SMALL-MOLECULE INHIBITORS OF PROTEIN–PROTEIN INTERACTIONS: PROGRESSING TOWARDS THE DREAM

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Protein–protein interactions have a key role in most biological processes, and offer attractive opportunities for therapeutic intervention. Developing small molecules that modulate protein–protein interactions is difficult, owing to issues such as the lack of well-defined binding pockets. Nevertheless, there has been important progress in this endeavour in recent years. Here, we use illustrative examples to discuss general strategies for addressing the challenges inherent in the discovery and characterization of small-molecule inhibitors of protein–protein interactions.

We dedicate this manuscript to our revered colleague and friend Andrew Braisted, Ph.D. (1964–2003).

Protein–protein interactions are central to most biological processes — from intercellular communication to programmed cell death — and therefore represent a large and important class of targets for human therapeutics. The current excitement surrounding therapeutic antibodies vividly demonstrates the value of such targets[1]. In the next year, US $5–7 billion will be spent on antibody-based antagonists, the fastest growing segment of the prescription-drug market[2,3]. As a compound class, therapeutic antibodies have some excellent properties: they are highly specific for their molecular targets and they tend to be very stable in human serum. On the other hand, antibodies suffer from difficulties in manufacture, high costs of goods and the lack of oral bioavailability. In addition, antibodies are not cell-permeable, and antagonism of intracellular protein–protein systems has so far been limited to antisense therapies, which block the expression of the targeted protein. For these reasons, protein– protein interactions have been of great interest to drug discovery; however, developing small-molecule antagonists has been difficult (for recent reviews see REFS 4–9).

Here, we review some of the issues and challenges associated with finding and characterizing small-molecule antagonists. First, we describe some of the general features of protein–protein interfaces. Next, we consider examples of the discovery of antagonists that bind directly to the 'hot spot' of a protein–protein interface, followed by examples of compounds that bind at allosteric sites distal from the protein–protein interface. In the final section, we discuss some of the general features of successful discovery programmes.

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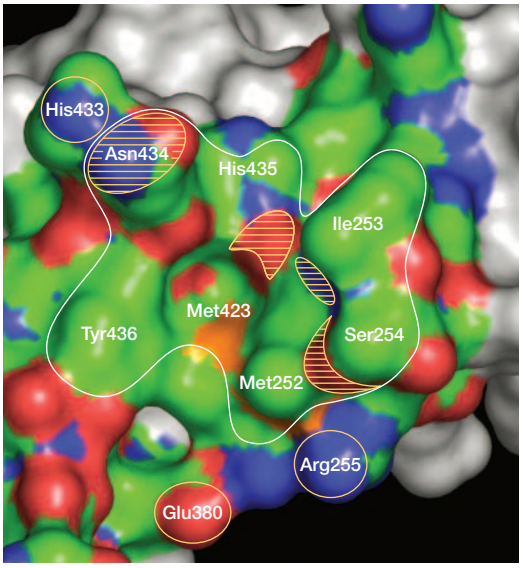
Protein–protein interfaces: the challenges

A number of factors can contribute to the challenge of identifying small, organic compounds that inhibit protein–protein interactions. These include the general lack of small-molecule starting points for drug design, the typical flatness of the interface, the difficulty of distinguishing real from artefactual binding, and the size and character of typical small-molecule libraries.

Natural small molecules known to bind at protein–protein interfaces are rare, whereas drug-friendly enzymes often have small-molecule substrates that can serve as templates for designing antagonists. One approach to getting a small-molecule starting point for a protein–protein interface has been to map the epitope of one of the proteins onto a small peptide or peptidomimetic. This approach has been especially successful for small, continuous peptide epitopes, such as for the integrins GPIIbIIIa, αvβ3 and α4β1 (REFS 10–19). Random screening occasionally identifies compounds that bind and even recruit protein–protein complexes. For example,

Box 1 | **Hot spots at protein–protein interfaces**



By combining X-ray crystallography with site-directed mutagenesis, researchers have analysed how protein–protein interfaces function[22,150,151]. Scanning mutagenesis methods, in which subsets of the protein surface are systematically mutated, indicate that many protein–protein interfaces contain compact, centralized regions of residues that are crucial for the affinity of the interaction[23,24,152]. These regions, called 'hot spots', tend to be found on both sides of the protein–protein interface. In crystal structures, hot spots look highly complementary to each other, with buried charged residues forming salt bridges and hydrophobic residues from one surface fitting into small nooks on the opposite face.

One of the interesting features of hot spots is their functional and structural adaptivity[28,30,31,153]. Many proteins function by binding to multiple partners. An important point is that these proteins tend to use the same hot spot, which adapts to present the same residues in different structural contexts. Structural changes are observed in side-chain rotations and in the protein backbone, usually in loops and in the hinge regions between domains. These observations have been most thoroughly characterized for the Fc domain of immunoglobulin (IgG)[28]. Fc has been co-crystallized with three protein ligands and one phage-optimized peptide, and these X-ray structures indicate a common hot spot for the four ligands. The figure shows the structure of Fc in surface representation, with the binding site coloured by atom type and the consensus binding site circled. The central hydrophobic site is circled in white, with hydrogen-bonding interactions shown by hashed lines and salt bridges circled in yellow. Statistical analysis suggests that this spot is more highly accessible, hydrophobic and flexible than most of the protein surface. To summarize data from numerous studies, hot spots seem to be special sites on a protein surface, and might be inherently good for binding ligands.

the vinca alkaloids were discovered as cytotoxic compounds, and were later found to affect the polymerization of tubulin[20]. Cyclosporins cause the formation of novel protein–protein complexes[21]; drugs themselves can therefore provide starting points for further small-molecule design on these targets. However, these are rare examples, and a general strategy for tackling protein interfaces is still being sought.

The shape of a typical protein–protein interface adds to the difficulty of drug discovery. Approximately 750–1,500 Å^2 of surface area is buried on each side of the interface[22], and X-ray structures of protein–protein pairs do not usually show small, deep cavities that look like small-molecule-binding sites. However, it might not be necessary for a small molecule to cover the entire protein-binding surface, because the subset of the interface that contributes to high-affinity binding (the 'hot spot') is often much smaller (BOX 1)[23–26]. In addition, for many protein–protein interactions, the apparent complementarity between the two surfaces involves a significant degree of protein flexibility and adaptivity[27,28]. Therefore there might be binding-site conformations that are well-suited to small-molecule binding yet are not visible in a single crystal structure[29–31]. Several studies have reported phage-display selection of small peptides that bind to protein hormones or receptors[32–40]. Strikingly, these randomly selected peptides usually bind at the protein hot spot, even though they were not selected for protein–protein inhibition. These results suggest that hot spots at a protein interface are particularly adept at binding to proteins, peptides and perhaps even small molecules.

Another problem with discovering drug-like small molecules to protein–protein targets is characterizing the stoichiometry and site of binding. Shoichet and co-workers have described a surprisingly common inhibitory mechanism that arises when hydrophobic or amphipathic small molecules form large aggregates, micelles or liposomes[41–43]. Additionally, some compounds act as protein denaturants or covalent inhibitors[44–46]. In these cases, the compounds might inhibit the function of a number of proteins without binding to a discrete site.

Although artefacts might arise for any protein target, they are more difficult to exclude when the target is a protein–protein interaction. These systems are usually screened using an inhibition assay, in which the ratio of compound/protein is very large. In addition, whereas enzyme inhibitors can be characterized for competitive versus ALLOSTERIC binding mechanisms using enzyme kinetics, such experiments are difficult to envision for protein–protein systems. It can even be complex to determine which of the two protein partners binds to the inhibitor, although fitting algorithms are available[47]. The overall problem is that a plurality of mechanisms might account for the observed inhibition.

Structural and biophysical methods can clarify the binding mechanism. Boehm *et al.* have demonstrated an efficient use of biophysical methods to characterize the mechanism of inhibitors of DNA gyrase[48]. Possible inhibitors of this enzyme were identified by *in silico* screening of the ATP-binding site; 'active' compounds were then screened in a second enzyme assay and analysed qualitatively by methods that address the

ALLOSTERIC PROTEIN
A protein containing two or more topologically distinct binding sites that interact functionally with each other.

binding of the compound to protein. These binding methods included analytical ultracentrifugation (AUC), SURFACE PLASMON RESONANCE (SPR), nuclear magnetic resonance (NMR) and X-ray crystallography. Following this battery of tests, seven out of fourteen classes of initial hits were determined to be non-drug-like inhibitors. The same methods have been used qualitatively and quantitatively to define the binding mechanism for protein–protein inhibitors. Additionally, a number of biological experiments, including antibody inhibition and site-directed mutagenesis, have been used to determine whether a series of small-molecule inhibitors of a protein–protein interaction show promise as leads.

Historically, drugs have been highly biased towards a small number of protein classes. It is possible that protein–protein interactions will become more tractable when larger libraries and different types of compounds are tested. This general notion has led to research focused on non-high-throughput-screening (non-HTS) approaches to drug discovery, including structure-based design, *in silico* screening, and fragment-based discovery. Each of these approaches, including HTS, has been used to identify initial small-molecule antagonists of protein–protein interactions, and these methods have been recently reviewed and evaluated[4,8,9]. The examples described below focus instead on how compounds are characterized, evaluated and developed into drugs and drug leads.

Competitive antagonists: IL-2 receptor

The cytokine interleukin-2 (IL-2) is a principal mediator of the T-helper immune response. Binding of IL-2 to its trimeric receptor (IL-2R), which contains α, β and γ chains, causes proliferation and differentiation of activated cells[49]. Clinical data have demonstrated the importance of IL-2 and IL-2Rα in mediating immune disorders; two anti-IL-2Rα antibodies have been approved for use in transplant rejection[50,51], and clinical trials for autoimmune diseases are ongoing. The structure of IL-2 is a four-helix bundle. The hot spot for IL-2Rα has been mapped onto the surface of IL-2 by site-directed mutagenesis[52,53] (C. Thanos, J. Hyde, J.A.W., personal communication) and NMR[54] (FIG. 1a). This binding site has an amphipathic character; one side (to the right in FIG. 1a) is largely hydrophilic and acidic, whereas the other side of the site (to the left in FIG. 1a) is hydrophobic and basic. Recent work indicates that the IL-2Rα binding surface binds to small molecules that complement this amphipathic structure[54–59].

One of the first examples of a small-molecule inhibitor of a cytokine–receptor interaction is Ro26-4550 (REF. 55), shown in FIG. 2. This compound was designed as a peptidomimetic of IL-2 and therefore was expected to bind to IL-2Rα. However, careful enzyme-linked immunosorbent assays (ELISA) and ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) NMR experiments (BOX 2) demonstrated that the compound bound to IL-2 itself, at the IL-2Rα-binding site. Ro26-4550, which has moderate affinity (IC_{50} = 3–6 μM) for IL-2, was not advanced further, perhaps because it lacked cell-based activity[59]. Nevertheless, this molecule was the first biophysically characterized inhibitor of a cytokine–receptor interaction and clearly indicated that a small molecule could bind at a protein hot spot and inhibit hormone-receptor binding.

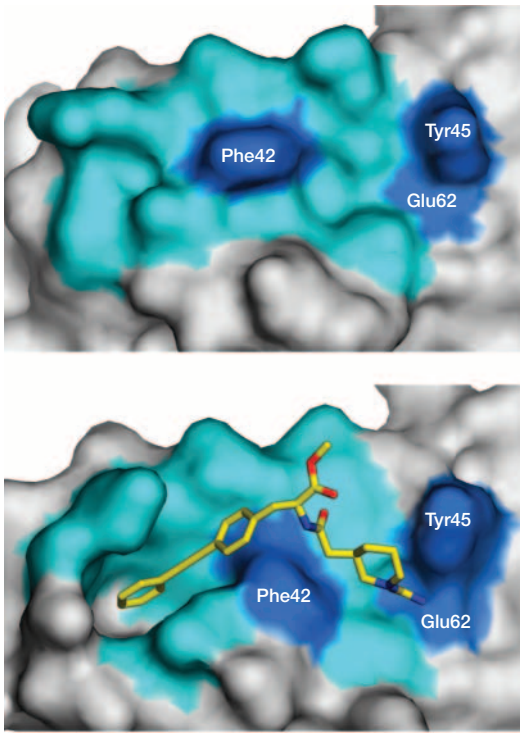


Figure 1 | **Structures of unliganded IL-2, and Ro26-4550 bound to IL-2. a** | Unliganded interleukin-2 (IL-2)[60]. **b** | Ro26-4550 bound to IL-2 (REF. 56). Hot-spot residues for binding to IL-2Rα are shown in light blue (moderately important) and dark blue (very important), as determined by site-directed mutagenesis[52,53]. Ro26-4550 is seen to bind at the same hot spot (**b**), which seems to be highly complementary to the Ro26-4550 structure. On binding of Ro26-4550 and other small-molecule inhibitors, the IL-2 binding surface undergoes a conformational change in the hydrophobic portion of the site (to the left of the structures), while remaining relatively fixed in the guanidine-binding portion of the site (to the right of the structures). The structural mobility of the hydrophobic site has been observed in several structures of IL-2 (REFS 56,58,62).

IL-2: structural evidence for binding-site adaptivity. FIGURE 1 compares the X-ray crystal structures of Ro26-4550 bound to IL-2 and unliganded IL-2 (REFS 56,60). The functionality on Ro26-4550 is complementary to the IL-2Rα hot spot; the positively charged guanidine makes two hydrogen bonds with glutamate 62, whereas the hydrophobic biaryl acetylene moiety fits into a groove on the hydrophobic side of the binding site. Importantly, the Ro26-4550-bound structure shows striking changes in the protein conformation in the hydrophobic region, as no groove is apparent in the unliganded structure of IL-2.

Structural and thermodynamic studies indicate that this portion of the protein is inherently flexible; NMR studies of IL-2 indicate that two loops adjacent to this region are somewhat disordered in solution[61]; several X-ray crystal structures of IL-2 further support this

SURFACE PLASMON RESONANCE
A method for measuring binding interactions between a surface-immobilized molecule and a solution-phase analyte. The technology measures changes in refractive index caused by a change in mass at the surface.

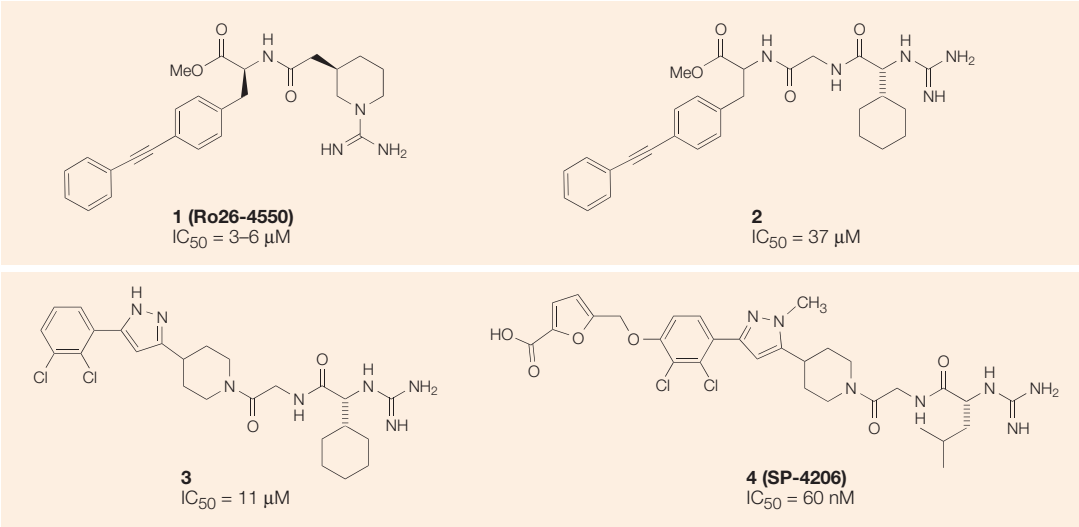
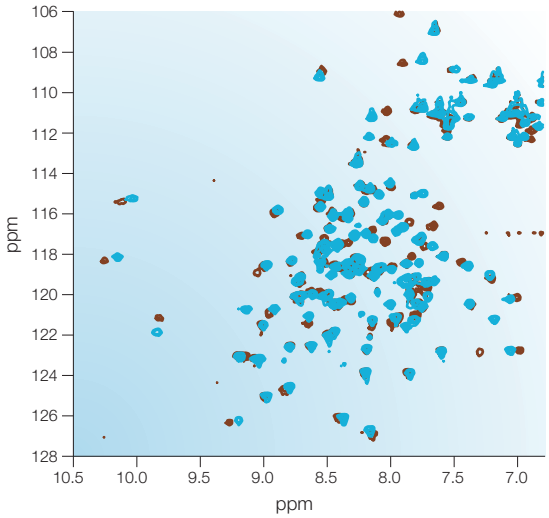


Figure 2 | **Small-molecule inhibitors of interleukin-2.** Compound 1 (Ro 26-4550) is the first well-characterized small-molecule inhibitor of a cytokine[54,55]. It also served as the starting point for the fragment-based synthesis of compounds 2–4 (REFS 57,59).

notion, because the structures vary significantly in the hydrophobic portion of the binding site and the adjacent loops[56,58,60,62]. Finally, thermodynamic measurements of the Ro26-4550–IL-2 interaction indicate that binding is enthalpically driven, with only moderately unfavourable entropy[56]. So, the structural and thermodynamic experiments indicate that the conformational freedom of IL-2 is reduced by ligand binding, but that this energetic penalty is not large. Binding probably 'captures' a low-energy conformation rather than 'inducing' a high-energy one. The high degree of adaptivity in the hydrophobic portion of the binding site allows for the creation of pockets and grooves in which small molecules can bind.

IL-2: fragment-based discovery of antagonists. A fragment-based approach was used to develop new compounds from Ro26-4550 (BOX 3)[57,59]. The process was devised in four steps. First, Ro26-4550 was divided into its component fragments, a biaryl acetylene amino acid and a piperidyl guanidine. The aromatic fragment was a weak inhibitor (IC_{50} = 2 mM) and bound to IL-2 as monitored by SPR; the guanidine moiety, by contrast, showed no binding or inhibition up to 10 mM. Previous studies have shown that weakly binding compounds can be productively utilized as chemical starting points, provided that something is known about their binding mechanism and, preferably, their binding location. So,

Box 2 | ^{1}H-^{15}N HSQC NMR



The ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) nuclear magnetic resonance (NMR) experiment has been used to characterize the binding sites of small molecules in many protein–protein systems (see main text). The method uses ^{15}N-labelled protein and measures the NMR resonance, or 'cross peak,' associated with the N-H bonds in the protein backbone. Each amino acid has a single resonance in the HSQC spectrum, except for asparagine and glutamine, which have two, and proline, which has no N-H bond. Because these N-H cross peaks are highly sensitive to perturbations in the nearby environment, they are good reporters of changes in local conformation and solvent exposure — the types of environmental effects caused by ligand binding.

The figure shows an example of an ^{1}H-^{15}N HSQC spectrum of ^{15}N-labelled interleukin-2 (IL-2) in the absence (blue) and presence (red) of a small-molecule inhibitor of IL-2 (compound 1, FIG. 2). The two-dimensional spectra are plotted with the ^{1}H chemical shift on the *x*-axis and the ^{15}N chemical shift on the *y*-axis. Some cross peaks are significantly shifted in the presence of the small molecule, and these correspond to amino acids that are located at the IL-2Rα binding site identified by mutagenesis and NMR[52–54,59].

in the second step, the aromatic amino acid was used as the basis fragment to evolve a new linker and guanidine moiety (FIG. 2, compound 2). In the third step, the optimized linker–guanidine fragment formed the basis for a small compound library from which a novel replacement for the aromatic group was found (compound 3).

At each of these three stages, compounds were characterized by a primary inhibition assay, a second inhibition assay (to test for assay-dependent artefacts) and by a panel of biophysical methods (to ensure stoichiometric binding at the IL-2Rα hot-spot)[59]. TABLE 1 compares the data from the biophysical methods for a representative set of compounds. SPR and AUC measurements (BOX 4) were done at multiple compound/protein ratios, allowing calculation of the dissociation constant K_d. ^{1}H-^{15}N HSQC measurements (BOX 2) demonstrated that compounds bound in approximately the same site as Ro26-4550, and the degree of signal

Box 3 | Fragment-discovery approaches

'Fragment assembly' has recently been proposed as a method for probing a large chemical space while synthesizing a minimal number of compounds[48,154,155]. Generally speaking, fragments are organic compounds, typically less than 200 Da in mass, that are screened for binding to a protein target. Active fragments are then linked or otherwise optimized to generate a small set of drug-sized molecules that are then screened for improved function. Relative to traditional library approaches, compounds derived from fragment discovery should be highly enriched for binding to the target. Additionally, it has been argued that fragments are more 'lead-like' than traditional screening libraries, in that they are smaller and less hydrophobic, therefore allowing more room to evolve 'drug-like' characteristics as the potency is improved[156,157]. However, because fragments are small, they tend to bind weakly, making discovery and characterization of weak hits a challenge. Approaches that have been successful at identifying and optimizing fragments include nuclear magnetic resonance (NMR)[87,158–161], particularly a method termed structure–activity relationships (SAR) by NMR[154,155,162], X-ray crystallography[163,164] and Tethering (the term Tethering is a service mark of Sunesis Pharmaceuticals Inc. for its fragment-based drug discovery)[165,166].

SAR by NMR uses ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) NMR (BOX 2) as a screening tool for identifying fragments that bind to a site of interest on a protein[160]. A combination of structure-based design and SAR is then used to link or merge the fragments. SAR by NMR has also been used to optimize the physico-chemical properties of an allosteric inhibitor of the I-domain of leukocyte-function-associated antigen-1 (LFA1) (REF. 110) (**a**). Compound 24 inhibited LFA1 function with roughly 100 nM potency, but showed poor solubility and pharmacokinetic properties. HSQC screening was done in the presence of compound 25, a minimized version of compound 24, and several fragments were found to bind to compound-25–I-domain complex with K_d values in the millimolar range. Linking these fragments to compound 25 gave compound 26, which had improved potency and drug-like properties.

The Tethering method of fragment discovery uses disulphide-bond formation to capture fragments that bind to a site of interest[155] (**b**). A cysteine mutation is placed near the small-molecule-binding site, and this mutant protein is interrogated with a mixture of disulphide-containing fragments. At equilibrium, those fragments that bind to the target and form a disulphide will be highly bound to the protein; mass spectrometry of the protein/disulphide mixture readily identifies the protein–fragment conjugates. Tethering has been particularly valuable for interrogating adaptive regions of protein structure, as seen for interleukin-2 (REFS 56–58). These two examples suggest that fragment-based methods hold promise for drug discovery for difficult targets such as protein–protein interactions.

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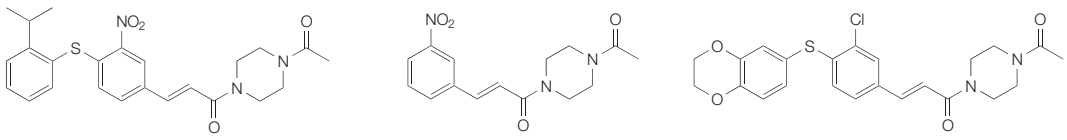

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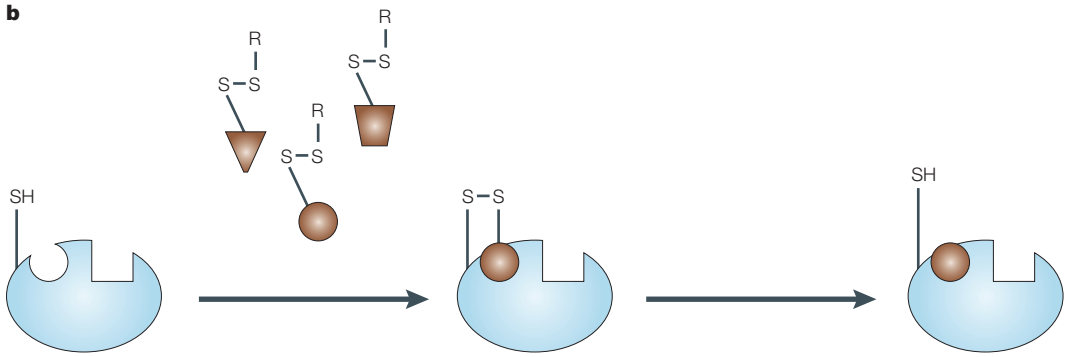


Table 1 | **Biophysical validation of inhibitors of IL-2***

Compound	IC_{50} (μM)‡	$K_{d,SPR}$ (μM)§	$K_{d,AUC}$ (μM)‖	^{1}H-^{15}N HSQC¶
1 (Ro26-4550)	7	19	20	+
5	280	150	400	+
6	26	70	50	NT
2	9	15	4	+
7	11	18	NT	+
3	4	7	8#	+
4 (SP-4206)	0.06	0.10	NT**	NT

*Adapted from REF. 59. ‡IC_{50} measured by IL-2/IL-2Rα ELISA; §$K_{d,SPR}$ measured by surface plasmon resonance (SPR) signal at equilibrium; ‖$K_{d,AUC}$ measured by sedimentation equilibrium analytical ultracentrifugation (AUC), data fit by Hetfitter[167]; ¶Binding at the IL-2Rα binding site assessed by NMR shifts of diagnostic residues, including Phe42 and Glu62 (FIG. 1). #$K_{d,AUC}$ measured for very similar structure. **Binding affinity too tight to measure by AUC. ELISA, enzyme-linked immunosorbent assay; HSQC, heteronuclear single quantum correlation; IL, interleukin; IL-2R, IL-2 receptor; NMR, nuclear magnetic resonance; NT, not tested.

perturbation was correlated with the binding affinity of the compounds. Importantly, compounds as weak as 300 μM demonstrated the same general behaviour as compounds with 3 μM IC_{50} values, and the rank-order for the binding was the same as that found by inhibition assays. X-ray crystallography of compound 3 verified that the series bound analogously to Ro26-4550. Chemistry, biophysics and structural biology were therefore used together to advance a fragment with millimolar binding affinity into a validated hit with low micromolar activity.

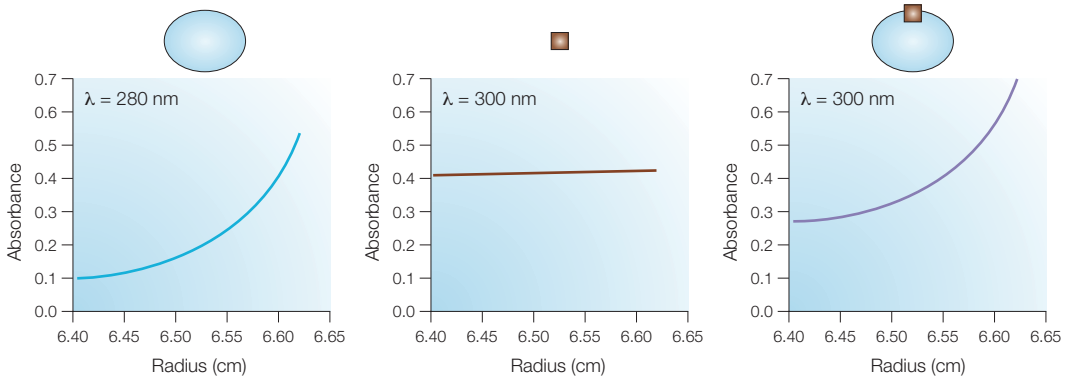
In the fourth step, compound optimization turned to Tethering (BOX 3)[57,59]. Cysteine mutations were placed in the adaptive region of IL-2, adjacent to the binding site for compound 3, and disulphide-containing fragments were screened for binding. Selected fragments tended to be hydrophobic and acidic; X-ray crystallography demonstrated that these fragments bound in more than one sub-site within the hydrophobic region[58]. Modelling of the fragments into the co-crystal structure of compound 3 and IL-2 suggested that small aromatic acids could be attached to the tri-cyclic compound via a two-atom spacer. A small number of aromatic acids were appended to an analogue of compound 3, and most of these compounds yielded at least a fivefold improvement in inhibition relative to the unmodified tri-cycle. The strongest inhibitor, containing a furanoic acid (SP-4206; FIG. 2), showed 60–100 nM activity in both a protein inhibition assay and an IL-2-binding assay (SPR). The X-ray crystal structure of SP-4206 bound to IL-2 shows the aromatic acid nestled in a positively charged pocket adjacent to the hydrophobic groove occupied by the tri-cycle[62]. Again, both this hydrophobic pocket and the basic groove are found to be highly adaptive, with both loop movements and side-chain rotations adjusting for optimal compound–protein complementarity.

Box 4 | **Analytical ultracentrifugation**

Analytical ultracentrifugation (AUC) is commonly used to measure the self-association of biomolecules in solution[167]. The centrifuge is equipped with an absorbance detector that measures the ultraviolet-visible spectrum of a sample placed in a specially designed rotor. The protein is centrifuged for several hours at 3,000–50,000 r.p.m., until equilibrium is reached. From the shape of the protein sedimentation curve at equilibrium, the molecular weight of the protein species can be determined.

Recently, investigators have used AUC to measure the association of proteins with small molecules[48,59,147,168,169]. The experiment is described schematically in the figure. The left panel shows the protein's sedimentation profile, detected by optical absorbance at 280 nm. The centre panel shows that the small molecule does not sediment when subjected to the same rotor speed, because it has a much lower molecular mass. Note that this small molecule can be detected at 300 nm, where the protein would have little detectable absorbance. The right panel shows the sedimentation profile of the small molecule (detected at 300 nm) in the presence of the protein. The small molecule now sediments with an apparent molecular weight equal to that of the protein.

For molecules with K_d <1 μM, AUC can be used to measure the stoichiometry of the compound–protein interaction. For molecules that bind in the micromolar range, fitting algorithms have been developed for determining the K_d of the interaction, even when the absorbance spectra of the compound and protein overlap[168,169]. These two regimes — stoichiometry versus K_d — are due to the concentration of reagents used in the experiment. If the K_d is much lower than the protein concentration (typically ~10 μM), then all added compound will bind to the protein until the binding sites are saturated (stoichiometry). The converse is true if the K_d is higher than the experimental concentrations.



In summary, biophysical and structural studies were brought together to characterize the mechanism of action for a series of small molecules, built up from fragments, with affinities ranging from 2 mM to 60 nM. Focusing on biophysics in the early stages of discovery ensured that chemical optimization was on the right track, and demonstrated that even weakly binding compounds can show drug-like binding at a protein–protein interface. Furthermore, due to the highly adaptive nature of the IL-2Rα binding site, it would have been difficult to predict the compound optimization pathway, and fragment-based screening proved to be an efficient tool for discovering new binding elements and increasing binding affinity.

Competitive antagonists: B7/CD28

The cell-surface proteins B7-1 and B7-2, found on ANTIGEN-PRESENTING cells, are important modulators of T-cell activation. Binding of the B7 proteins to CD28, found on the T cell, augments activation, whereas binding of B7 to cytotoxic T lymphocyte antigen-4 (CTLA4) reduces activation[63,64]. One of the complexities of inhibiting B7 function is the high local concentration of ligands at the cell–cell interface. Although the affinity of the B7/CD28 and B7/CTLA4 interactions are low (0.2–20 μM), the high valency ('Velcro effect') caused by receptor clustering might help stabilize cell–cell interactions[65–68]. The crystal structure of B7-1 bound to CTLA4 emphasizes the possible multivalency of this protein–protein interaction[69,70]. Both B7-1 and CTLA4 are homodimers, and they form a periodic, zipper-like pattern in the crystal lattice. Interestingly, there is less evidence for oligomerization of B7-1/CD28. The structure of B7-1 bound to CTLA4 reveals a highly complementary and hydrophobic protein–protein interface with a relatively small buried-surface area (roughly 600 Å^2 for each protein; FIG. 3a)[69].

Wyeth Research has reported the discovery and characterization of small-molecule ligands that bind to B7-1 (FIG. 3b) at the protein–protein hot spot[71,72]. Compounds 8 and 9, identified through HTS, are described as reversible inhibitors of B7-1/CD28, with IC_{50} values in the 4–50 nM range. The mechanism of inhibition was investigated using a number of biochemical assays. Initially, binding reversibility[71] was determined by dilution experiments (D. Erbe, personal communication). In these experiments, the compound and protein are incubated at high concentrations; when the compound/protein mixture is diluted, the concentration of compound should be too dilute to inhibit the protein's function. Retention of protein activity in these experiments indicates that the compound binds reversibly, but does not address whether the compound inhibits by forming micelles, liposomes or other aggregates. However, three experiments indicate that the compounds are not aggregators or denaturants: first, they are highly specific for human B7-1; second, their activity is not affected by the addition of detergent[43] (D. Erbe, personal communication); and third, the compounds bind to human B7-1, but not to proteins that they do not inhibit.

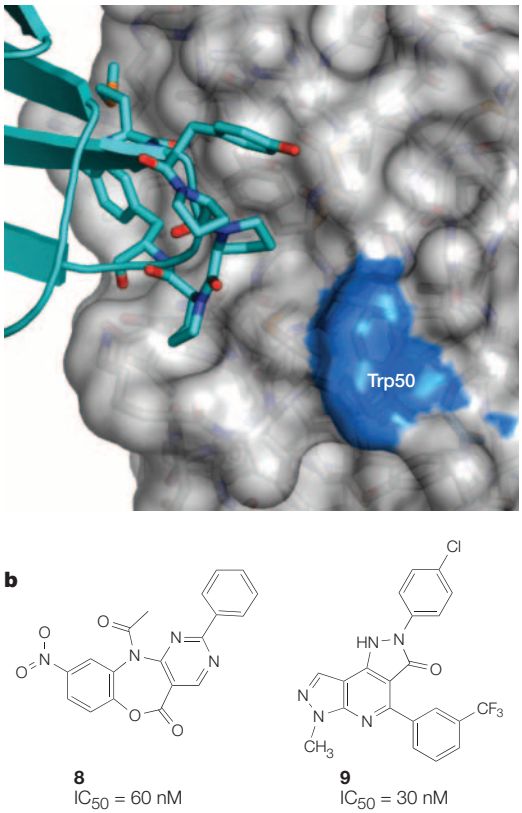


Figure 3 | **Protein and small-molecule ligands for B7-1.** **a** | Structure of B7-1 (surface) bound to CTLA4 (ribbon)[69]. Both CTLA4 and CD28 bind to B7-1 using an MYPPPY motif (shown in tubes, coloured by atom type). In the co-crystal structure, these residues are found to bind in a shallow depression in the surface of B7-1, creating a highly complementary and compact binding interface. Mutation of Trp 50 (shown in blue) abolishes binding to CD28, CTLA4, and compounds 8 and 9 (REF. 71). **b** | Structure of small-molecule inhibitors identified through high-throughput screening[71,72].

The strongest evidence in support of bona fide hot-spot binding comes from site-directed mutagenesis. Inhibition assays show that compounds 8 and 9 are selective for human B7-1 over mouse B7-1 and human B7-2. In addition, the binding of the compounds to several proteins was monitored by equilibrium dialysis. In equilibrium dialysis, the protein and small molecule are separated by a membrane with a low-molecular-mass cutoff. If the small molecule binds to the protein, it diffuses through the membrane and becomes concentrated on the side containing protein. Quantitative equilibrium dialysis can be used to obtain stoichiometry and dissociation constants; in the present study, dialysis measurements were done for qualitative and comparative purposes. The authors prepared a number of chimeric B7-1 proteins in which regions of the human (hu) sequence were exchanged for the murine amino acids[73]. This 'homologue scan' demonstrated that sequences needed for huCD28 binding are also required for compound binding[71]. In addition, a single amino-acid mutation that results in loss of binding of

ANTIGEN-PRESENTING CELL
An immune system cell that takes up antigens, processes them, and presents them to T cells, which are then stimulated to mount an immune response.

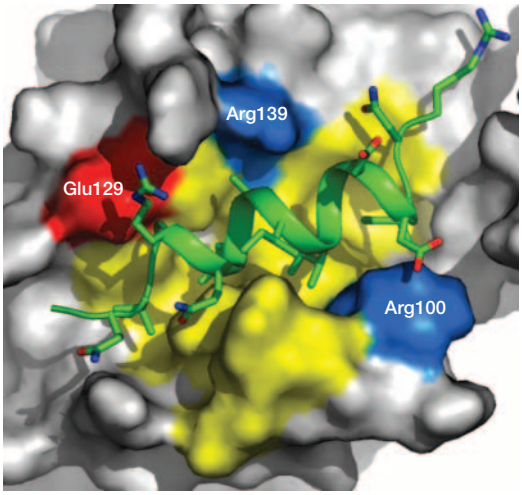


Figure 4 | **Structure of BCL-X_L (surface representation) bound to a sixteen-residue peptide derived from BAK (ribbon representation)[79].** The α-helical peptide binds in a long, hydrophobic groove in the surface of BCL-X_L; hydrophobic residues in the binding site are shown in yellow, whereas charged residues are shown in red (negative charge) and blue (positive charges). The peptide is largely hydrophobic, but contains charged residues that complement the three charged residues in the binding site. Mutagenesis data indicate that several hydrophobic residues and two of the charge–charge interactions are important for binding affinity. BCL, B-cell lymphoma.

CTLA4 and CD28 also negated binding of the small molecules. Taken together, these experiments indicate that the small molecules bind to B7-1 with a low stoichiometry at the receptor-binding hot spot.

Other binding and activity data make it unclear whether compounds are behaving in a drug-like manner. First, there is a discrepancy between the extent of inhibition and the extent of binding[71]. Because dialysis measurements were run with protein concentrations well above the IC_{50} values, the enrichment of compound on the protein side of the dialysis membrane should have been higher than the two- to fivefold observed. Second, although the compounds seem to bind at the CTLA4 binding site, they inhibit the B7-1–CTLA4 interaction very weakly (~ 10 μM). The authors propose that the difference between the inhibition of CD28 and CTLA4 could be due to the higher valency of the CTLA4–B7-1 interaction[65]. This explanation is consistent with the lack of inhibition observed in a cell-adhesion assay, which involves highly avid protein–cell interactions. On the other hand, a B7-1-binding Fab fragment was found to inhibit in the adhesion assay, albeit with a 200-fold reduction in activity[71]. Last, the binding of inhibitors is time-dependent, which could be due to a slow conformational change in the B7-1 binding site, as the authors suggest, or to a non-standard binding mechanism.

On balance, more than one mechanism of action might be needed to explain the data. A direct determination of stoichiometry of binding, and ideally a structure of the complex, would be very helpful for determining how compounds 8 and 9 inhibit the effects of B7-1.

APOPTOSIS
Programmed cell death.

ISOTHERMAL CALORIMETRY
A method for measuring the change in heat that occurs when a protein–ligand complex is formed. From these data, the thermodynamic parameters for the interaction can be determined.

Competitive antagonists: BCL family

B-cell lymphoma-2 (BCL2) and BCL-X_L are anti-APOPTOTIC proteins whose function is regulated by the binding of anti- or pro-apoptotic factors such as BAK[74–77]. BAK is a member of the pro-apoptotic proteins known as 'BH3 only' proteins because they share homology with the BCL proteins only in the third homology domain[78]. The binding of BCL-X_L to the 16-residue BH3 domain from BAK has been characterized by NMR (FIG. 4)[79]. The NMR structure indicates that the BAK-derived peptide forms an α-helix and binds in a hydrophobic groove formed by the seven α-helices of BCL-X_L. Several laboratories have suggested that small molecules could bind in this hydrophobic groove and inhibit BCL function. It is noteworthy that this site seems to bind amino naphthalene sulphonic acid (ANS), a hydrophobic dye used to detect exposed hydrophobic areas and partially denatured proteins[80]. It is possible that ANS binding does not occur in the BAK-binding groove (for example, it does not induce apoptosis). Nevertheless, this observation raises the possibility that molecules developed as drug leads might also have issues with low binding specificity.

Several laboratories have reported the identification of 100 nM–10 μM small-molecule ligands for BCL2 and/or BCL-X_L (FIG. 5)[80–87]. Discovery methods include virtual screening[81,84], HTS[82,87], ligand-based design[86] and mechanistic analysis of a known compound[83]. In each case, compounds were shown to inhibit binding of BAK peptide and to induce apoptosis in BCL-expressing cell lines. In addition, several compounds were tested in mechanistic and biophysical assays. Degterev *et al.* showed that their inhibitors (BH3I-1 and -2) are selective for BCL2 and BCL-X_L over unrelated proteins[82]. Tzung, Kim and co-workers used changes in compound fluorescence and ISOTHERMAL CALORIMETRY to show that antimycin A binds to BCL2 and BCL-X_L stoichiometrically, with a K_d that is comparable to the IC_{50}[80,83].

Four research teams have monitored the ^1H-^{15}N HSQC spectrum of BCL-X_L in the presence of their compounds. Enyedy and co-workers found that addition of compound 14 causes small and localized perturbations of residues on one end of the peptide-binding groove (the left side in FIG. 4)[84]; Kutzki *et al.* identified the other end of the groove as the binding site for their peptidomimetic compound 15 (REF. 86). Degterev, Lugovskoy and co-workers extended the HSQC experiment to develop a binding structure–activity relationship (SAR) for related compounds[82,85]. Coupling multiple HSQC measurements with computational modelling, they propose that compounds BH3I-1 and -2 cause the same conformational change in BCL-X_L that is observed on binding of BAK peptide. In a novel application of the technology, Jahnke *et al.* used a combination of HSQC spectroscopy with spin-label enhanced relaxation to demonstrate the binding orientation of compound 16 on BCL-X_L[88]. Spin labels contain unpaired electrons, which dramatically enhance the relaxation of NMR signals in a distance-dependent manner[87]. Therefore, HSQC cross-peaks near the spin label are no longer observed in the NMR spectrum, and the binding site and

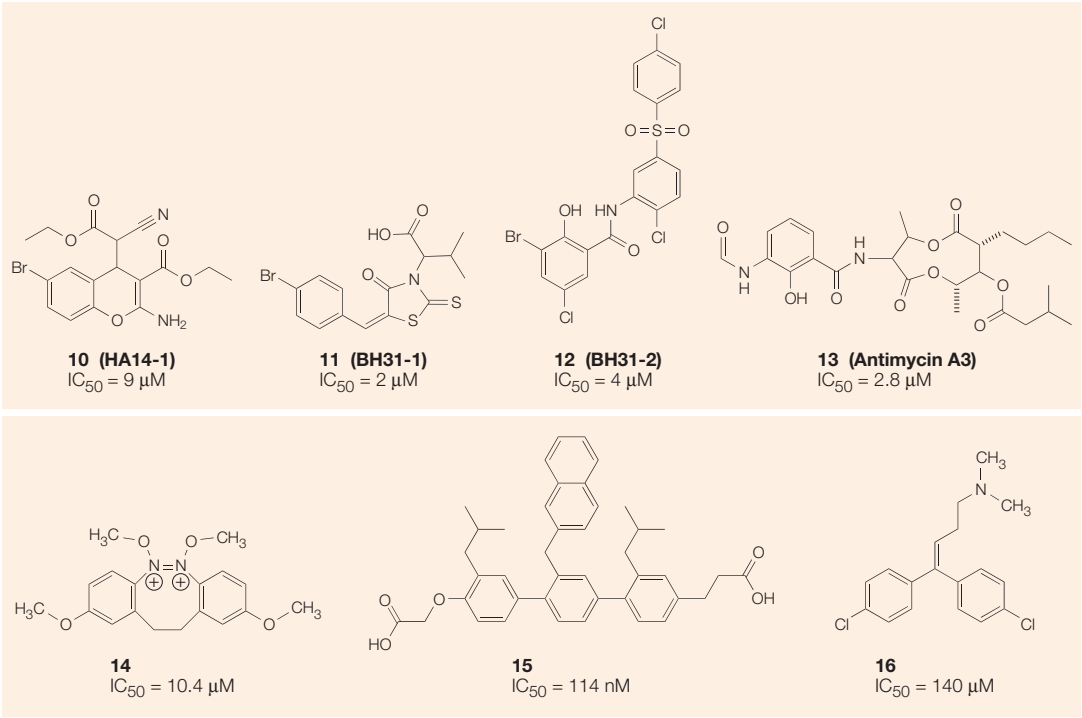


Figure 5 | **Small-molecule inhibitors of B-cell lymphoma 2 (BCL-2) and BCL-X_L[81–84,86,87].**

orientation of the spin-labelled compound can be estimated. Jahnke *et al.* found that compound 16 bound in two sites on BCL-X_L; using this information, the authors prepared a dimeric molecule with a 10 μM IC_{50}. Taken together, the NMR and other biophysical data make a strong case for the ability for small molecules to bind in the protein-binding groove and to affect the biological function of BCL2 and BCL-X_L.

Similarly to the BCL family, the interaction between the oncoproteins p53 and murine double-minute-2 (MDM2) involves the binding of a single α-helix (from p53) in a hydrophobic groove formed by three α-helices (from MDM2). Recent reports show that this protein–protein interaction is also amenable to inhibition by small molecules[89–92]. The allosteric binding site in the I-domain of leukocyte function-associated antigen-1 (LFA1, see below) also bears a structural resemblance to the BCL2 and MDM2 binding sites. Therefore, protein–protein interfaces that utilize an α-helix binding groove might be particularly amenable to small-molecule drug discovery.

Allosteric inhibitors: LFA1

Molecules that undergo large conformational changes offer the possibility of allosteric inhibition. Many cell-surface receptors undergo such conformational changes. The insulin receptor, for example, is activated by a conformational change induced by the binding of insulin[93]; the extracellular protein LFA1 is elaborately regulated by metal ions outside the cell and by signalling pathways inside the cell[94]. Furthermore, cell-surface receptors, hormones such as nerve growth factor (NGF)[95] and enzymes such as nitric oxide synthase (NOS)[96] are regulated by oligomerization. These allosteric mechanisms could provide alternative, even multiple, opportunities for small-molecule antagonism.

LFA1 is a member of the integrin family, a well-studied class of cell-surface proteins found primarily on immune cells. By binding to other cell-surface molecules called cell-adhesion molecules (CAMs), integrins mediate cell–cell adhesion, extravasation and T-cell activation[97,98]. The integrin/CAM family is so far unique among protein–protein systems in that small-molecule antagonists have been discovered for many members. For integrins lacking a 180-amino-acid domain called the 'inserted domain' (I-domain), these antagonists mimic the CAM-binding epitope and bind to the integrin at the receptor's binding site[10–19]. LFA1 contains an I-domain, which serves as the binding site for its ligand, intercellular adhesion molecule-1 (ICAM1) (REFS 99,100). Although no small molecules have been shown to bind to LFA1 at the ICAM1 binding site, two classes of allosteric inhibitors have been identified[101]. (In addition, Sanfilippo *et al.* have reported a series of tri-cyclic compounds that inhibit the LFA1/ICAM1 interaction, but the mechanism has not been characterized[102].)

Binding of ICAM1 to LFA1 seems to be regulated by conformational changes in LFA1 and by clustering of LFA1 on the surface of activated cells[99,103,104]. The current model for conformational regulation proposes that changes in the ICAM-binding I-domain are coupled to the rest of the protein through the interaction of the α7 helix in the I-domain with the 'I-like' domain in the β-chain of LFA1 (FIG. 6a)[94,105–107]. One class of allosteric small-molecule inhibitors binds to the inactive conformation of the I-domain, in a deep, hydrophobic cleft

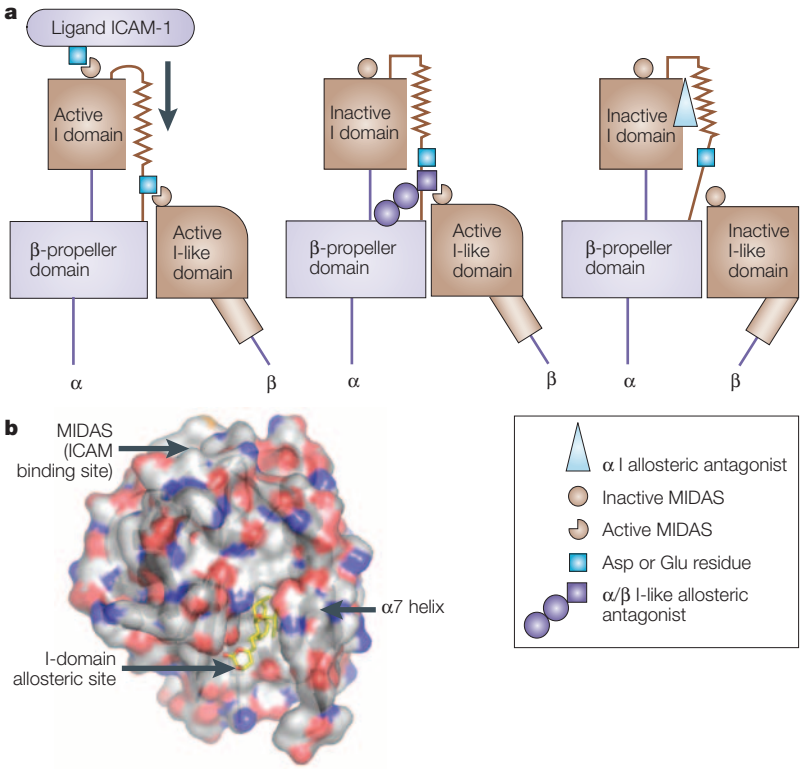


Figure 6 | **Mechanism of inhibition of LFA1 by small molecules. a** | Models for allosteric inhibition[94]. The α- and β- chains of LFA1 (αL and CD18, respectively) interact with each other at the headpiece domains. In the active conformation of LFA1, ICAM1 binds to the I-domain, which is held in the active conformation through interactions between the α7 helix with the I-like domain. α/β I-like domain antagonists such as compound 20 (FIG. 7) bind at the junction between the α and β chains, interrupting the α7/I-like domain interaction. I-domain antagonists bind in a hydrophobic groove next to the α7 helix, which also interrupts the α7/I-like domain interaction. **b** | Structure of lovastatin (yellow tubes, coloured by atom type; FIG. 7) bound to the I-domain of LFA-1 (surface; coloured by atom type)[108]. Lovastatin is found in a deep, hydrophobic pocket adjacent to the α7 helix. Part **a** reproduced with permission from REF. 94 © Macmillan Magazines Ltd (2003).

next to the α7 helix[108–110]. The other class of allosteric inhibitors seems to bind at the junction between the I-domain and the I-like domain, blocking the interaction of the α7 helix with the I-like domain[111–113].

I-domain allosteric antagonists. Three distinct series of compounds have been shown to bind in the hydrophobic cleft in the I-domain of LFA1 (FIG. 7). A high-throughput screen identified lovastatin as a low-micromolar inhibitor of the LFA1–ICAM interaction[108]. Optimization of this scaffold and a diazapane scaffold resulted in compounds with IC_{50} values in the 100 nM range[114,115]. A hydantoin series, represented by BIRT377, was found through HTS to be a nanomolar inhibitor of LFA1–ICAM[116]. A third series, represented by compound 19, was initially discovered through HTS and then optimized through medicinal chemistry and a fragment-discovery method called SAR by NMR (BOX 3)[110,117–120]. Because these compounds did not resemble known integrin antagonists, significant effort went into understanding how they bound to and inhibited LFA1.

Structural biology and I-domain antagonists. The isolated I-domain of LFA1 has been recombinantly expressed and characterized by NMR[121] and X-ray crystallography[122]. ^{1}H-^{15}N HSQC NMR spectroscopy of the I-domain in the presence and absence of lovastatin shows that the residues near the ICAM-binding metal-ion binding site (MIDAS) are not affected by lovastatin, whereas the residues in the hydrophobic crevice between helix 1 and helix 7 are strongly affected[108]. The X-ray crystal structure of the lovastatin–I-domain complex was also solved, verifying the NMR results and providing a detailed description of the small-molecule-binding site (FIG. 6b). A compound analogous to BIRT377 was also crystallized and was shown to bind in the same region of the I-domain[109]. By analogy to other I-domains, it was proposed that this hydrophobic pocket is filled by α7 in the active, ICAM-binding conformation of LFA1, and that the presence of the compound locks the I-domain — and LFA1 — into the inactive conformation[108,110,113]. This hypothesis is consistent with the rigid-body motion of the α7 helix observed by NMR and crystallography[105,121,123]. The hydrophobic groove created by movement of the α7 helix is, like IL-2, an example of an adventitious small-molecule-binding site resulting from the protein's conformational flexibility.

Mapping the binding of I-domain antagonists. For many small-molecule–protein interactions, high-resolution structural data are difficult to obtain. The research team at Boehringer Ingelheim has developed mass spectrometry (MS)-based methods to obtain binding-site information for hydantoin antagonists of LFA1 in the absence of NMR or crystallography[109,124]. A BIRT377 analogue with a photo-affinity label was synthesized and reacted with the LFA1 I-domain under ultraviolet light. The protein was then digested with trypsin and analysed by MS; careful studies indicated that the probe specifically labelled the I-domain at proline 281. This information led to a binding hypothesis that was refined by computational modelling and later verified by X-ray crystallography[109].

An MS method was also used to measure the noncovalent binding of molecules to the I-domain in the gas phase[124]. The observed mass spectrum correlated with 1:1 binding of the compound and protein, but the ratio of bound: unbound protein did not correlate with binding affinity. This result is not surprising, because gas-phase stability is often very different from solution-phase affinity. An interesting observation from these studies was the change in charge distributions in the MS signal. When a protein is ionized by electrospray ionization, a series of charge states, corresponding to different protonation states of the protein, are observed. Changes in this distribution are indicative of changes in protein structure due to denaturation, changes in conformation or masking of ionizable groups. The binding of hydantoin compounds reduced the amount of denatured and oligomerized I-domains, and reduced the yield of the +9 charged state in favour of the +8. Taken together, these changes suggest that the compounds rigidify the I-domain structure, consistent with their function as allosteric inhibitors.

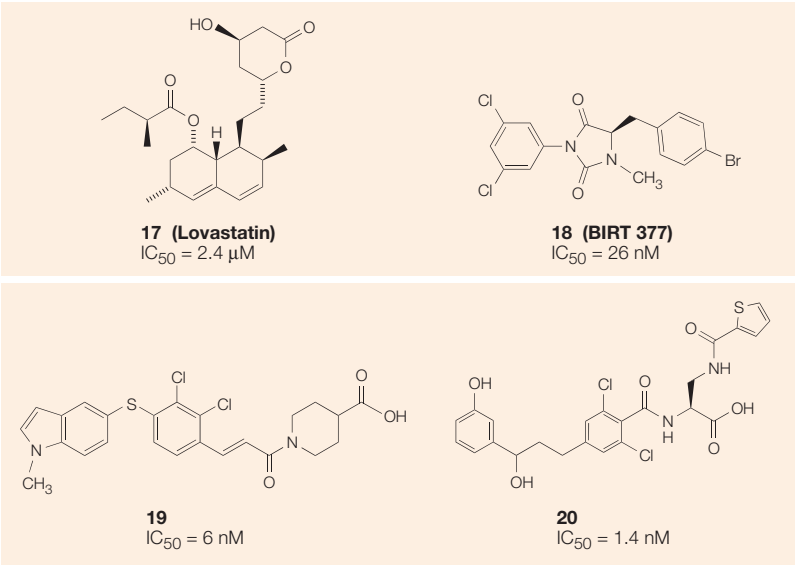


Figure 7 | **Allosteric inhibitors of LFA1.** Compounds 17–19 (REFS 108,116,119) are examples of I-domain antagonists. Compound 20 represents a class of inhibitors recently termed 'α/β I-like domain antagonists[111,112].

Antibodies have also been used to assess the allosteric effects of small molecules on the conformation of full-length LFA1. The binding of antibodies to the I-like domain, the I-domain and other regions of LFA1 were monitored in the presence of a series of lovastatin derivatives[113] and BIRT377 (REFS 116,125,126). These compounds were found to inhibit antibody binding to certain epitopes on the I-domain, but to have no effect on other portions of the protein. One of the lovastatin derivatives inhibited the binding of certain I-like domain antibodies, suggesting that this compound accesses new binding pockets that are closely linked to the I-like domain[113]. Using antibodies that recognize the active conformation of LFA1, Woska *et al.* demonstrated that the I-domain antagonists maintain LFA1 in an inactive state, even in the presence of activating metal ions[125]. In summary, antibody mapping and MS studies demonstrate that valuable information about the binding of small-molecules can be obtained in the absence of high-resolution structures.

Mapping the binding of I-domain antagonists: α/β I-like allosteric antagonists. The series of compounds represented by compound 20 (FIG. 7)[111,127] has a different mechanism of action from the I-domain ligands described above. It was suggested that these compounds mimic the binding epitope of ICAM1 by mapping side-chain functionality onto a small molecule scaffold[111]. However, biochemical and biophysical measurements do not support the proposal that the compounds bind at the ICAM1-binding site[112,113]. For example, ^{1}H-^{15}N HSQC NMR[113] and SPR measurements[112] do not show direct binding of the compounds to the I-domain. Furthermore, compounds bind to a version of LFA1 in which the I-domain has been deleted. Nevertheless, these compounds are highly potent inhibitors, and it is therefore interesting to determine their mechanism of action.

A combination of biochemical measurements indicate that compounds in this series bind at the junction between the I- and I-like domains of LFA1 and function as allosteric antagonists[112]. Ligand-like antagonists of integrins lacking an I-domain[128] typically stabilize the integrin heterodimer towards dissociation in sodium dodecyl (lauryl) sulphate (SDS) solution. When compound-20-like molecules were tested in this assay, gel electrophoresis showed that LFA1 was similarly stabilized, suggesting that compounds bound to a site at the dimer interface[112]. Next, Shimaoka *et al.* prepared disulphide mutations that lock the I-domain into the active or inactive conformation. Compounds bound to both conformations but did not block ICAM1 binding to the activated mutant, suggesting that inhibition occurs by an allosteric mechanism. Finally, the binding of LFA1 antibodies in the presence of compound 20 and its analogues was monitored[112,113]. In contrast to the I-domain ligands, these compounds did not inhibit binding of I-domain antibodies, but did inhibit binding of I-like domain antibodies. Taken together, these data suggest that compound 20 and its analogues bind at the bridge between the α- and β-chains, blocking the interaction between the I-like domain and the α7 helix in the I-domain (FIG. 6a). This binding site is analogous to the ligand-mimetic antagonists of integrins not containing an I-domain.

Drug-development studies with I-domain and α/β I-like domain antagonists demonstrate the potential for small-molecule inhibition through allosteric regulation. The fact that three binding sites (the active site and two allosteric sites) have been found for LFA1 indicates that there might even be several ways to inhibit complex protein signalling systems. In addition, research into the mechanisms of action of these inhibitors underscores the power of combining biochemistry, biophysics and structural biology. Several of the molecular series were identified through functional screens; nevertheless, uncovering the mechanism of binding was important for advancing the initial compounds. Even when the structure of a compound suggests a binding hypothesis, it is important to validate the actual binding site through biophysical and structural means. Finally, both classes of allosteric compounds provide useful drug leads for LFA1, and have furthermore served as probes for understanding the allosteric regulation of this important class of adhesion proteins.

Allosteric inhibitors: inducible NOS

Nitric oxide synthase (NOS) enzymes are multidomain, haem-containing proteins that generate NO from L-arginine[96]. Two isoforms of NOS are constitutively expressed in endothelium, where NO radical plays a positive role in angiogenesis and vascular health[129], and in neurons, where both positive and negative effects have been observed[130,131]. NO produced by inducible NOS (iNOS), on the other hand, is implicated in tissue damage

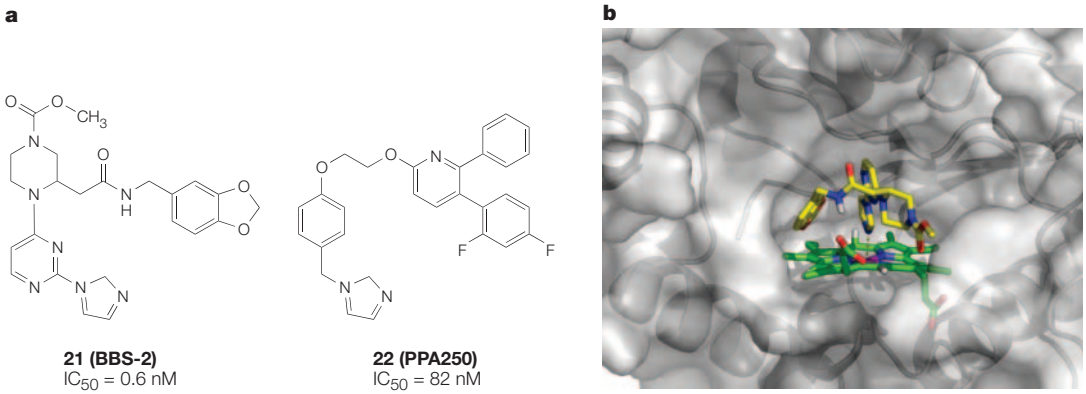


Figure 8 | **Small-molecule allosteric antagonists of inducible nitric oxide synthase. a** | BBS-2 and PPA250 are potent inhibitors of inducible nitric oxide synthase (iNOS) dimerization during protein synthesis[137,138]. **b** | Structure of BBS-2 bound to iNOS, viewed from the dimerization face[137]. Monomeric iNOS (white ribbon/surface) was made by deletion mutagenesis. BBS-2 (yellow tubes, coloured by atom type) binds to the iron atom in the haem cofactor (green tubes coloured by atom type) and causes helices at the dimerization face to become disordered.

during inflammation[132,133], prompting researchers to look for inhibitors that are selective for iNOS[134–136]. Two laboratories have reported the identification of molecules (FIG. 8) that allosterically inhibit the function of iNOS by binding to the haem cofactor in the protein active site and disrupting protein dimerization[137–140].

PPA250, BBS-2 and related compounds were found to inhibit dimerization during protein synthesis with inhibition constants (K_i) in the low nanomolar range, but not to disrupt preformed iNOS dimers[137,138]. The unusual mechanism of BBS-2 activity was further characterized by radioligand binding, absorbance spectroscopy and X-ray crystallography. First, radiolabelled BBS-2 was shown to bind to haem-containing iNOS monomers stoichiometrically and reversibly, with a dissociation rate of 200 minutes at 22 ^{O}C[139]. Absorbance spectroscopy indicated that the imidazole moiety of BBS-2 bound directly to the haem cofactor, as expected. The X-ray crystal structure of BBS-2 bound to the monomeric oxygenase domain of iNOS (FIG. 8) was consistent with the proposed mechanism, and provided clues as to how ligand binding could disrupt dimerization by disrupting the structure of α-helices near the protein–protein interface[137]. Both BBS-2 and PPA250 were shown to be active in animal models of disease[138,140–142]. Again, mechanistic, structural and biological studies worked together to validate the allosteric inhibition of this protein–protein interaction.

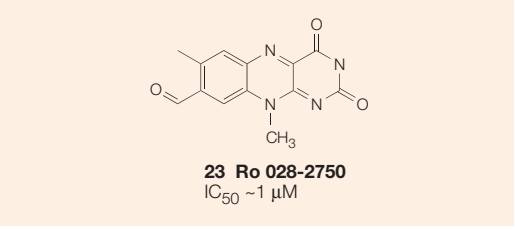


Figure 9 | **Small-molecule antagonist of nerve growth factor[147].**

Allosteric inhibitors: nerve growth factor

Nerve-growth factor (NGF) is a 27-kDa homodimer whose binding to neuronal cells can cause neurite growth or cell death, depending on the expression of two cell-surface receptors, TRKA (pro-growth) and p75^{NTR} (pro-apoptotic)[95,143]. The hot spots for binding to the two receptors are distinct, and the two receptors can bind simultaneously[144–146]. Niederhauser *et al.* have published the characterization of a small molecule, Ro 028-2750 (FIG. 9), which binds to NGF (IC_{50} ~ 1 μM) and inhibits binding to p75^{NTR} selectively over TRKA[147]. This selectivity was then used to dissect the functions of p75^{NTR} and TRKA in neuronal differentiation, apoptosis and neurite outgrowth.

The authors propose that Ro 028-2750 causes a conformational change in NGF that abolishes binding selectively to p75^{NTR} at submicromolar concentrations and to both p75^{NTR} and TRKA at higher concentrations[147]. AUC and fluorescence quenching measurements demonstrated ~1:1 binding between the small molecule and the NGF dimer. Inhibition of NGF–p75^{NTR} binding was measured by crosslinking ^{125}I-NGF to cells expressing p75^{NTR} followed by gel electrophoresis. Inhibition of the hormone–receptor interaction was found to be time-dependent, leading the authors to conclude that Ro 028-2750 causes a conformational change in NGF over time. No direct evidence is provided for a conformational change or for binding at the dimer interface.

The interesting biological effects of Ro 028-2750 would be augmented by further clarification of the molecule's mechanism of action. To use the compound as a tool for understanding biology, it would be relevant to understand the selectivity of Ro 028-2750 for NGF over other proteins. In addition, time-dependent inhibition can be due to conformational change, but can also be a symptom of covalent binding. Ro 028-2750 contains an aldehyde, which can covalently modify proteins[148,149]; it would therefore be valuable to demonstrate that Ro 028-2750 binds to NGF reversibly[45]. Evidence for binding to the dimer interface would also be of interest. Finally, as it

Box 5 | **From screening to validated hit: defining the mechanism of inhibition**

Many approaches have been used to identify new compounds that can serve as starting points for a drug discovery programme. Once these new compounds, or 'hits,' have been identified, the research team must decide whether these hits should be pursued. A high degree of validation is recommended for protein–protein interactions, because the targets are typically new to small-molecule discovery and the initial compounds are often hydrophobic and have weak activities. In TABLE 2, we outline a series of steps for validating and characterizing a new series of inhibitors; these same steps should be considered not only for protein–protein interactions, but whenever the mechanism of action of a small-molecule series is in question.

Typically, compounds are first identified as hits when they show activity in a functional screen (TABLE 2; step 1). It is usually straightforward to follow-up with a secondary functional assay that has a different method of detection (TABLE 2; step 2) to rule out assay-specific artefacts. Varying the conditions of the functional assay(s) can also flag two common mechanisms — aggregation[43] and irreversible binding[44,45] (TABLE 2; steps 3 and 4). Aggregators often show low specificity and extreme sensitivity to the presence of blocking proteins or detergents. Such compounds can also be very sensitive to the concentration of the target protein in the assay. These screening results provide 'red flags' for ill-behaving compounds, but do not necessarily demonstrate that compounds are behaving in a drug-like fashion.

Qualitative biophysical assays can be used in a screening mode to identify high-stoichiometry binding and unusual binding kinetics directly. In recent years, a number of nuclear magnetic resonance (NMR) experiments have been developed for qualitatively measuring the binding of small molecules to proteins; many of these methods also require fast binding kinetics[87,158,170–172]. If accessible, analytical ultracentrifugation (AUC)[48,147,168,169] can be a very valuable method because it provides a positive signal for the compound whether or not it is bound; AUC also detects changes in the aggregation state of the compound and/or protein and can allow determination of the stoichiometry or K_d (TABLE 2; step 5). Surface plasmon resonance (SPR)[173,174] and fluorescence[83,147] are two other common methods for looking at a drug–protein interactions qualitatively and quantitatively. If the drug target is an enzyme, then kinetic measurements can also address issues of aggregation and reversibility. In general, a compound series is reasonably well validated when assay-dependent artefacts, aggregation and irreversible binding are ruled out. Biophysical methods further allow the researcher to view the binding event itself, providing strong, positive evidence that a compound is a bona fide hit.

For new biological systems or chemical series, it can be valuable to measure the stoichiometry of the protein–ligand interaction and to demonstrate that the observed inhibition is related to the binding interaction by relating the IC_{50} to the K_d. A number of biophysical methods are available for addressing these issues, and the most appropriate approach depends on the availability of instrumentation, the expected affinity of the interaction and the solubility and availability of the reagents. For instance, isothermal calorimetry (ITC) requires a significant amount of protein (on the order of several hundred micrograms) and high compound solubility in buffer (typically >100 μM). When these requirements are met, ITC provides a wealth of valuable data, including the K_d, the enthalpy (and entropy) of the interaction and the binding stoichiometry[175].

The most detailed level of characterization obtained for a protein–ligand interaction is structural, usually from NMR or X-ray crystallography (TABLE 2; step 6). Until recently, these high-resolution methods often lagged behind the hit-validation process, but advances in NMR sensitivity[176] and X-ray crystallography throughput[177] have made these approaches valuable even in the early stages of a drug-discovery programme. Binding-site information can be obtained at lower resolution by mapping the binding epitope through site-directed mutagenesis or antibody inhibition. In addition to validating the utility of a small-molecule hit, binding-site characterization can have a major impact on compound optimization.

is known that the two receptors for NGF have different binding epitopes[146], site-directed mutagenesis might further clarify the small-molecule binding site.

Conclusions

There are now a number of reports of small-molecule inhibitors of protein–protein interactions. Have patterns emerged? It seems that the approaches for initial compound discovery — HTS, computational screening, fragment discovery — have many of the same advantages, and encounter the same hurdles, for protein–protein systems as for more traditional therapeutic targets. It is likely that, as with traditional targets, a variety of discovery approaches will be needed. Given the general difficulty of this class, selection of a tractable protein–protein system is also important. On the basis of the data so far, good targets for small-molecule inhibition are those that have small hot spots that can be covered by a drug-sized molecule, and perhaps those hot spots that have demonstrated binding to small peptides. Furthermore, although it seems obvious that proteins must have cavities for molecules to bind, the cavities themselves might not be obvious from an initial inspection. They might be found away from the binding interface in an allosteric site, or they might be found in cryptic sites within the adaptive regions of the protein hot spot.

Drug discovery is also crucially augmented by the availability of orthogonal methods of characterization; such methods include biophysics, mutagenesis, epitope mapping and structural biology. BOX 5 and TABLE 2 outline some of these experiments, their uses and their limitations. In general, a novel molecule can be described as 'validated' when it has been shown to bind noncovalently with 1:1 binding stoichiometry to the target of interest. In the cases in which the data are unexpected — as when the compound inhibits in cells but not *in vitro*, or vice versa — added emphasis should be placed on understanding the mechanism of inhibition. Knowledge

Table 2 | **From screening to validated hit: defining the mechanism of inhibition**

Step	Property of inhibitor	Suggested methods	Comments
1	Functional inhibition	Typically how compounds are identified, by ELISA, SPA, DELFIA.	Deglycosylation of target proteins and conjugation of reporter proteins can reduce protein solubility and introduce artefacts; proteins should be kept as close to native state as possible.
2	Inhibition in orthogonal assay	SPA, FRET, inhibition of antibody binding.	Eliminates detection-specific artefacts from primary screen.
3	Aggregation of compound and/or protein	Extreme sensitivity to conditions,for example detergents, concentration of protein.	Bona fide, hydrophobic or amphiphilic compounds may also be sensitive to detergents, so can produce false negatives.
		Functional inhibition of unrelated proteins.	Non-selectivity is often a sign of aggregation or denaturation.
		AUC	Equilibrium sedimentation diagnoses aggregation of compound and compound-dependent aggregation of proteins. Requires optical absorbance of compound for direct detection of protein–compound interaction.
4	Reversibility	Surface plasmon resonance (SPR).	Allows measurement of kinetic constants; can be sensitive to nonspecific binding to the SPR surface.
		Mass spectrometry.	Allows detection of covalently bound compounds.
		Radioligand binding.	Measuring the binding kinetics of a radiolabelled compound is most useful for tight-binding compounds.
		Concentration/dilution.	Compound is incubated with concentrated solution of protein; mixture is then diluted so that the compound concentration is well below the IC_{50} and the activity of the protein is measured. Identifies covalent modifiers, but might not identify aggregators (which are sensitive to compound/protein ratio).
5	Binding stoichiometry, K_d	Isothermal calorimetry.	Gives highly validated stoichiometry and/or K_d, but requires high compound solubility and can be difficult for weak binders or entropy-driven binding.
		SPR, radioligand binding.	See step 4 above.
		Fluorescence.	Straightforward method when either compound or protein have significant fluorescence that changes on compound binding.
		Equilibrium dialysis.	Compound detected by UV, HPLC, or radioligand binding.
		NMR	Ligand-based methods monitor binding of ligands with weak–moderate affinity (10^{-3} – 10^{-6} M); can be used even with large proteins.
		AUC	Can determine K_d for moderate affinities (10^{-4} –10^{-6}) and stoichiometry for higher affinities.
		Mass spectrometry of complex.	Not always feasible, especially for binding driven by hydrophobic interactions.
6	Binding site	X-ray crystallography.	Gives high-resolution image of binding interactions; a static view of binding.
		NMR	HSQC identifies protein residues affected by compound binding; NOESY experiments can lead to a three-dimensional model of the interaction in solution. These NMR methods require well-expressed, soluble, small proteins.
		Functional inhibition of known ligands.	Inhibiting the binding of antibodies, peptides or other small molecules with known binding sites could indicate same-site binding, but is subject to interpretation.
		Site-directed mutagenesis.	Mutations that do not affect the structure of the protein but alter binding could indicate that the residue is in the small-molecule binding site.
		Photoaffinity labelling/MS.	Compound is conjugated to a photoaffinity label in a way that does not affect its binding to the protein. The compound and protein are incubated in the presence of light, the protein is digested by a protease, and the location of the label is determined by changes in the digestion pattern. Incomplete or multiple labelling might complicate the analysis.

AUC, Analytical ultracentrifugation; DELFIA, brand name for Perkin Elmer plate-based assay format that uses lanthanide chelates and time-gated emission detection; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; HPLC, high performance liquid chromatography; HRP, horse radish peroxidase; HSQC, heteronuclear single quantum correlation; MS, mass spectrometry; NOESY, nuclear Overhauser effect spectroscopy; NMR, nuclear magnetic resonance; SPA, scintillation proximity assay; SPR, surface plasmon resonance; UV, ultraviolet.

of the precise binding site is also advantageous, and therefore NMR and X-ray crystallography have key roles in several of the examples described in this review. In some cases, crystallography is used to identify the binding site or to generate hypotheses for structure-based design. In other cases, high-resolution structural data back up results from other methods. Such confidence building is important, especially when the methods are new and the drug targets novel, but it should be noted that a combination of chemical and biological approaches do provide valuable information. Often, it is the combination of methods, rather than any one experiment, that propel a drug-discovery project forward.

Protein–protein interactions are clearly more challenging than drug targets that naturally bind small molecules. However, there have been clear inroads into these targets. The drug discovery community understands more about what kinds of binding sites might be more tractable than others. We have developed better ways of screening diversity space (both empirically and computationally) for new compounds that interact with these sites. We are also improving screening and characterization protocols that allow more accurate selection of advance-able molecules. This field is still in its infancy, but given the progress that has been made and the importance of this target class, it is likely to receive increased attention in the future.

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Competing interests statement

The authors declare that they have competing financial interests; see Web version for details.

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