistent with results of alanine scanning experiments with the Keap1 protein[139]. (C) A screen of the Evotec Lead Discovery Library using a homogeneous confocal fluorescence anisotropy assay (two-dimensional fluorescence intensity distribution analysis, 2D-FIDA) identified 18 validated hits[144], most notably **5** shown as an X-ray structure bound to Keap1 viewed from same orientation as B (pdb code: 4iqk). Compound **5** makes four π-π stacking interactions, three with tyrosine

residues and the fourth with Arg415: the unsubstituted ring of the naphthalene is inserted deep into the polar pocket in order to make this latter interaction (D) Chemical structure of compound **5** and further developed inhibitor **6**.

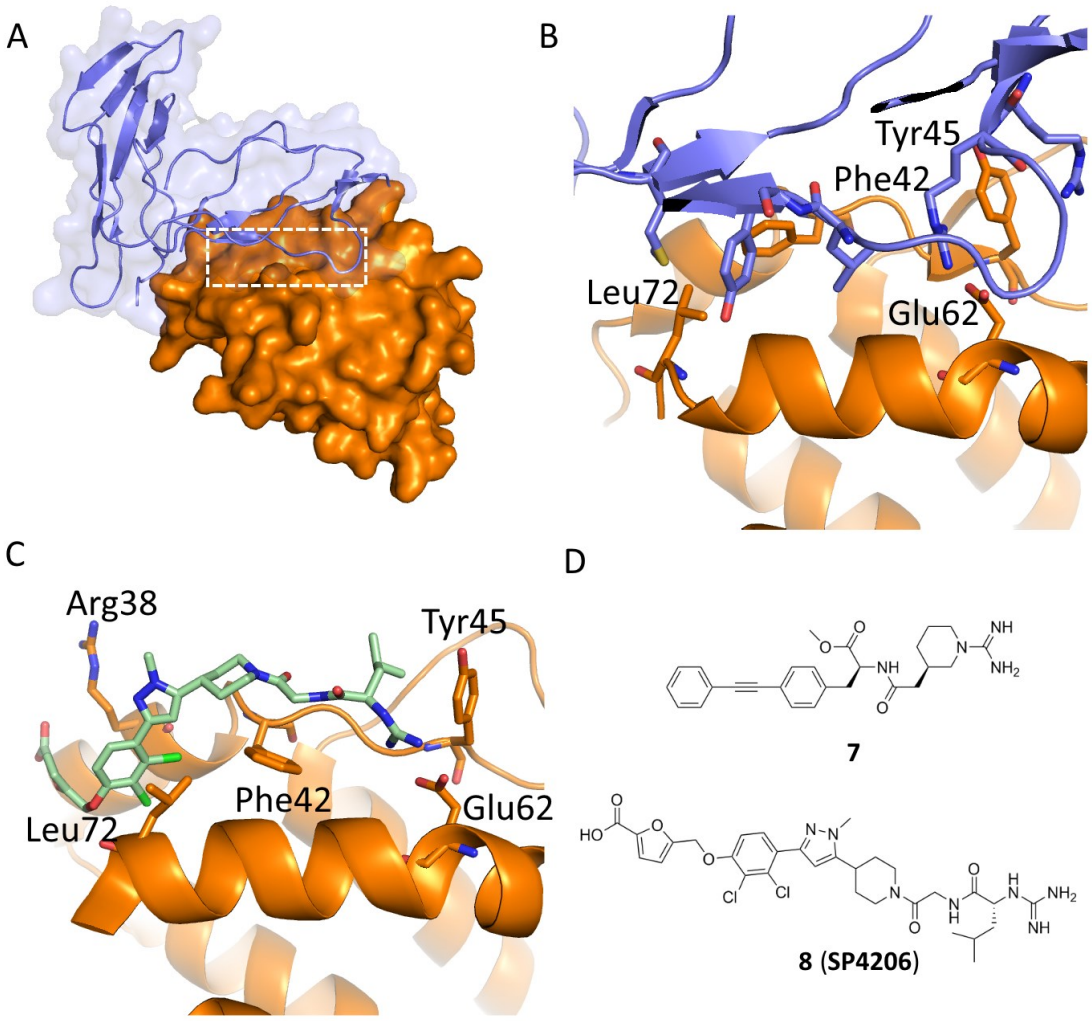


Figure 6: (A) Crystal structure of IL-2Rα (blue cartoon and transparent surface) bound to IL-2 (orange surface) pdb code: 1z92. (B) Detail of this interaction with IL-2 shown as orange cartoon and key residues on IL-2 and IL-2Rα as sticks. Labelled residues of IL-2 are indicated. Overall 20 IL-2 and 21 IL-2Rα residues interact to bury 1868 Å^2. In one patch, Tyr45 on IL-2 packs into a pocket formed by the methylenes of IL-2Rα Arg35 and Arg36. In a second patch Phe42 and Leu72 on IL-2 insert into a pocket on IL-2Rα assembled from Leu42, Tyr43 and Met25. This latter patch is surrounded by salt-bridges and hydrogen bonds and has been proposed to be the most important energetically[28]. The residues highlighted above were among those identified as important for binding from earlier mutation studies carried out on both IL-2Rα[167] and IL-2[168]. (C) X-ray structure of Sunesis compound **8** (**SP4206**) bound to IL-2 viewed from same orientation as B (pdb code: 1py2). Comparison of the binding of **8** (**SP4206**) and IL-2Rα by X-ray crystallography reveals conformational changes, with IL-2Rα covering approximately twice the area, making around three times as many heavy atom contacts, and being 2- to 4-fold less ligand efficient than **8** (**SP4206**)[169]. From an electrostatic

perspective, both IL-2Rα and **8** (**SP4206**) show a distinctive zwitterionic character. The small molecules showing a much smaller electrostatic field due to the presence of a much simplified network of charge-charge interactions. An experiment, in which the residues on IL-2 known to contact the ligand were individually mutated to alanine, demonstrated that the same IL-2 residues are most important for the affinity of **8** (**SP4206**) and IL-2Rα. (D) Chemical structure of Roche compound **7** and Sunesis compound **8** (**SP4206**).

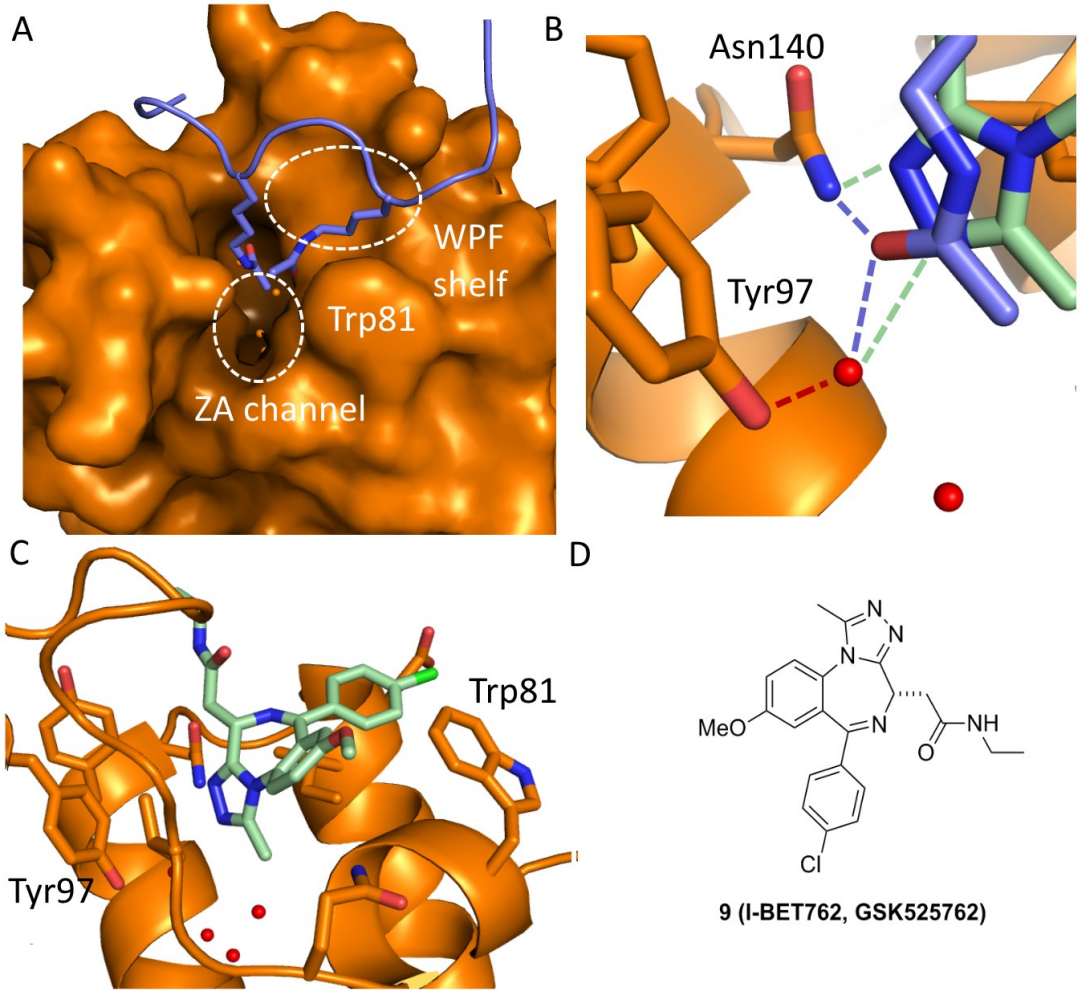


Figure 7: (A) Crystal structure of BRD4 bromodomain 1 bound to a diacetylated histone 4 peptide (pdb code: 3uvw). In addition to the acetyl lysine recognition site, two important structural features of the BET family of bromodomains are indicated; the WPF shelf and the ZA channel. In BRD4, the WPF shelf comprises three stacked amino acid side chains; Trp81, Pro82 and Phe83. The ZA channel is a narrow hydrophobic region that joins the Z and A helices. BET inhibitors typically interact with these hydrophobic areas to drive increased potency. (B) Detail of acetylated lysine of histone 4 interacting with BRD4 (pdb code: 3uvw), overlaid with compound **9** (**I-BET762**, **GSK525762** pale green carbons, pdb code: 3p5o), demonstrating molecular mimicry of the key interaction.[163] The acetyl lysine is recognised by a tunnel lined with hydrophobic side chains, including Leu94, Ile146, Tyr139 and Tyr97, with bound waters at the base. The acetyl group interacts with the side chain of Tyr97 via a water molecule and the side chain of Asn140. (C) Crystal structure of compound **9** (**I-BET762, GSK525762**) bound to BRD4 bromodomain 1. The side chain of Trp81 forms the edge of the binding pocket, packing against the *para*-chloro benzene group of **9** as part of

the WPF shelf. The para-methoxy benzene group is directed towards the ZA channel. (D) Chemical structure of inhibitor **9** (**I-BET762, GSK525762**).

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Glossary

Globular protein - literally a protein whose peptide chains are folded to form a broadly-spherical shape, often extended to mean a non-membrane-bound protein in which multiple regions of the peptide chain combine to give a defined tertiary structure with one or more binding or active sites.

Hot-spot – a region of a binding surface contributing a disproportionately large amount to the interaction-energy of a pair of proteins or a protein and a ligand.

Alanine-scanning – a set of experiments in which amino acids in a protein are sequentially mutated to alanine in order to estimate the contribution of the individual side chains to the binding energy of a protein-protein interaction.

Discontinuous epitope – a binding site in which amino acids that occur in different regions of the protein sequence combine to interact with the partner protein, these might be periodic side-chains on one face of a helix or residues from a group of neighbouring chains in a globular protein.

Intrinsically disordered protein/peptide – a protein or peptide lacking a fixed three-dimensional structure when in a monomeric state. Such species may adopt a more ordered structure when interacting with other proteins or ligands.

Box – Biophysical methods

Due to the low affinity of fragment hits for their protein targets structural or biophysical methods are often used to detect these interactions. The most important methods of detection include:

Thermal shift (TS)[54]– the stabilization associated with binding of a ligand to a protein leads to an increase in the unfolding-temperature of the protein that can be detected by heating the protein in the presence of a dye sensitive to protein-denaturation.

Surface plasmon resonance (SPR)[55]– A method which detects changes in the refractive index of a surface layer caused by binding events, enabling the measurement of ligand affinities and in some cases the determination of binding kinetics.

Ligand- or protein-based NMR[56]– ligand binding is detected through changes in the nuclear magnetic resonance signals of either the ligand or the protein respectively.

X-ray crystallography[57] is used to determine the structure of ligands bound to proteins; most commonly preformed crystals of the protein are soaked in a high-concentration solution of the ligand prior to data collection.

Isothermal titration calorimetry – the heat associated with the binding of a ligand to the protein is measured and used to determine both the dissociation constant and potentially the thermodynamics of binding.

Druggability – an assessment of the ease with which a drug can be developed to interact with a given protein target. In this case a relatively narrow meaning, namely the ease of generating a small molecule with reasonable affinity, is implied.

Pharmacophore – the features in a ligand responsible for its binding to a protein, reduced to an abstract representation lacking an underlying framework of bonds or rings.

Apoptosis – programmed cell death, controlled by a complex network of interactions including PPIs.

Ubiquitination – the attachment of a small protein, ubiquitin, onto another protein often as a prelude to controlled degradation of the labeled protein.

ADMET – Absorption, Distribution, Metabolism, Excretion and Toxicity - key properties of a potential drug depending in part on the physicochemical properties of a molecule.

PDZ domain – a protein domain containing a series of Gly-Leu-Gly-Phe repeating units. Named after the post-synaptic density-95 protein (PSD-95), the *Drosophilia* discs large tumour suppressor (Dlg-A), and the tight-junction associated protein Zonula occludens-1 (ZO-1).[148]

Author biographies

Chris Abell is the Professor of Biological Chemistry at the University of Cambridge. He leads a large multidisciplinary group interested in biological chemistry. His main research focus is on using fragment-based methods in drug discovery and chemical biology. Major interests include inhibiting enzymes in *Mycobacterium tuberculosis*, and developing modulators of protein-protein interactions. In 1999, he co-founded Astex, the world-leading company in fragment-based drug discovery. Chris also has research interests in developing microdroplets as a novel experimental platform (spin outs: Sphere Fluidics (2010) and Aqdot (2013)). In 2012 he was elected to the Academy of Medical Sciences.

John Skidmore received a B.A. from the University of Oxford where he stayed for his D.Phil supervised by Jo Peach. He then carried out a post doc at the University of Liverpool with Prof Stan Roberts. Between 2001 and 2010 John worked as a medicinal chemist and project leader at GlaxoSmithKline. In 2010 John moved to the University of Cambridge, where he led protein-protein interaction drug discovery projects funded by the Wellcome Trust. In 2015 John moved within the University, to his present position as the CSO of the Alzheimer's Research UK Cambridge Drug Discovery Institute

Duncan Scott is currently a Senior Post-Doctoral Research Associate at the University of Cambridge. He received an MSci in Chemistry in 2003 and subsequently gained a PhD investigating inhibitors of pantothenate synthetase in the laboratory of Professor Chris Abell in 2007. His interests lie in biophysics, medicinal chemistry and developing inhibitors against protein-protein interactions. Duncan is currently leading a medicinal chemistry team on a protein-protein interaction project, in a University-wide collaboration funded by the Wellcome Trust.

Andrew Bayly received his Ph.D in 2011 from Imperial College, London, where he worked towards the synthesis of alpha-helix mimetics targeting nuclear receptor-cofactor interactions under the supervision of Alan Spivey. He then received his postdoctoral training in the laboratory or Chris Abell in a team developing inhibitors of the RAD51-BRCA2 interaction before moving to Vertex Pharmaceuticals (Abingdon, UK) in 2013 where he has been working as a medicinal chemist.

Online summary

- Protein-protein interactions are increasingly being targeted by drug discovery groups and there exists great scope for therapeutic modulation of this target class in disease.
- The array of structurally interacting elements by which proteins interact with one another is wide and resists clear-cut classification. However broad divisions can be made by grouping interactions based upon the globular or peptidic nature of the proteins.
- Some strategies for developing inhibitors against a given PPI may have more traction against certain classes of PPIs than others; for example fragment based drug discovery has shown particular promise in targeting bromodomains and peptide mimetics in mimicking beta-strands.
- We examine case studies representative of the various structural-types of PPI and discuss lessons learnt from each.
- A summary of current status of inhibitors in clinical trials against different targets is presented.