Small-Molecule Inhibitors of IL-2/IL-2R: Lessons Learned and Applied

C.G.M. Wilson and M.R. Arkin

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Abstract The IL-2:IL-2R protein–protein interaction is of central importance to both healthy and diseased immune responses, and is one of the earliest examples of successful small-molecule inhibitor discovery against this target class. Drug-like

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We dedicate this manuscript to the memory of Dr Warren DeLano, who taught us how to look at structures.

L. Vassilev and D. Fry (eds.). *Small-Molecule Inhibitors of Protein-Protein Interactions*, Current Topics in Microbiology and Immunology 348, DOI 10.1007/82_2010_93

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inhibitors of IL-2 have been identified through a combination of fragment discovery, structure-based design, and medicinal chemistry; this discovery approach illustrates the importance of using a diverse range of complementary screening methods and analytical tools to achieve a comprehensive understanding of molecular recognition. The IL-2 story also provides insight into the dynamic nature of protein–protein interaction surfaces, their potential druggability, and the physical and chemical properties of effective small-molecule ligands. These lessons, from IL-2 and similar discovery programs, underscore an increasing awareness of the principles governing the development of drugs for protein–protein interactions.

1 Introduction

Protein–protein interactions (PPIs) are a new class of drug target, and their inhibition presents a challenging mechanism of action by which to affect therapeutic change (Arkin and Wells 2004; Wells and McClendon 2007; Betzi et al. 2009). In contrast to enzyme–substrate interactions, protein–protein recognition frequently occurs through flat surfaces or wide, shallow grooves that bind large (>750 $Å^2$) peptide epitopes (Fig. 1). Several features can influence the likely druggability of a given PPI. First, peptide epitopes can involve a single region of a polypeptide – such as an α-helix from one face lying in a groove on the opposite face – or a series of discontinuous segments from one or more protein domains. Second, while the physical interface is typified by a large number of polar and nonpolar interactions, their individual contributions are not uniform (Ma et al. 2003; Cunningham et al. 1989; DeLano 2002a; Ofran and Rost 2007; London et al. 2010). PPIs tend to contain a small number of residues – termed hotspots – that are responsible for the majority of binding strength (Fig. 1). In some cases, hotspots contain regions of structural flexibility, allowing the same protein surface to bind multiple partners (DeLano et al. 2000), or the PPI to allosterically alter protein function (Gold et al. 2006; del Sol et al. 2009). Finally, PPI affinity varies from the micromolar to the picomolar range, implying a wide range of interaction dynamics and perhaps a range of inherent binding energy of the protein surfaces. The nature of the interface, the structure and dynamics of the hotspot, and the PPI affinity will help predict the druggability of a PPI, the best approaches to take toward compound discovery, and the kinds of molecules likely to be identified in the discovery effort.

In addition to the real challenges of developing small-molecule inhibitors for PPI, there is also a bias in the chemical libraries and experimental methodologies that we use to interrogate this new class of targets. Most current drugs target G-coupled protein receptors and enzymes, and our knowledge of "drug-like" synthetic molecules is largely informed by this experience (Lagerstrom and Schioth 2008; Lipinski et al. 2001). For instance, the molecular weight of current, orally available drugs is generally less than 500 Da; if this is a pharmacological requirement, then the ligand efficiency – the ΔG/number of heavy atoms – must be ~0.3 for a 10 nM inhibitor containing 38 non-hydrogen atoms (Hopkins et al. 2004).

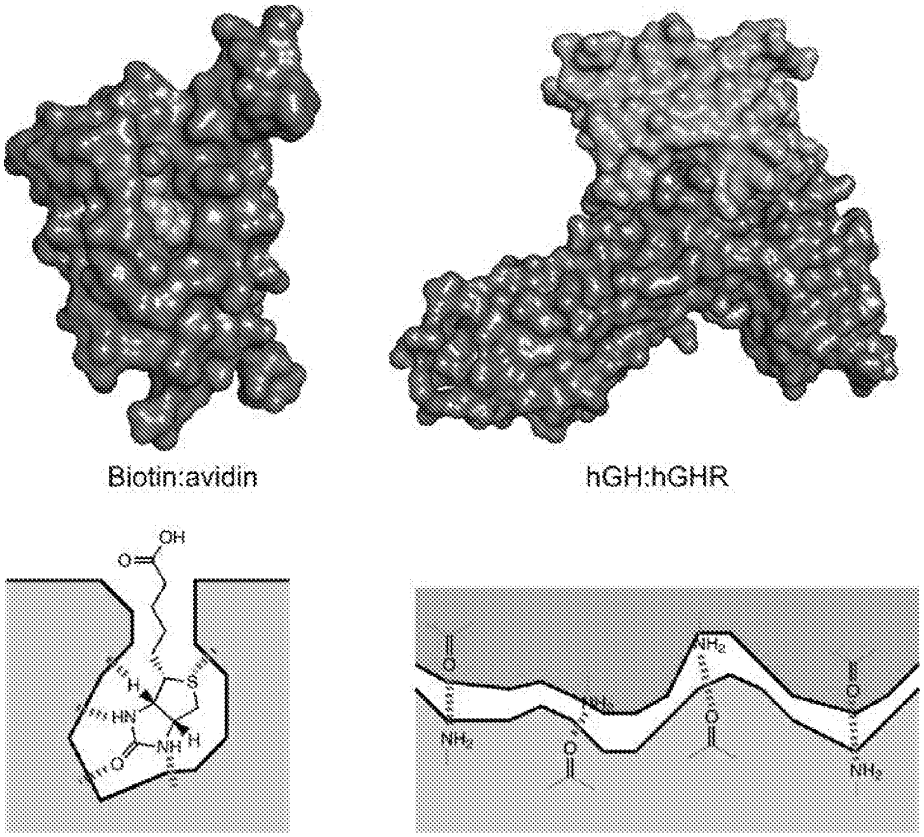


Fig. 1 Small molecule and protein–protein recognition. Binding of biotin by avidin (1stp) illustrates the deep grooves and localized interactions that characterize classic, high-affinity ($K_d \sim 10^{-15}$) small-molecule recognition. In contrast, the interaction between growth hormone and growth hormone receptor (1axi) is spread across a greater area, with a lower density of interactions ($K_d \sim 10^{-9}$). Except where otherwise indicated, surface and cartoon representations were prepared using PyMOL (DeLano 2002b)

Whether this ligand efficiency is achievable for a PPI inhibitor and, if not, whether we can make larger compounds into drugs remain open questions. Our knowledge bias may even be apparent within the handful of successful PPI inhibitor discoveries to-date – our present understanding is almost exclusively based on compounds that act against comparatively small, continuous PPI epitopes (Vassilev et al. 2004; Lee et al. 2007). By virtue of their limited size, these PPI surfaces could represent a subset of PPI targets that are more readily inhibited by classically oriented chemical libraries. Nevertheless, most compound collections available to drug discovery scientists are highly biased toward compounds that are lead-like for enzyme targets, and perhaps away from compounds that are PPI inhibitor-like (Sperandio et al. 2010).

There is also a knowledge gap in selecting the best approaches for finding inhibitors, though a range of methods – both computational and experimental – have been successfully used (Betzi et al. 2009; Trosset et al. 2006; Ciulli and Abell 2007; Fattori et al. 2008; Pellecchia et al. 2008; Casey et al. 2009). Regardless of the primary screening method used, it is crucial to validate primary hits using orthogonal methods. In the past decade, numerous structural and biophysical approaches that measure the small molecule/protein interaction directly have come of age. In combination, methods such as SPR, NMR, and analytical centrifugation cross-validate compounds for further investigation and generate a clear understanding of the mechanism of inhibition at work (Arkin and Wells 2004; Boehm et al. 2000; Giannetti et al. 2008). These methods can also reveal general properties of the molecule, facilitating early removal of problematic aggregators or promiscuous inhibitors from compound sets. The increasing use of biophysical methods reflects a growing awareness of the unique challenges posed by PPIs, both in terms of targets themselves and the chemical characteristics of small-molecule ligands.

Fragment-based lead discovery (Arkin and Wells 2004; Erlanson and Hansen 2004; Jhoti et al. 2007; Schulz and Hubbard 2009; Fischer and Hubbard 2009; Chessari and Woodhead 2009) has gained popularity for PPI inhibitors due to its potential to identify highly ligand-efficient and novel small-molecule hits. In fragment-based discovery, a compound half the size of a traditional drug (e.g., 250 Da) is screened for binding to a target of interest. Biophysical approaches are generally favored over functional assays because fragment/protein interactions tend to have low affinity, and binding methods – such as NMR (Shuker et al. 1996), X-ray (Carr and Jhoti 2002), surface plasmon resonance (SPR) (Hamalainen et al. 2008), or Tethering (Erlanson et al. 2000) – provide additional information about binding site and/or binding stoichiometry. Active fragments are then linked or evolved in a second step and retested for binding or inhibition of the target. Fragment-based approaches might be especially suitable for PPI because they favor binding sites with multiple and nearby subsites, which appears to be the trend for PPI/small-molecule systems (Fuller et al. 2009). The sophistication of fragment linking and growing also potentially enables the assembly of small molecules that explore and capture available surface features for high-affinity binding. Finally, the emphasis on data-rich biophysical assays allows investigators to assess the potential druggability of a PPI (Brown and Hajduk 2006; Hajduk et al. 2005), while helping to identify and eliminate problem molecules (or fragment pharmacophores) early in the discovery process.

The search for small-molecule PPI inhibitors has been underway for little more than 20 years. Several glimpses of their potential have emerged, with notable successes and a few compounds in clinical testing. Together with our growing understanding of the trends and rules for small-molecule PPI inhibitor design, these give cause for optimism. Interleukin-2 was among the first successful demonstrations that small molecules could inhibit PPIs (Braisted et al. 2003; Hyde et al. 2003; Raimundo et al. 2004; Waal et al. 2005; Arkin et al. 2003; Thanos et al. 2003, 2006), and it remains one of the few examples of a small-molecule mimicking a highly discontinuous epitope. The wealth of structural data gathered in the

exploration of IL-2 small-molecule inhibitors has revealed surprising complexity at protein–protein interfaces and serves as a rich model that continues to guide screening and inhibitor design.

2 IL-2 Biology

2.1 Ligand and Receptor Biology

IL-2, the first interleukin peptide hormone discovered, is characterized by its ability to stimulate T-cell proliferation (Nowell 1960; Morgan et al. 1976; Smith 1980; Gillis et al. 1982; Greene et al. 1984; Robb et al. 1984a). Mature IL-2, a secreted glycoprotein of 133 amino acids (15.5 kDa), is a single chain polypeptide produced by T cells in response to immune stimuli mediated by the T-cell receptor (TCR) and major histocompatibility complexes (MHC) I and II (Nelson and Willerford 1998; Malek 2008). In the resting immune system of healthy individuals, circulating IL-2 levels are extremely low or undetectable, while raised levels follow infection and accompany normal immune response.

The IL-2 receptor family comprises three single-pass transmembrane proteins, IL-2Rα (p55, CD25), IL-2Rβ (p75, CD122), and IL-2Rγ (p64, CD132) (Robb et al. 1981, 1984b; Leonard et al. 1984, 1985; Greene et al. 1986; Hatakeyama et al. 1989; Tsudo et al. 1990; Takeshita et al. 1992; Noguchi et al. 1993). IL-2Rα is present at low concentrations on T cells and is expressed along with IL-2 following TCR activation, forming a transient autocrine/paracrine signaling loop. IL-2Rβ is constitutively expressed on resting T cells and is also a component of the IL-15 receptor (Grabstein et al. 1994). The widespread expression of IL-2Rγ on immune cells and its presence in several other interleukin receptor complexes (IL-4, IL-7, IL-9, IL-15, and IL-21) lead to the alternative name of common gamma chain (γc) (Sugamura et al. 1996; Lai et al. 1996; Kovanen and Leonard 2004; Alves et al. 2007; Wang et al. 2009). Receptor monomers bind IL-2 with a wide range of affinities (α = 10 nM, β = 100 nM, γ ~ 0.7 mM), while complexes exist as an intermediate affinity dimer (β- and γ-subunits, ~1 nM) and a high-affinity trimer (α-, β-, and γ-subunits, K_d ~ 5 pM). The α-chain has a short C-terminal intracellular segment. Only IL-2Rβ and IL-2Rγ contribute to intracellular signaling through their long cytoplasmic tails, which contain Box domains that are constitutively associated with inactive Janus kinases JAK1 (IL-2Rβ) and JAK3 (IL-2Rγ) (Russell et al. 1994; Taniguchi et al. 1995; Gesbert et al. 1998). Capture of IL-2 and binding to/formation of the high-affinity trimer results in activation of JAKs and downstream activation of MAP and STAT5 signaling pathways (Fig. 2) (Lin and Leonard 2000; Gaffen 2001).

In the generalized immune response to foreign antigen, IL-2 and IL-2Rα are coexpressed by activated T cells. Secreted IL-2 is bound by the monomeric IL-2Rα and concentrated at the cell surface. IL-2 bound to IL-2Rα is presented to β- and γ-subunits (on the same or adjacent cell), triggering intracellular signaling and changes in transcription. IL-2 receptor activation leads to reduced expression of

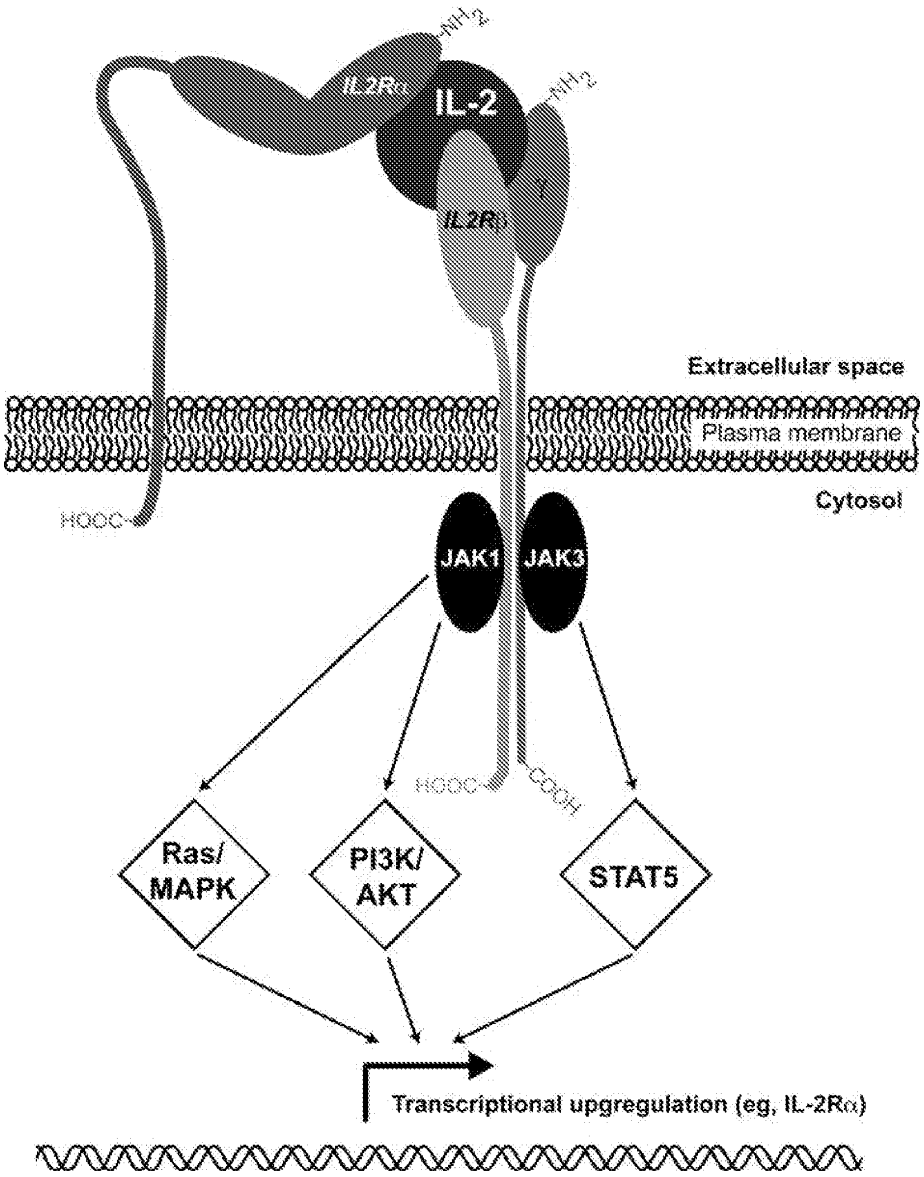


Fig. 2 IL-2 receptor complex and signaling. Capture of IL-2 by the receptor α-subunit leads to high-affinity complex formation, which leads to cross-phosphorylation of JAK kinases associated with IL-2β and IL-2γ. Changes in gene expression take place through MAP, AKT, and STAT5 pathways

IL-2, restricting autocrine stimulation, while levels of IL-2Rα remain high for continued paracrine surveillance (Smith and Popmihajlov 2008; Crispin and Tsokos 2009). Depending on co-receptor stimuli, activated T cells proliferate and undergo commitment into CD4+ (helper, Th) or CD8+ (cytotoxic T lymphocyte, Tc)

lineages (Williams and Bevan 2007; Busse et al. 2010). The level of IL-2 signaling has also been shown to affect the development of non-terminally differentiated (quiescent) CD4+ and CD8+ memory cells, which are necessary for effective immune recall upon reinfection. Highly specialized regulatory T cells (Tregs) are dependent on IL-2 for survival and express large numbers of IL-2Rα, but they are unable to produce IL-2. These cells are thought to mediate active but selective immune suppression and are critical in the maintenance of self-tolerance (Rouse and Suvas 2004; Burchill et al. 2007; Josefowicz and Rudensky 2009; Pipkin et al. 2010; Kalia et al. 2010).

2.2 Diseases and Therapies

Due to the central importance of IL-2 signaling in T-cell activation and proliferation, IL-2 agonists and antagonists have been investigated as treatments for a range of immune-cell disorders.

2.2.1 IL-2 Agonists: Oncology and Infectious Disease

Aldesleukin (Proleukin; Chiron) is a recombinant IL-2 used in the treatment of kidney cancer (Kintzel and Calis 1991; Bukowski et al. 1997; Reeves and Liu 2009). Renal carcinomas shed soluble IL-2Rα that blocks normal IL-2 responses by titrating free IL-2 (Bien and Balcerska 2008). Exogenously administered IL-2 restores normal T-cell response, combating tumor growth. Denileukin diftitox (Ontak; Eisai) is a recombinant IL-2-diptheria toxin conjugate used as a cytotoxic orphan drug in the treatment of IL-2Rα over-expressing T-cell lymphomas (Manoukian and Hagemeister 2009).

In its capacity as a potent immunostimulator, IL-2 has also been explored as an ameliorative treatment for T-cell loss as a consequence of HIV infection (Lotze et al. 1984; Kovacs et al. 1996). In spite of early promise, a recently reported long-term study suggests no real improvement in disease progression, immune function, or eventual outcome (Abrams et al. 2009). The precise reasons for this failure are not understood and may reflect the underlying complexity of normal T-cell activation or interference by the HIV itself.

2.2.2 IL-2Rα Antagonists

IL-2Rα antagonists have been considered as agents for restricting the immune response, since IL-2Rα is strongly upregulated during the immune response and establishes the IL-2-selective high-affinity receptor complex (Malek 2008). Both therapeutic antibody and small-molecule discovery programs have sought to develop IL-2Rα-selective inhibitors.

Allograft Transplantation

Anti-IL-2α treatment has found an FDA-approved home in allograft transplantation. Nonself MHC human leukocyte antigens that accompany allografts provoke a strong cell-mediated immunity response in the host. IL-2 secretion is followed by infiltration of graft tissue by cytotoxic effector T cells, leading to cytokine release that induces necrosis (Lynch and Platt 2009).

Blockade of IL-2 signaling has long been recognized as a route to achieving immune suppression and prolonging graft survival (Kirkman et al. 1985; Reed et al. 1989; Masri 2003). Prior to the discovery of therapeutic antibodies, IL-2 signaling was prevented by corticosteroids, cyclosporine (via calcineurin inhibition), and rapamycin (through the mTOR pathway) (Hardinger et al. 2004; Ponticelli 2005; Geissler et al. 2008). The current best therapies for acute rejection prophylaxis include the anti-IL-2Rα therapeutic antibodies dadizumab (Zenapax; Hoffmann-La Roche) and basiliximab (Simulect; Novartis) (Church 2003; Vincenti et al. 2004; Sandrini 2005; McKeage and McCormack 2010). These agents act through a combination of direct ligand: receptor blockade, receptor down regulation (triggering removal of IL-2Rα from the cell surface without signaling), and antibody-dependent cell-mediated cytotoxicity (ADCC) through activation and recognition by NK cells. First approved for kidney grafts in 1997, both antibodies continue to undergo clinical trials for various transplant indications (Poirier 2004; Delgado and Ross 2004; Van Gelder et al. 2004; Ensor et al. 2009).

Although they are used for pre-transplantation prophylaxis and during the acute phase of transplant rejection, dadizumab and basiliximab are not currently used for long-term maintenance of immune suppression. This usage is due to two factors. First, IL-2 signaling and CD25+ (IL-2Rα expressing) T cells are probably secondary to other cytokines and co-receptors in coordinating long-term graft survival (Nashan 1999; Kishimoto et al. 2000; Waldmann 2002; Larsen et al. 2006; Racape et al. 2009; Benghiat et al. 2009; Xu and Cao 2010). Second, it reflects an important cost–benefit relationship in transplant management. Monoclonal antibody therapeutics have proven to be enormously successful for many hard-to-treat diseases, many of which involve PPIs (Chan and Carter 2010). However, they come with several limitations and liabilities, including cost of goods, convenience of administration, potential side effects, and eventual loss of efficacy due to the production of anti-drug antibodies (De Groot and Scott 2007; Hansel et al. 2010). The slow clearance of immunosuppressive monoclonals (basiliximab half-life is ~7 days) is advantageous from a dosing perspective, but highly problematic in the event of opportunistic infection or adverse reaction. In contrast, small-molecule agents are generally cheaper, easier to administer, and easier to dose correctly. Hence, restricted use of basiliximab and dadizumab also reflects their higher cost burden in the context of effective and established small-molecule combinations. A case can therefore be made for developing selective small-molecule IL-2Rα inhibitors that would simultaneously capture the mechanistic advantages of anti-IL-2Rα therapeutic antibodies, with the convenience and lower expense of the current small-molecule drugs. On the other hand, the recent crystal structure of basiliximab in complex with IL-2Rα reveals that ~66% of the IL-2 binding

site is occupied by the antibody (Du et al. 2010). This poses the key question: can a small molecule that replicates this functionality be found?

Autoimmune Disease

The involvement of IL-2 signaling in chronic inflammatory and autoimmune diseases (rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes) is controversial (Ridderstad et al. 1991; Sadlack et al. 1993; Parkes et al. 1998; Brennan and McInnes 2008; Chistiakov et al. 2008). Certain autoimmune conditions, notably those involving inflammation of neurological tissues such as multiple sclerosis (MS) and uveitis, have been shown to be responsive to anti-IL2Rα antibodies (Dunn 2004; Martin 2008; Yeh et al. 2008; Kim 2009; Bielekova and Becker 2010). Daclizumab and basiliximab, two anti-IL2Rα antibodies approved for graft rejection, have recently been in phase II trials for MS and uveitis. Neither of these indications has reached the market, however.

3 Protein Structures

3.1 IL-2

The gene for IL-2 was cloned in 1983 (Degrave et al. 1983; Taniguchi et al. 1983), and the crystal structure (Fig. 3) determined in 1987 (Brandhuber et al. 1987). IL-2 is a compact globular protein, composed of four tightly packed α-helices adopting a down–down–up–up configuration (cytokine fold) common to many interleukins and

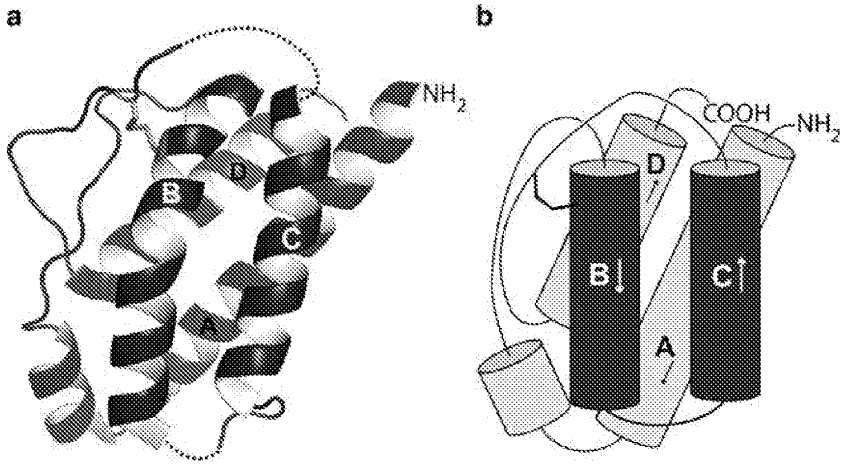


Fig. 3 Structure of IL-2. (**a**) Cartoon model of IL-2 crystal structure 1m47. (**b**) Topology of IL-2 four-helix (down–down–up–up) bundle with linking turns and extended loops

growth factors (Bazan 1990; Rozwarski et al. 1994). A single disulfide bond establishes a covalent link between helix A and the middle of a 13 residue stretch of extended peptide preceding helix D. Site-specific mutagenesis identified a set of surface residues (Lys 35, Arg 38, Phe 42, Lys 43) critical for receptor binding; these residues lie on a concave face of IL-2 whose character (hydrophobic and basic) and location were consistent with a ligand–receptor hotspot for the PPI (Sauve et al. 1991).

3.2 IL-2 Receptor

The co-structure of IL-2: IL-2Rα was determined in 2005 (Rickert et al. 2005) and confirmed the IL-2 hotspot. IL-2Rα is an elbow-shaped protein consisting of two β-sheet sushi domains – a structural fold seen frequently in complement-related proteins but rarely among cytokines (Fig. 4). The PPI is defined by a near parallel packing of IL-2 and IL-2Rα secondary structures, with 20 IL-2 ligand side chains and 21 IL-2Rα receptor residues burying an area of ~1,900 $Å^2$. The residues contributed by each protein to the interface are derived from sequential and nonsequential portions of their primary sequences, leading to a discontinuous contact surface.

The IL-2: IL2Rα hotpot is composed of hydrophobic patches, including IL-2 side chains Phe 42 and Leu 72, projecting into a complementary cavity formed by Leu 42, Tyr 43, and Met 25 on the surface of IL-2Rα, and a buried salt-bridge between Glu 62 (IL-2) and Arg 36 (receptor). Numerous polar and salt-bridge interactions surround the hotspot. There are few differences between the free and receptor-bound IL-2 backbone (Cα main chain) conformations, with the exception

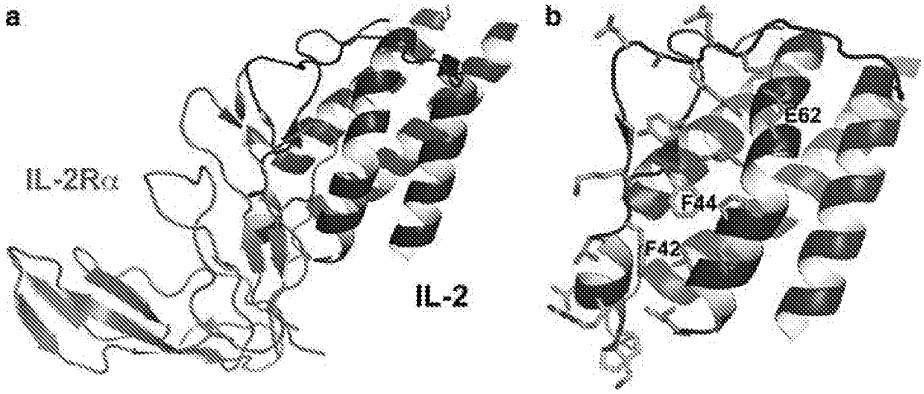


Fig. 4 IL-2: IL-2Rα cocrystal structure 1z92. (**a**) The two sushi domains of IL-2Rα (*blue*) forming an elbow structure. (**b**) Key side chains that form the IL-2 portion of the protein–protein interface. Phe 42 and 44 define a hydrophobic center, surrounded by polar and charged groups, including Glu 62

of an extended solvent-exposed loop (containing Cys 105) that becomes ordered and contributes polar residues in the ligand:receptor complex. Subtle changes are observed, however, for several IL-2 side chains that flip into new conformations in the complex; for example, Lys 35 forms a salt-bridge with the receptor side chain of Asp 4 and main chain of Leu 2.

A similar cytokine:sushi domain interaction defines the recognition of IL-15 by IL-15-Rα, but the binding surface is physically and chemically very different (Chirifu et al. 2007). The contact area is ~30% smaller and is much more hydrophilic, with a pronounced acidic groove on IL-15 binding to a highly basic protrusion on IL-15Rα. Several water molecules are thought to enhance the interaction, resulting in much tighter (picomolar versus nanomolar for the IL-2:IL-2Rα) association. The IL-2:IL2Rα interface appears, therefore, to be unique among the interleukins.

The structure of the quaternary, high-affinity, and biologically active complex (IL-2:IL-2Rα:IL-2Rβ:IL-2γ) was published 5 months after the IL-2:IL-2Rα structure, and shed unprecedented light on the organization, physical inter-relationships, and likely mechanism of IL-2 activity (Fig. 5) (Wang et al. 2005). The IL-2:IL-2Rα interaction is unchanged from the previously described binary ligand:receptor structure. It is significant that IL-2Rα does not interact with IL-2Rβ or IL-2Rγ; it functions to present the IL-2 ligand to the β- and γ-receptor subunits responsible for signal initiation.

The β-subunit consists of an immunoglobin-like fibronectin type III β-sandwich fold common to cytokine receptors (Wang et al. 2009). The IL-2:β-subunit interface covers a smaller surface area (~1,350 $Å^2$) and contains numerous buried water molecules. IL-2Rγ also adopts an immunoglobulin-like fold. The IL-2:γ-subunit interface is smaller still (~970 $Å^2$) and comparatively flat; the interaction surface lacks specific side chain contacts and provides generic interaction features in keeping with its role as a common chain shared by many interleukin receptors. β- and

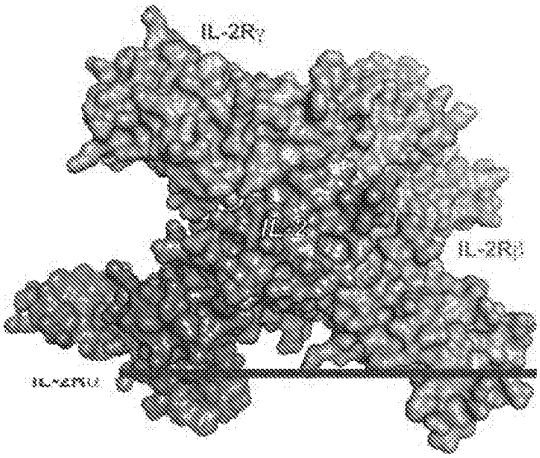


Fig. 5 IL-2: receptor quaternary complex (2erj). In contrast to the β- and γ-subunits (*green* and *pink*, respectively), IL-2Rα (*blue*) interacts exclusively with IL-2 cytokine

γ-receptor subunits also share an interface consisting of a core hydrophobic strip surrounded by hydrogen bonds. However, since isolated IL-2Rβ and IL-2Rγ extracellular domains have no measurable affinity (Rickert et al. 2004), this interaction may depend on cooperative associations with IL-2, β-, and γ-subunits within the context of the plasma membrane. With unique structural and functional roles, the IL-2:IL-2Rα interaction appears to be both the most tractable and biologically relevant for small molecule intervention and discovery.

4 IL-2 Small-Molecule Inhibitors

4.1 Discovery of Ro26-4550

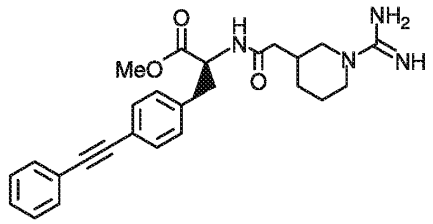
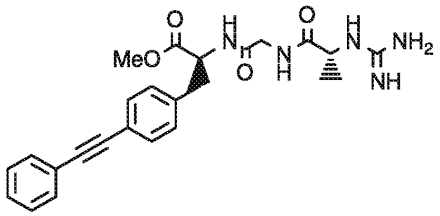
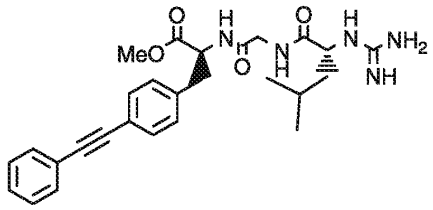
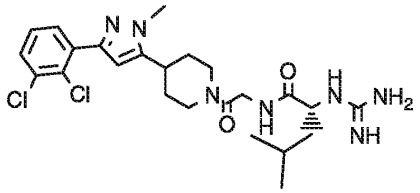
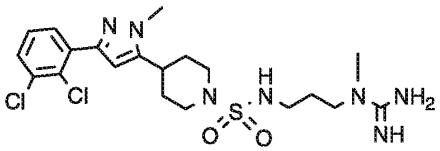
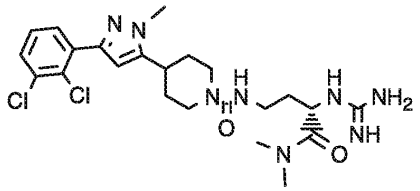
The first small molecule shown to inhibit the IL-2:IL-2Rα interaction was reported by Roche (Nutley, NJ) (Tilley et al. 1997). Acylphenylalanine analogs were designed to mimic the binding regions of IL-2 identified by mutagenesis and therefore to bind to IL-2Rα. However, HSQC NMR showed that these compounds bound to IL-2 itself (Emerson et al. 2003). The published example, Ro26-4550 (Table 1, compound **1**), was an enantiomer-specific, competitive inhibitor of IL-2Rα with an IC_{50} of 3 μM. In addition to HSQC NMR (Tilley et al. 1997), hydrogen/deuterium exchange measurements established that the compound interacted with a specific subset of residues defined by the IL-2Rα contact site, while pH titrations indicated a likely orientation with the guanidinium group forming a salt-bridge with Glu 62 (Emerson et al. 2003). Although of modest affinity and limited development potential as a therapeutic, Ro26-4550 was the first example of a small-molecule cytokine/receptor PPI inhibitor, and a major proof-of-principle that such interactions could potentially be drugged.

4.2 Structural Characterization of Ro26-4550 and the Importance of Protein Dynamics

Ro26-4550 was taken up by Sunesis Pharmaceuticals (South San Francisco, CA) as the starting point for detailed structural characterization and PPI inhibitor discovery. Through these studies, the small-molecule-binding potential of IL-2 was revealed to be more subtle and dynamic than anticipated (Braisted et al. 2003; Hyde et al. 2003; Raimundo et al. 2004; Waal et al. 2005; Arkin et al. 2003; Thanos et al. 2003, 2006).

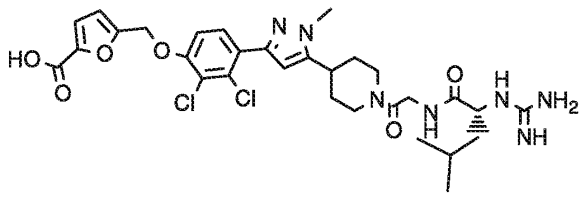
The X-ray structure of the IL-2:Ro26-4550 complex displays marked alterations in protein conformation from unliganded IL-2 (Arkin et al. 2003). This result contrasts with the determined protein co-structures described above, which show few differences in IL-2 upon binding receptor subunits. Where the free- and receptor-bound IL-2

Table 1 Structure–activity relationships among IL-2 inhibitors. Exploration of hydrophobic and polar (basic) groups of the initial Roche hit (1) established chemical and structural preferences, which led to compound SP4206 (7)

Compound number	Structure	IC_{50} (μM)
1 (Ro26-4550)		6
2		280
3		9
4		2
5		14
6		7

(*continued*)

Table 1 (continued)

Compound number	Structure	IC_{50} (μM)
7 (SP4206)		0.06

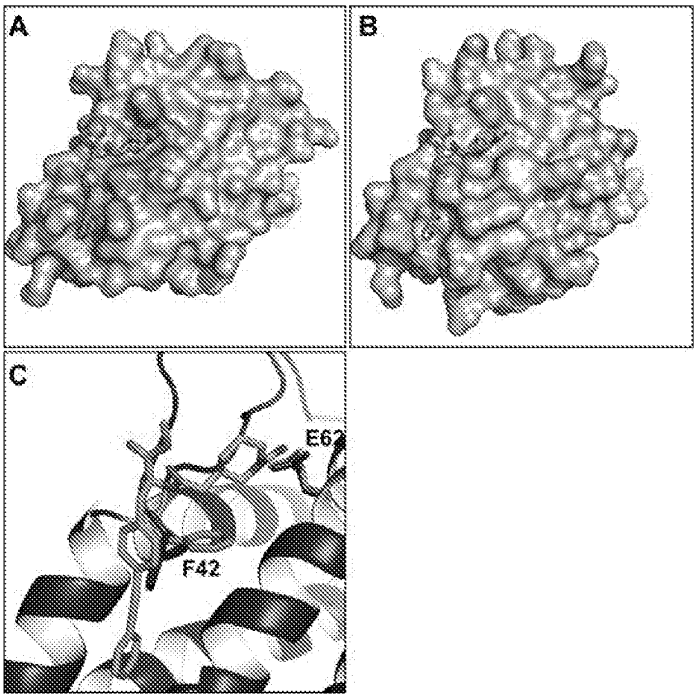


Fig. 6 Binding of Roche small-molecule inhibitor. (**a**) Structure of Ro26-4550 bound to IL-2 (1m48). Protein atoms in contact with the small molecule are colored *orange*. (**b**) Ro26-4550 mapped onto unliganded coordinates 1m47, illustrating the significant surface remodeling that takes place upon small-molecule binding. (**c**) Changes in Phe 42 and Glu 62 side chain conformations from unliganded (*black*) to Ro26-4550 bound (*orange*)

presents a largely flat surface, Ro26-4550 binds in a groove on IL-2 formed primarily by surface side chain rearrangements, notably Phe 42, and slight main chain movement in the short helix associated with Lys 35. This new IL-2 surface buries ~60% (450 $Å^2$, Fig. 6) of the small-molecule surface area. The biaryl alkyne motif is bound in a hydrophobic pocket composed of Arg 38, Met 39, Phe 42, Leu 72, and Lys 43, which are also critical to IL2-Rα recognition. A second, acidic subsite is centered on glutamate 62. Glu 62 forms the anticipated salt-bridge with the compound's piperidyl

guanidine group, which acts as surrogate for IL2-Rα Arg 36. Unlike the hydrophobic pocket, this region does not undergo significant conformational change.

Although the core of IL-2 is essentially rigid, the protein surface is comparatively dynamic and accommodates a set of selective small-molecule interactions. Significantly, these contacts involve the same residues that interact with the IL-2 receptor (Rickert et al. 2005). Thus, the structure of IL-2 bound to Ro26-4550 indicates that small molecules can bind to protein–protein interface at the hotspot, using regions of the hotspot that are inherently adaptive. This theme had recently been appreciated for Fc domains, which were shown to bind several protein and peptide ligands using various conformations of the same hotspot residues (DeLano et al. 2000; Ma et al. 2002).

4.3 Ligand-Binding Potential and Surface Plasticity of IL-2

The ligand-binding potential of the hydrophobic and acidic subsites was explored using the tethering method of fragment discovery (Erlanson and Hansen 2004; Erlanson et al. 2000). Tethering uses a library of disulfide-exchangeable fragments to select compounds that bind to a site of interest near a native or engineered cysteine residue (Fig. 7). Because the binding of the fragment is stabilized by the reversible disulfide bond, tethering is especially powerful for identifying very weak interactions and enables the exploration of binding sites not easily accessible to NMR, SPR, or functional screening approaches. Sunesis used the disulfide-trapping

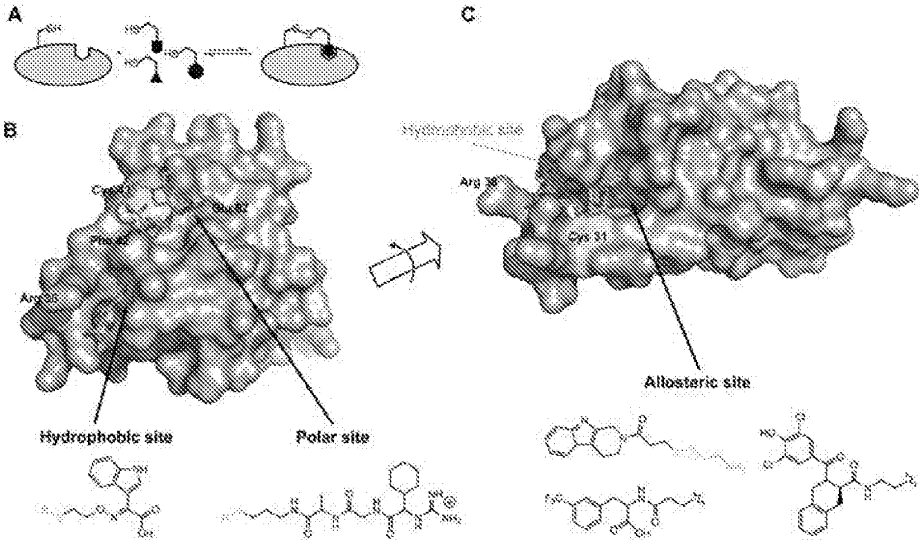


Fig. 7 Small-molecule-binding sites of IL-2 explored through tethering. (**a**) Principle of tethering through disulfide exchange. (**b**) Representative tethering hits and binding sites superimposed on IL-2 (composite of structures 1m4a, 1m4b, 1nbp). (**c**) Model rotated to reveal the allosteric site

method extensively in lead discovery (Erlanson et al. 2003a; Yang et al. 2009; Cancilla et al. 2008) and also reported examples in which tethering identified previously unknown sites that allosterically regulated protein activity (Erlanson et al. 2003b; Hardy and Wells 2009).

Using a series of functionally benign cysteine substitutions that surround the hydrophobic (Phe 42) or acidic (Glu 62) sites on IL-2, libraries of thiol fragments were screened for disulfide exchange labeling by mass spectrometry (Arkin et al. 2003). Tethered fragment hits were rare (0.1–1%), with the hydrophobic subsite showing a preference for small aromatics and particularly for aromatic acids (Fig. 7; see Sect. 4.4). In contrast, the polar site displayed strict linker length and basic group requirements, consistent with angle and distance constraints associated with forming the Glu 62 salt-bridge.

Following the theme of structural adaptivity, Sunesis researchers then investigated whether Ro26-4550 was capable of inducing structural changes within IL-2 that enabled allosteric binding of a new set of tethered fragments at a theoretical "cryptic" site (Fig. 7b) (Hyde et al. 2003). In these screens, fragments were selected for binding to one of three cysteine mutants in the hydrophobic subsite (N30C, Y31C, or N33C), in the presence and absence of Ro26-4550. Forty-four fragments showed increased tethering in the presence of Ro26-4550. A series of disulfide-trapping experiments demonstrated that these fragments and Ro26-4550 were mutually synergistic, increasing the binding affinities of fragment and R026-4550 by 3- to 30-fold. When the exchangeable disulfide was removed from these fragments, SPR data confirmed the tethering experiments; fragments bound stoichiometrically and reversibly to IL-2 only in the presence of Ro26-4550. Crystals were then grown in the presence of Ro26-4550 and a synergistic fragment. Interestingly, the structure solved from these crystals showed no density for the Ro26-4550, and the IL-2Rα-binding site was in an intermediate conformation between unliganded and small-molecule liganded structures. Nevertheless, the tethered fragment showed strong density and was deeply buried in a previously unknown hydrophobic site formed by helix A residues Met 23, Ile 24, Gly 28 and the following loop, and helix D residues Leu 70, Leu 80, Pro 82, and Leu 85. This new fragment-binding site overlapped with the IL-2Rβ-binding site defined by helix A residues Leu 12, Leu 19, and Met 23 and helix D residues Leu 85, Val 91, and Glu 95.

Computational simulations of IL-2 have provided important clues toward understanding the ability of Ro26-4550 and fragments to bind synergistically (McClendon et al. 2009). Equilibrium molecular dynamics (MD) simulations revealed highly correlated side chain and subtle backbone movements that form tightly coupled networks. For example, simulation of a fragment occupying the allosteric site led to conformational changes in Phe 42 that effectively order the Ro26-4550 interaction site and facilitate binding. This observation was consistent with the X-ray structure that showed Phe 42 in an intermediate position in the presence of the synergistic fragment. Similarly, the MD simulations suggested that binding at the IL-2Rα-binding hotspot triggered a cascade of small conformational changes that led to the opening of the cryptic site.

While the biological significance of small-molecule-induced allostery is unclear, it is interesting to speculate on the overlap between the allosteric site and the IL-2Rβ recognition surface (Wang et al. 2005). The transmission of binding events across IL-2 suggests the possibility of an IL-2Rα interaction facilitating the subsequent binding of IL-2Rβ. IL-2 might therefore act as an active, rather than passive mediator of receptor complex assembly. Inspection of available structures is inconclusive, largely because there is no IL-2:IL-2Rβ binary structure for comparison. The available IL-2 structures reveal no major differences beyond a handful of alternative side chain rotamers (notably His 16) in the IL-2Rβ-binding region. In a more general sense, however, these discoveries raise the enticing possibility that other PPI domains possess hidden allosteric potential that can be exploited for drug discovery (del Sol et al. 2009).

4.4 Medicinal Chemistry Optimization of IL-2 Antagonists: SP4206

A fragment-minded approach was used to evolve the Ro26-4550 scaffold into a more potent and drug-like inhibitor of IL-2:IL-2Rα (Table 1) (Raimundo et al. 2004). Ro26-4550 could be seen as two fragments – a hydrophobe and a piperidinyl guanidine – linked by an amide bond. When deconstructed, the guanidine fragment did not show measureable binding to IL-2, while the hydrophobic biaryl fragment bound with a $K_d \sim 3$ mM. Starting with this weakly bound fragment, the piperidinyl moiety was replaced by attaching a small library of new linker/guanidine fragments to the biaryl hydrophobe. The resulting chemical series, with a guanidine-containing unnatural dipeptide, was equipotent to Ro26-4550 and bound in the same site (compound **3**). This dipeptide fragment was then used as the anchor for a hydrophobic library. Again, the most potent compounds from this series bound analogously to the parent compound, but the resulting series was much more synthetically tractable and served as a promising point for lead optimization (compound **4**). A third set of libraries sought to remove the peptidic character of the linker and to improve potency. Waal et al. tested a series of aliphatic linkers to connect the hydrophobic tricycle and the guanidine; although two aliphatic series showed similar levels of binding to IL-2, tighter binding compounds were not identified (compounds **5**, **6**) (Waal et al. 2005). Throughout the optimization process, compounds were tested for both activity (inhibition of IL-2:IL-2Rα) and binding to IL-2. Binding measurements included biophysical methods (SPR, analytical ultracentrifugation) and structural approaches (^{1}H-^{15}N HSQC NMR, X-ray crystallography) (Braisted et al. 2003; Raimundo et al. 2004; Arkin and Lear 2001).

Three observations from this scaffold-hopping exercise are noteworthy for PPI inhibitor discovery. First, the initial hits inhibited IL-2 weakly (compound **2** IC_{50} ~300 μM) yet showed distinct structure–activity relationships and biophysical properties that were entirely consistent with the more potent analogs. Thus, by monitoring both the activity and biophysical properties of ligands, one had confidence that the series was "real" and not an artifact. The solubility afforded by the

guanidine also greatly facilitated these measurements. Second, the affinities of the two fragments were very weak, but both were critical to achieving tight binding in the linked series. Significant structural changes could be made to both halves, provided that the distance and conformation between them allowed deep hydrophobic contact on one side and strong hydrogen bonding on the other. The details of hydrogen bonding and linker chemistry were less important. Third, the highest potency achieved after extensive medicinal chemistry was ~2 μM. This affinity appeared to represent the maximum binding energy inherent in these two binding sites.

To improve binding affinity, the researchers sought to identify a third pharmacophore that could be appended to compounds such as **6** (Table 1). Using the disulfide-trapping (tethering) approach, ten individual cysteine mutations surrounding compound **6**'s binding site were screened against 7,000 disulfide-containing fragments (Braisted et al. 2003). The most hit-rich cysteines were those near the adaptive hydrophobic region; in particular, Leu72Cys was found to select a high percentage of aromatic acid fragments. Modeling fragments tethered to Leu 72 in the **6**:IL-2 structure suggested that these aromatic acids sat in a pocket adjacent to the terminal phenyl ring of compound **6**, and that these pharmacophores could be linked through a two-atom spacer. A small library was thus prepared, and the hypothesis was borne out; all compounds containing an aromatic acid linked by two atoms had sub-micromolar activity (Braisted et al. 2003; Raimundo et al. 2004). The most active compound **7** (SP4206) had an IC_{50} = 60 nM. As shown by the X-ray structure of SP4206:IL-2, this compound preserves the key guanidinium and hydrophobic regions, with a second charged group (furanoic acid) contributing further electrostatic interactions within the Lys 32, Lys 35, Arg 38, and Lys 43 region (Fig. 8) (Thanos et al. 2003). SP4206 has the striking appearance of a molecule tailored to embed in the IL-2 surface.

Initial studies of SP4206 as a drug lead were promising (Raimundo et al. 2004). SP4206 inhibited intracellular phosphorylation of STAT5 mediated by IL-2 with an EC_{50} = 3 μM; the compound series showed cell-based activity that tracked with binding affinity. SP4206 also showed promising early PK properties (terminal half-life of 2.1 h in mice, clearance 13.6 ml/min/kg, and volume of distribution of 1.1 l/kg). The lead compound did not show significant cell permeability, perhaps due to its high molecular weight (662 Da) and zwitterionic nature. Unfortunately, concerns about the clinical value of IL-2Rα antagonists led to closure of this lead discovery program before the researchers were able to assess whether they could surmount the known liabilities and create a small-molecule inhibitor of IL-2 that had in vivo activity.

4.5 Comparison of Small-Molecule and Protein Interactions with IL-2

The Roche and Sunesis small molecules were among the first PPI inhibitors to have their mechanism of action understood in atomic detail, and it is very instructive to

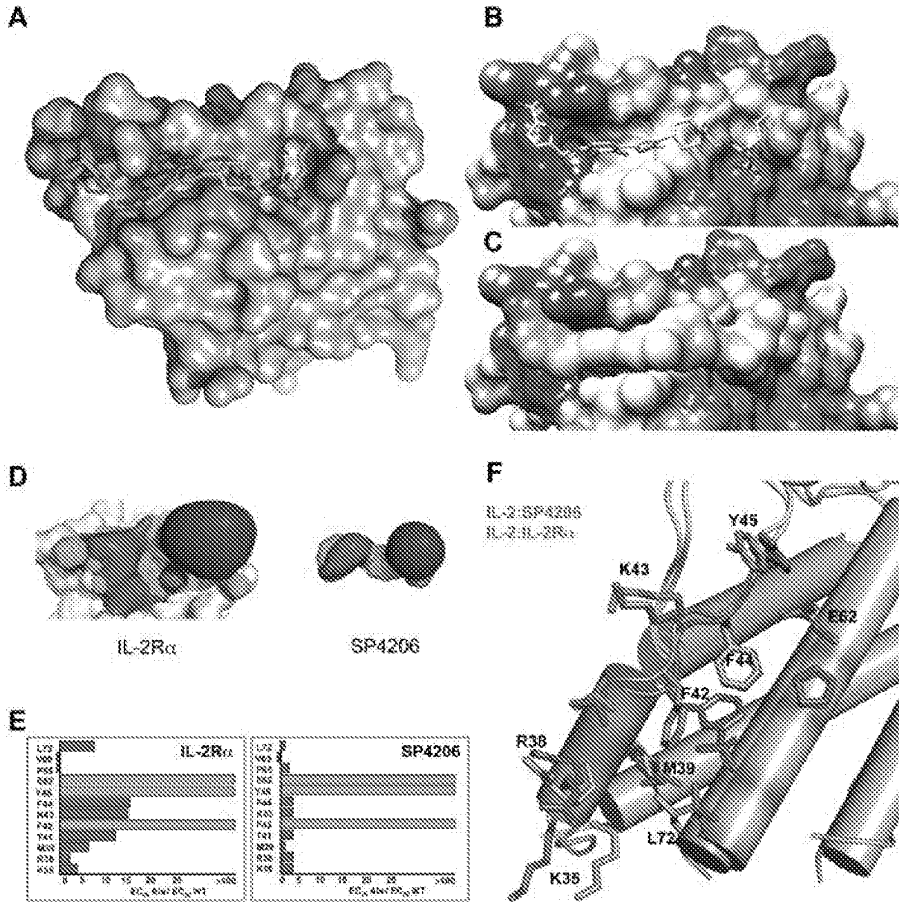


Fig. 8 Chemical and structural mimicry of IL-2 small-molecule inhibitors. (**a**) Crystal structure of Sunesis compound SP4206 bound to IL-2 (1py2). (**b**) Surface electrostatic potential of IL-2 with SP4206 shown as a *stick cartoon* (*blue* = basic, *red* = acidic), surfaces generated using a default Amber force field (Cornell et al. 1995) with Chimera (Pettersen et al. 2004). (**c**) Complementarity of IL-2 and SP4206 surface potentials. (**d**) Similarity in surface potential between the biological receptor IL-2Ra and the small molecule SP4206. (**e**) Hot spot analysis of IL-2 receptor and SP4206 binding by alanine-scanning mutagenesis. The same key residues (Phe 42, Tyr 45, Glu 62) of IL-2 are critical to both protein and small-molecule recognition. (**f**) Overlay of interface side chain conformations (SP4206 bound, 1py2; IL-2Rα bound, 1z92). Receptor and small-molecule recognition exploit a range of similar (Phe 44, Glu 62) and highly dissimilar (Arg 38, Lys 35, Met 39, Phe 42, Leu 72) side chain rotamers to achieve binding, which approximate to the polar and hydrophobic binding pockets

compare these small molecule:protein interactions to the IL-2:IL-2Rα interaction itself. Beyond occupying a common region of the IL-2 surface, receptor and inhibitors appear at first to share little, and their gross differences (e.g., the receptor domain is an order of magnitude greater in mass) might be expected to dominate the

interaction mechanism. In fact, a number of critical similarities, combined with subtle differences, collectively enable the small molecules to recapitulate key properties of the larger protein interaction partner.

As ligands of IL-2, both IL-2 receptor and small-molecule compounds use combinations of peripheral, complementary polar interactions linked by central hydrophobic groups. In spite of their large size disparity, the polar charge distributions of receptor and small molecule interfaces are very similar (Fig. 8b–d) (Thanos et al. 2006). In the receptor:IL-2 interaction, the electrostatic fields are defined by a string of acidic groups (glutamate and aspartic acid) on IL-2Rα and a basic Arg 36 that map onto complementary basic (Lys 35, Arg 38) and acidic (Glu 62) patches, respectively, on the surface of IL-2. In the SP4206:IL-2 complex, furanoic acid takes the place of the IL-2Rα acidic patch, and the guanidinium group takes the places of the basic Arg 36. Hence, SP4206 and IL-2Rα supply similar electrostatic groups and form analogous salt-bridge interactions. While the receptor presents a larger surface area, SP4206 effectively mimics the spatial distribution that is critical for charge-based interactions.

Structural adaptivity is also a major theme illustrated by IL-2 small molecules. While receptor binding is accompanied by few changes in IL-2 structure, small-molecule binding results in more significant remodeling of the cytokine surface (Fig. 8f). The most noticeable is Phe 42, which flips from an "outward" rotamer in both apo and receptor-bound structures to an "inward" conformation with SP4206 bound. Phe 42 defines critical hydrophobic interactions in both PPI and protein: drug complexes, but does so through completely different conformations. With IL-2Rα, Phe 42 binds a hydrophobic cavity on the receptor and undergoes no apparent motion. In the case of SP4206, the inward rotation of Phe 42 opens a hydrophobic channel that is filled by the apolar linker of SP4206. Tyr 45 displays a similar tendency to occupy a unique conformation in the presence of small molecule, although the shift is less dramatic. Some IL-2 side chain movements appear to be shared by receptor and small molecule, notably the orientations of Lys 35 and Arg 38 that contribute to the basic patch exploited by both ligands. IL-2 small-molecule inhibitors can be thought of as exploiting a combination of receptor-like mimicry and novel means to achieve binding, through repurposing and inducing new structural features.

These similarities and differences are underscored by alanine-scanning mutagenesis data for receptor and small-molecule binding (Fig. 8e) (Thanos et al. 2006). Both share the same key hotspot residues on IL-2 (red bars). On the other hand, the receptor displays graded effects for peripheral interface residues (blue bars, e.g., Met 39, K43, and Phe 44) reflecting more distributed and potentially redundant interactions, while the effect of mutations on SP4206 binding is bimodal and restricted to its smaller footprint. The hotspot for SP4206 on IL-2 is therefore more concentrated than for IL-2Rα.

This observation is especially noteworthy when considered from the perspective of ligand efficiency (LE, the binding energy per heavy atom) (Hopkins et al. 2004). Compared to the biological receptor, IL-2 small-molecule inhibitors are significantly more efficient agents of molecular recognition. With a K_i of 68 nM and heavy

atom count of 45, SP4206 has an LE value of 0.22, which is comparable with mean values for typical small-molecule ligands (0.24–0.26) (Wells and McClendon 2007; Reynolds et al. 2007). In contrast, the contact interface of IL-2Rα has a calculated LE of 0.08, a typical value for PPIs. The higher ligand efficiency of IL-2 inhibitors demonstrates the potential for small molecules to capture equivalent – or at least representative – properties of much larger ligands, and occasionally to uncover entirely new interaction modes inaccessible to proteins (Wells and McClendon 2007).

5 Themes from IL-2 Small-Molecule PPI Inhibitors: Lessons Learned and Applied

5.1 Target Dynamics and Surface Plasticity

The importance of conformational change has long been understood as a defining feature of molecular recognition. Recently, biophysics, protein folding, and MD simulation have exposed the significance of coupled interaction networks and conformational ensembles to PPIs (Betzi et al. 2009; McClendon et al. 2009; Eyrisch and Helms 2007, 2009; Boehr et al. 2009). Rather than exploring a continuum of random, independent conformation states, protein surfaces are now known to display coupled motions that define ordered ensembles. Transient pockets and cavities, opening and closing on rapid (picosecond) timescales, have been detected. The earliest events in ligand recognition appear to exploit these preexisting distributions and induce further concerted shifts in side chain conformations that favor binding. These principles are demonstrated by both the primary (SP4206) and secondary (allosteric) binding sites of IL-2 (Hyde et al. 2003; Arkin et al. 2003; McClendon et al. 2009); interaction between inhibitor and the protein surface triggers local, as well as distant, changes in structure without incurring large, energetically expensive motions. Ligand binding is therefore less a matter inducing fit on a conformational blank canvas, and more a selection process that realizes latent potential within the protein surface.

5.2 Inhibitable Surface Epitopes

PPIs were long thought to be undruggable by small molecules because of their highly dispersed binding contacts. The discovery of interaction hotspots indicated that smaller – and therefore more druggable – sites might exist within PPI interfaces (Arkin and Wells 2004; Wells and McClendon 2007). Analysis of PPI surfaces has established general trends in composition, suggesting branched hydrophobic side chains and aromatic residues are preferred, with lower percentages observed for

charged and polar groups (Sperandio et al. 2010; Pagliaro et al. 2004; Fry 2006). However, the context of polar groups can be critical for establishing affinity and specificity, as with the Glu 62 buried salt-bridge in the IL2:IL-2Rα and IL-2: SP4206 complexes. The secondary structure of the protein interface can also be important for small-molecule binding; the IL-2 inhibitors bind at a preexisting shallow groove arising from secondary structure interfaces (strand and helix packing against helix) and access a dynamic region made of loops and clusters of long (Arg, Lys), branched (Met, Leu, Val), and aromatic (Phe, Tyr) side chains. Other small molecules make similar use of grooves and dynamic regions at the junction of two structural elements (Laskowski et al. 1996; Glaser et al. 2006).

In the literature, most small-molecule inhibitors of PPIs target interfaces that are comprised of short linear peptide sequences or helical motifs (Wells and McClendon 2007; Fuller et al. 2009). Peptide-binding surfaces might be inherently more druggable than other PPI because they are more concave or smaller, or because small-molecule scaffolds can be designed to mimic the periodic display of peptide side chains. By contrast, the IL-2:IL-2Rα interaction is an example of a much larger, highly discontinuous PPI epitope involving at least 13 residues from three separate IL-2Rα peptide segments. The interaction hotspot is, however, defined by only three amino acids from two segments (Phe 42, Tyr 45, and Glu 62). The overall size, discontinuity, flexibility, and complexity of an interface do not necessarily limit the potential of a small molecule in preventing recognition, but could make ligand design much more difficult.

5.3 Inhibitor Ligands: Shape, Composition, and Construction

The lead molecules in the IL-2:IL-2Rα story highlight a number of themes common to nearly all PPI inhibitor problems. Hit identification and development frequently results in molecules that possess characteristic shapes, a tendency toward significant hydrophobicity, and the linkage of several low-affinity pharmacophores.

5.3.1 Shapes

The topology of inhibitors has emerged as one of the strongest themes from PPI inhibitor discovery, with linear I- and L-shaped structures or branched E-, X-, and T-shaped molecules being common (Sperandio et al. 2010; Fuller et al. 2009; Reynes et al. 2010). These multipronged scaffolds are consistent with binding to several smaller subsites found on PPI. In the case of IL-2, small-molecule inhibitors are V- and L-shaped molecules that satisfy complementary features (explicit or latent) on the protein surface.

Comparison of two small-molecule mimics of α-helical ligands is also instructive. Bcl-xL and MDM2 both recognize helical motifs in their partner proteins (pro-apoptotic Bak1 and p53) and act to prevent apoptosis of damaged cells

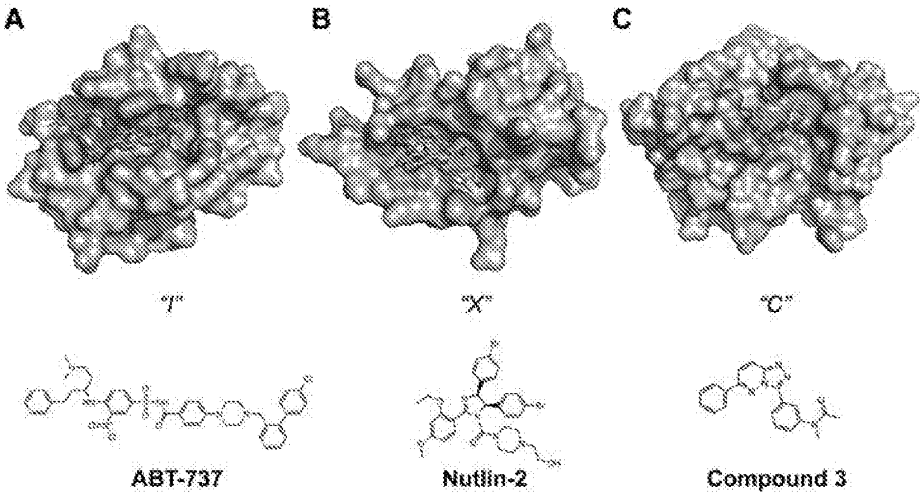


Fig. 9 Protein–protein interaction inhibitors and their structures. Inhibitors of (**a**) Bcl-xL (2yxj), (**b**) MDM2 (1rv1), and (**c**) ZipA (1y2g) illustrate the recurring chemical and topological themes of hydrophobicity, aromaticity, and extended, star-shaped and C-shaped compounds

(Lomonosova and Chinnadurai 2008; Kruse and Gu 2009). They are both therefore potentially valuable targets for inducing death in tumor cells. Small-molecule inhibitors have been identified for both targets, using either NMR-based fragment screening and fragment linking (for Bcl-xL) (Oltersdorf et al. 2005; Stauffer 2007) or a combination of computer-aided ligand searches and HTS (for MDM2) (Vassilev et al. 2004; Vassilev 2007). Crystallized inhibitors of BCL-xL (ABT-737) and MDM2 (Nutlin-3) share some general features with each other and with SP4206: they mimic the key interactions of their natural helical ligands, bind through predominantly hydrophobic interactions, and induce new structural features (widened grooves and deeper cavities) in their receptors. However, the shapes of these inhibitors are very different; ABT-737's extended shape corresponds with the long, but shallow groove in the surface of Bcl-xL, while Nutlin-2 (and many other MDM2 inhibitors) is star-shaped and reflects the smaller binding pocket of the target protein (Fig. 9). Contrasting both of these, the small-molecule inhibitors of the bacterial ZipA/FtsZ protein–protein interaction are compact, C-shaped molecules that bind within the shallow surface curvature of an antiparallel β-sheet (Rush et al. 2005).

5.3.2 Chemical Character and Ligand Efficiency

The SAR of IL-2 inhibitors shows an established tendency for affinity to increase with both molecular weight and hydrophobicity. This trend is often true during small-molecule lead optimization, and reviewers have noted that ligand efficiency remains similar throughout optimization (Hopkins et al. 2004). Furthermore, LE is

similar for leads within a target class, irrespective of the structure or the specific protein; thus, LE for PPI is ~0.24, whereas LE for kinase inhibitors ranges from 0.35 to 0.45. LE might be particularly low for PPI surfaces because they have relatively few of the combined chemical (such as buried salt bridges) and physical features (cavities) necessary for the highest affinity interactions (Wells and McClendon 2007). Similarly, the observation that PPI contain multiple, small subsites suggests that larger compounds will be needed to link fragments across these sites (Fuller et al. 2009).

Increasing hydrophobicity can, however, lead to nonspecific interactions with the target and/or with other molecules. For instance, Shoichet and others have thoroughly characterized an aggregation phenomenon, whereby compounds form large, spherical structures that adsorb and inhibit proteins with low specificity (Coan and Shoichet 2008; Coan et al. 2009). In the case of the IL-2 inhibitors, systematic medicinal chemistry revealed strong structure–activity relationships in terms of functional groups, stereochemistry, linker length, and linker composition, all of which could be rationalized through experimentally determined crystal structures. Even in the absence of crystallography, the binding affinity, stoichiometry, and general binding site were closely monitored by biophysical assays (e.g., SPR and NMR). We strongly advocate for careful analysis of the mechanism of inhibition early in any lead discovery process, but this analysis is particularly important for PPI, where early hits tend to be larger and more hydrophobic than average.

5.3.3 Cooperativity Through Fragment Linking

SP4206 and related molecules contain the hallmark features of successful PPI inhibitors built through the assembly of low molecular weight, low-affinity compounds (Arkin and Wells 2004; Erlanson and Hansen 2004; Coyne et al. 2010). The key pharmacophores for IL-2 (guanidinium, extended aromatic group, and furanoic acid) are covalently connected so that their binding is coupled and individually weak interactions act cooperatively. While the guanidine and acid fragments exploit electrostatic complementarity, the contributions of the linkers are also highly significant. First, they impose distance, angle, and stereochemical constraints on the linked fragments, giving rise to a highly specific interaction. Second, linkers are far from passive connectors and can contribute directly to the efficacy of the molecule (Schuffenhauer et al. 2005; Rohrig et al. 2007; Chung et al. 2009).

Finding routine approaches for linking fragments remains an important challenge. Linker groups frequently reflect available chemical building blocks, with comparatively easy ligations (e.g., acylations that yield amide bonds) appearing as a common feature in early fragment optimization. In the IL-2 case, unnatural peptidic linkers allowed rapid parallel synthesis and yielded novel inhibitors; however, more complex chemical strategies are sometimes required. Structure-based design and computational modeling of linkers can identify potential solutions (Mauser and Stahl 2007;

Law et al. 2009; Ertl and Schuffenhauer 2009); however, synthetic tractability is still a significant hurdle.

5.4 Screening and Characterization Tools

The development and widespread availability of biophysical methods have clearly facilitated small-molecule PPI inhibitor discovery (Arkin and Wells 2004; Boehm et al. 2000; Carr et al. 2005; Renaud and Delsuc 2009). The examples described above have made extensive use of the structure and mechanism-oriented approaches to build a sophisticated quantitative understanding of the small-molecule inhibitor interaction. Approaches – such as X-ray crystallography, ITC, SPR, or NMR – that were once considered a luxury to be used late in lead development are now valued as integral to the earliest stages of ligand discovery. Furthermore, these methods are increasingly used in a complementary manner: while crystallography remains the gold standard for structural description, NMR and SPR enable access to dynamics, stoichiometry, and binding kinetics of small molecule–protein interactions. Together with binding thermodynamics derived from isothermal titration calorimetry, these collectively form a more complete picture of the inhibitory mechanism that informs rational ligand optimization.

5.5 Computational Methods

Virtual screening has long been an appealing strategy for drug discovery, but has proven complicated for even classical enzyme active sites (Brewerton 2008; Zoete et al. 2009; van Montfort and Workman 2009; Villoutreix et al. 2009; Cross et al. 2009). Computational simulations have aided the rationalization of experimentally determined structure–activity relationships and have guided subsequent medicinal chemistry, but successful de novo prediction is rare. A majority of current approaches make extensive use of existing data from related or similar molecules, from which protein–ligand interaction fingerprints can be extracted and used to train docking procedures.

Due to their high value and detailed structural characterization, Bcl-xL, MDM2, and IL-2 have all been used for successful validation of virtual screening methods (Betzi et al. 2009; Sperandio et al. 2010; Casey et al. 2009; Fuller et al. 2009; Eyrisch and Helms 2007, 2009; Enyedy et al. 2001; Bowman et al. 2007; Mukherjee et al. 2010). Bcl-xL and MDM2, in particular, have attracted special attention for *in silico* screening against molecules in existing chemical libraries. Numerous small-molecule alternatives that mimic (at least computationally) lead compounds have been identified, and these await validation through experimental characterization. It is important to remember, however, that Bcl-xL and MDM2 both bind continuous helical epitopes and possess shallow grooves that are clearly visible in unliganded structures. In

contrast, IL-2 presents the more stereotypically flat, featureless PPI interface and is probably a more challenging virtual target.

In the general case, where small-molecule inhibitors are not yet known, computational approaches are still more challenging. Since PPI often lack obvious cavities, docking must be preceded by a search for potentially dockable (bindable) cavities and grooves. Virtual screening has demonstrated particular promise for docking fragments, and researchers are actively working to integrate virtual screening and fragment discovery for PPI inhibitors (Betzi et al. 2009; Fuller et al. 2009; Reynes et al. 2010; Vajda and Kozakov 2009; Brenke et al. 2009). The convergence of PPI surface simulation, target-optimized docking procedures, and PPI inhibitor-oriented virtual compound collections presents an exciting opportunity to drug the hardest of PPI targets.

6 Conclusions

The IL-2:IL-2Rα interaction inhibitors highlight several key advances in our understanding of PPIs and serve as a useful background against which to view wider developments in the territory of high-risk difficult-to-drug target discovery.

PPIs are clearly druggable (or at least inhibitable), and their surfaces are not nearly as featureless or barren to small-molecule interactions as was once thought. However, their druggability is highly variable, with some targets and perhaps entire target classes presenting a greater challenge by virtue of the structure and the complexity of their interfaces. General patterns, trends, and rules are beginning to emerge from biology and chemistry. These should soon enable the prediction of target druggability and facilitate the design, synthesis, and matching of PPI chemical libraries to desired targets.

The efficient discovery, development, and characterization of small-molecule PPI inhibitors are dependent on high-resolution techniques that bring insight at atomic resolution, or on timescales that detect rapid association and dissociation of ligands. Because no single method is sufficiently descriptive, or without caveats, the discovery process relies on the combination of these data in a detailed synthesis, one that places emphasis on clear mechanism as well as potency. Reference has been made to numerous structures and structural rationalizations (chemical SAR and proteins) throughout this review, which underscores our need to visualize complex relationships in order to understand them. Our present understanding of PPI inhibitors would also be impossible without highly sophisticated yet easy to use molecular graphics and analysis software (DeLano 2002b; Pettersen et al. 2004; Goddard and Ferrin 2007; Seeliger and de Groot 2010).

Beyond the intellectual understanding gained from the IL-2 inhibitor work, the story of their discovery, development, and the eventual decision to halt development has continuing relevance to contemporary programs. In spite of the challenges, the value of PPI inhibitors is acknowledged through continued investment in start-up companies with an explicit focus on PPIs and in specialized screening

facilities equipped to take on early-stage discovery. The risk associated with PPI targets is now better understood in terms of the investment (including commitment to the required screening and assay technology) and the longer journey toward a final product. Instrument and reagent manufacturers are clearly responding to a market need by offering new products and features oriented toward PPI inhibitor research, including enhanced sensitivity for small-molecule detection and protocols optimized for PPI-oriented assays. The increased interest in developing small-molecule inhibitors of PPI seems to reflect an industry-wide acknowledgment that proof-of-principle has been established and that PPIs are too valuable to be ignored. Our task, then, is to find ways to approach – and ultimately to solve – the high-risk/high-reward PPI inhibition equation.

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http://www.springer.com/978-3-642-17082-9

Small-Molecule Inhibitors of Protein-Protein Interactions
(Eds.)L. Vassilev, D. Fry
2011, X, 174 p. 63 illus., 20 in color., Hardcover
ISBN: 978-3-642-17082-9