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Perspective

Inhibition of Protein−Protein Association by Small Molecules: Approaches and Progress†

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Scientific discovery consists in the interpretation for our own convenience of a system of existence, which has been made with no eye to our convenience at all.

—Norbert Wiener

Introduction

The ability of proteins to associate rests at the core of biology. Cellular architecture, information transfer, and chemical specificity rely upon highly precise recognition events; frequently these events involve the assembly of two or more proteins. The binding of two proteins may occur with low or high affinity, but clearly protein association in cells does not occur in a random, disorganized way. Rather, protein association is carefully scripted to achieve specific goals, be they the assembly of particular subcellular architectures or the relay of information (signal transduction). Protein−protein association events may be recognized in all aspects of cell biochemistry. The mammalian immune response relies in large part upon the recognition of proteins and peptides by antibodies. Cell−cell recognition and attachment to the extracellular matrix is mediated by cell surface receptors (cadherins and integrins) that are ligated by protein partners such as actin and fibronection. Signal transduction from the cell surface to the nucleus is frequently mediated by one or more protein−protein associations, e.g., Grb-2 binding to p185^{erbB2} to recruit its downstream target SOS to the membrane. Then, transcription itself is orchestrated by a plethora of transcription factors, activators, and suppressors, whose assembly is poorly understood but clearly important. Given the ubiquitous nature of these relationships, and the knowledge that inappropriate protein−protein binding can lead to disease, it should not be surprising that protein−protein interactions have attracted the attention of scientists in the pharmaceutical industry and elsewhere who are interested in producing inhibitors for use as biochemical tools or therapeutic agents. Indeed, there are ample examples in the literature of the use of antibodies, dominant negative proteins, or medium-sized peptides to inhibit particular protein−protein assemblies. In contrast, the discovery of small "drug-like" molecules that can perform a similar function has proven difficult.

A number of special challenges are presented by targeting protein−protein binding in a drug discovery

† Abbreviations: AcpYEEK, *N*-acetyl-*O*-phospho-tyrosyl-glutamyl-glutamyl-lysine; Apaf-1, apoptotic protease activating factor-1; bcl-2, B-cell leukemia/lymphoma 2; bFGF, basic fibroblast growth factor; BH3, Bcl-2 homology domain 3; CHO, chinese hamster ovary; DHFR, dihydrofolate reductase; EGFr, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FKBP, FK506-binding protein; FRET, fluorescence resonance energy transfer; GA, geldanamycin; Grb2, Growth factor receptor bound protein 2; GST, glutathione S-transferase; HIV, human immunodeficiency virus; GH, growth hormone; HRP, horseradish peroxidase; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; MW, molecular weight; NGF, nerve growth factor; NMR, nuclear magnetic resonance; NO_x, nitric oxides; iNOS, inducible nitric oxide synthase; PARP, polyadenosylribose polymerase; PBMC, peripheral blood mononuclear cells; SAR, structure−activity relationship; SH2, src homology domain-2; PI, phosphatidyl-inositol; SOS, son-of-sevenless; TNF, tumor necrosis factor; zVADfmk, *N*-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethyl ketone..

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program, some of which may apply generally, and others of which almost certainly do not. Protein–protein binding typically occurs over a relatively large surface area (approximately 800 $Å^2$ per protein on average); however, it is a common misconception that binding must comprise many low affinity interactions rather than a small number of essential high affinity interactions.[1,2] In fact, in the association of human growth hormone (hGH) with its receptor, only 8 of 31 buried hGH residues at the binding interface account for approximately 85% of the binding energy.[3] These eight residues are matched by a complementary set of nine essential residues of 33 buried residues on the receptor side.

The overall binding affinity between two proteins depends on the function of the protein complex. For example, obligate homodimers usually associate strongly ($K_d = 10^{-9}–10^{-12}$ M), and their interfaces consist of large numbers of hydrophobic residues which disfavor the monomeric state.[2] An exception is the DNA helicase UvrD with $K_d = 1.4$ μM.[4] Both interface surface area and binding affinity generally increase with increasing molecular weight. In contrast, proteins that associate and dissociate in response to changes in their environment, which are likely to include the majority of signal transduction mediators, tend to bind more weakly and exhibit correspondingly more hydrophilic residues at the binding interface. The different character of the binding interface is dictated by the requirement for exposure of this patch to solvent in the monomeric state of the protein.

The relative affinity of protein–protein binding tends to be overemphasized when discussing the potential of small molecules to inhibit such interactions. High affinity binding itself has not presented an insurmountable obstacle to the discovery of enzyme inhibitors, and enzyme–ligand affinities span a similar range to protein–protein binding affinities. This is true even when the ligand itself is another protein—a protease substrate for example. Furthermore, even proteins that bind with a relatively high affinity will be out-competed by a comparatively weak small molecule ligand, if that small molecule is present at high enough concentration.[5] Another consideration is that, at least in some cases, the mere disruption of a binding equilibrium will be sufficient to produce a significant biochemical effect without the need to completely inhibit protein–protein binding.

A more pragmatic concern is that the binding surfaces between two proteins tend to be relatively flat, lacking crevices and pockets that might provide snug binding sites for small molecules. Although a structural characterization of protein homodimers indicates that a significant degree of protrusion is observed at some protein interfaces, this situation is less likely to be true at the interface between nonobligatory heterodimers.[2] Nonetheless, it is clear that protein–protein binding interfaces vary widely in nature and some are likely to present better targets than others for drug discovery.

In this review, I have attempted to present recent examples of small molecule protein–protein binding inhibitors (PPBIs) that demonstrate the progress that has been made in the last two years toward the goal of specifically inhibiting protein–protein binding for therapeutic advantage using a small molecule. I have avoided discussing peptide inhibitors[6] except in the context of their use as templates for the design of related nonpeptide molecules or where a particular example serves to illustrate important approaches to inhibitor discovery or design. Furthermore, I have endeavored not to duplicate the work of Cochran to whose review the reader is referred for a summary of work published prior to 1999.[5] Finally, I have chosen to omit any discussion of integrin inhibitors; recent reviews of the extensive literature in this area are provided by Hölzeman[7] and by Samanen.[8] In organizing this review, I have chosen to divide it into three broad sections, which I have loosely entitled “Inhibitors by Screening”, “Inhibitors by Design”, and “Future Directions”. This arbitrary division helped me to organize my thoughts although it has resulted in the separation of some accounts that are closely related, such as the many excellent approaches to the identification of SH2 inhibitors. Finally, I concede that this review is unlikely to be comprehensive, although I hope it is representative. Where pertinent work has been omitted I welcome the opportunity to expand my own knowledge of this exciting field of discovery.

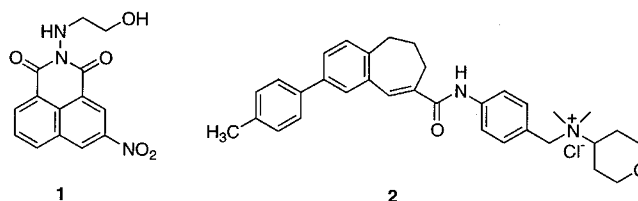

Figure 1. Compound **1**, ALE-0540; **2**, TAK-779.

Inhibitors by Screening

While many approaches to the discovery of small molecule ligands involve some element of screening, this section will describe recent work that has relied predominantly on screening to identify inhibitors of the targeted protein–protein binding interaction. These approaches will be further sub-divided by the nature of the assay employed in the screening process.

Competitive Binding. The most direct method that has been employed for the identification of small molecule inhibitors of a protein–protein association is a competitive binding assay in which one of the protein partners is labeled, typically with a radioisotope. One such example is the discovery by Owalabi et al. of a small, non-peptide inhibitor of the peptide nerve growth factor (NGF) binding to its receptor p75 and the kinase Trk A.[9] NGF is essential for the survival of sympathetic and immature sensory neurons and increases the pain response. Antagonists of NGF action are expected to behave as nonantiinflammatory analgesics.

To identify NGF antagonists, mouse NGF was labeled with iodine-125 to produce a reagent that could be detected using standard radiometric methods. In binding assays that employed either purified Trk A, or p75 and Trk A expressing PC12 cells, low volume screening of commercially available compound libraries identified *N*-{5-nitro-1*H*-benz[*de*]isoquinoline-1,3(2*H*)-dione}-2-aminoethanol (ALE–0540; **1**, Figure 1) as a competitive inhibitor of NGF binding with an $IC_{50} = 5.88 \pm 1.87$ μM for binding to Trk A and an $IC_{50} = 3.72 \pm 1.3$ μM for binding to p75 and Trk A bearing PC12 cells. ALE-

0540 did not bind to NGF and was further shown to be inactive at a selection of other receptors, e.g., 5HT-2A, endothelin, and opioid receptors. ALE-0540 was effective in cell-based assays as indicated by the inhibition of Trk A (auto)phosphorylation and inhibition of neurite outgrowth. In addition, ALE-0540 displayed antiallodynic effects in vivo in rat models of inflammatory and neuropathic pain.

A similar approach was taken by Baba et al. in the discovery of antagonists of HIV-1 cell attachment.[10] This group prepared CHO cells expressing the β-chemokine receptor CCR5, which is a target for HIV-1 and mediates membrane fusion and cell entry by this retrovirus. HIV-1 binding to CCR5 is inhibited by the natural receptor ligands which are large peptides such as RANTES. By employing a screen for ^{125}I-RANTES binding to CHO/CCR5 cells, Baba et al. identified from a Takeda compound library a small molecule inhibitor dubbed TAK-779 (**2**, Figure 1). This non-peptide inhibitor completely prevented the association of ^{125}I-RANTES with CHO/CCR5 cells at a concentration of 100 nM with IC_{50} = 1.4 nM. The inhibition was specific, and no effect was observed on ligands binding to CCR1, CCR3, or CCR4, although TAK779 did inhibit the binding of chemotactic protein to CCR2b with an IC_{50} = 27 nM. TAK779 inhibited RANTES-induced Ca^{2+} mobilization in CCR5 expressing CHO cells but not CCR1 expressing CHO cells. The goal of this study was to identify inhibitors of HIV replication; as desired, TAK779 potently inhibited the replication of HIV-1 in CCR5 expressing MAGI cells with an IC_{50} = 1.2 nM. No effect was observed on the replication of CCR4 specific HIV-1 with up to 20 μM TAK779. HIV-2 replication was similarly unaffected. The effect of TAK779 extended to the inhibition of CCR5 specific clinical isolates of HIV-1 in PBMCs.

Although the exact binding site for TAK779 on CCR has not been defined, it was found to inhibit the binding of only one of two monoclonal antibodies specific to the second extracellular loop, suggesting that this loop may associate directly with the inhibitor. Chemokine receptor antagonists, similar to other small molecule ligands for G-protein coupled receptors, are generally believed to function allosterically by binding to the receptor protein transmembrane region. A growing set of chemokine receptor antagonists has been identified, and although a small number of representative examples are included in this review, they are generally not regarded as authentic inhibitors of protein–protein binding.[11–13]

The groups of Boger and Cheresh recently collaborated to discover compounds that disrupt the binding of matrix metalloproteinase 2 (MMP2) to the integrin $\alpha v\beta 3$ as potential antiangiogenics.[14] Combinatorial mixtures of compounds designed to favor inhibition of protein–protein binding were screened using an ELISA type assay. To perform the screen, purified integrin was adsorbed onto microtiter plate wells and the MMP2 was labeled with biotin. Integrin-MMP2 association could then be detected using a horseradish peroxidase (HRP)-linked anti-biotin mAb and a peroxidase substrate. The initial inhibitors identified using this screen (e.g., A6B10C4; **3**, Figure 2) possessed a very high molecular weight and were unsuitable for biochemical studies; however, further refinement of the structure with a view to approaching more drug-like molecules resulted in the design of TSR1265 (**4**, Figure 2). Although this molecule is still large by pharmaceutical standards, it was suitable for demonstrating the principle that inhibition of MMP2-$\alpha v\beta 3$ binding in cells can disrupt angiogenesis.[15]

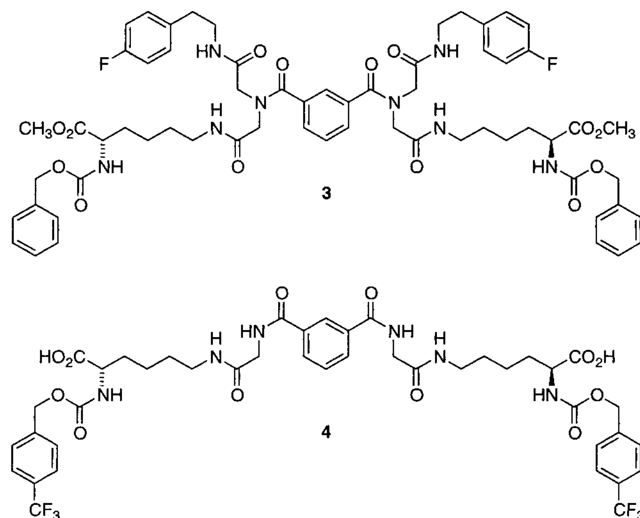

Figure 2. Compound **3**, A6B10C4; **4**, TSR1265.

TSR1265 labeled with carbon-14 was demonstrated to bind selectively to $\alpha v\beta 3$ but not $\alpha 5\beta 1$ or MMP2. Binding was independent of ligand binding to the high affinity RGD binding site, indicating that the two sites are different and not allosterically linked. In a cell-based assay, hamster CS-1 melanoma cells that had been transfected with the human $\beta 3$-integrin subunit were prevented by TSR1265 from degrading collagen IV. This effect was attributed to the inability of these cells to recruit MMP2 when TSR1265 was present. This hypothesis was supported by data indicating that TSR1265 inhibits the binding of MMP2 to $\beta 3$-integrin transfected cells. Significantly, TSR1265 did not prevent the degradation of collagen IV by MMP2 alone. In vivo studies were performed using 10-day old chick chorioallantoic membrane (CAM). In this model system, TSR1265 almost completely abolished bFGF-stimulated angiogenesis despite levels of MMP2 activation and expression equivalent to controls. Consistent with this result, the growth of $\alpha v\beta 3$-negative CS-1 tumors in chick CAM was reduced following a single IV injection of TSR1265, and treated tumors displayed less surface vasculature and overall blood vessel density than untreated tumors. The use of $\alpha v\beta 3$-negative tumors in this experiment supports the conclusion that the antiangiogenic effect arises through a specific effect on the vasculature rather than by direct inhibition of tumor growth.

Enzyme Assay. In instances where one of the partners in a protein–protein recognition event is an enzyme, the activity of this enzyme may be altered by association with its protein partner. Examples of this signaling mechanism are not uncommon, although they are frequently triggered by an initial catalyzed event such as phosphorylation. When the result of protein–protein association is a change in enzyme activity, then the level of this activity can be used to provide a readout of the extent to which protein–protein association has occurred. For example, the phenothiazine antipsychotic trifluoroperazine (**5**, Figure 3) was found to inhibit

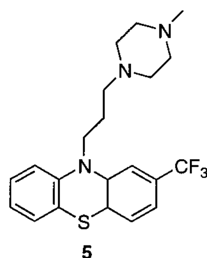

Figure 3. Trifluoroperazine.

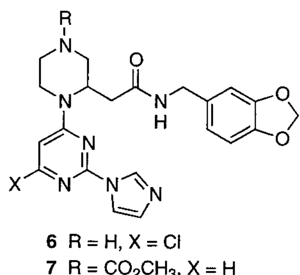

Figure 4. Inhibitors of iNOS.

(Ca^{2+}-Mg^{2+})-ATPase in a calcium/calmodulin-dependent manner consistent with an indirect action on the enzyme through binding to calmodulin.[16,17] The inhibitory effect of trifluoroperazine on the ATPase (and other downstream proteins) could be overcome by excess calmodulin.

For proteins that are catalytically active only in their homodimeric state, an inhibitor of dimerization may be detectable as an inhibitor of the catalytic activity.[18] Particular care is required in this case to employ control experiments to establish that inhibition is not of the more classical competitive or noncompetitive form. Scientists at Pharmacopeia and Berlex have successfully used this approach to discover small molecule inhibitors of inducible nitric oxide synthase (iNOS),[19] an enzyme that is implicated in the pathogenesis of inflammatory and autoimmune diseases. The assay consisted of A-172 cells that were induced to produce iNOS by the addition of interferon-γ, IL-β, and TNF-α; $NO^{\bullet}$ production was measured by standard methods 22 h following induction. Using this assay, workers screened an encoded solid-phase library of pyrimidine imidazoles, a structural class that was chosen based on prior knowledge of heme ligands that display selectivity for iNOS vs endothelial NOS. None of these compounds showed significant activity against partially purified human iNOS; however, in the cell-based assay 53 compounds were identified that displayed >60% inhibition of $NO^{\bullet}$ production. The most potent library inhibitor (**6**, Figure 4) and a related, less chemically reactive inhibitor (**7**, Figure 4) were measured to have $IC_{50} = 1.1$ nM and 0.6 nM, respectively. Control experiments indicated that these compounds did not alter iNOS transcription or translation and that the potency of inhibition increased with time following induction of iNOS ($t_{1/2} \sim 8$ h). Subsequent electrophoresis and size-exclusion chromatography experiments demonstrated that these inhibitors prevent dimerization of the iNOS monomers. Compound **7** bound to iNOS monomers with $K_i = 2.2$ nM but was inactive against dimeric iNOS in enzyme activity assays. This compound also was effective in vivo, suppressing LPS-stimulated plasma NO_x production in a dose-dependent manner in rats with $ED_{50} = 1.2$ mg/kg. Further chemical modification led to improved selectivity for iNOS vs neuronal and endothelial NOS.

Further structural insight into this inhibition of iNOS was derived from a crystal structure of an inhibitor–iNOS complex at 2.25 Å resolution. This structure revealed that the inhibitor bound via its imidazole ring to the sixth coordination position on the heme iron. This coordination appears to allosterically inhibit protein–protein interactions between monomers by disrupting helix 7a leading to partial disorder in helix 8, which is part of the dimer interface.

Fluorometry. The ability of certain molecules to fluoresce when irradiated with light may be exploited in a variety of ways in the development of binding assays.[20,21] For example, the act of sequestering a fluorophore from solvent upon binding to a protein can result in an increase in the emitted light. This technique can be used to observe the binding of small molecules but is generally rather insensitive. The proximity of two proteins can be observed by exploiting a phenomenon known as fluorescence resonance energy transfer or FRET. A fluorescence output from the detector fluorophore is only observed when it is sufficiently close to the donor fluorophore to absorb the light that it emits. Another elegant use of fluorescence employs probe molecules to observe and measure binding events by taking advantage of the slower tumbling of larger molecules or molecular complexes. When a fluorescent compound is irradiated with polarized light, the extent of polarization of the emitted light is a function of the tumbling rate of the molecule. Rapid tumbling leads to inhomogeneous fluorescence emission, causing a lower signal to be measured at a detector, which bears a polarizer corresponding to the irradiated light. In contrast, a molecule that tumbles more slowly will emit a higher proportion of its signal in the same plane as the irradiated light, leading to a higher signal at the detector. This very sensitive technique can be widely applied to the observation of binding through the employment of a probe fluorescent ligand that can be displaced into solution by experimental ligands or partner proteins.

Two examples of the use of fluorescence polarization to examine inhibition of protein–protein binding come from studies of the apoptosis regulating Bcl-2 family proteins.[22] Apoptosis is an energy requiring process of cellular suicide.[23,24] While our understanding of the events involved in apoptosis is still growing, it is clear that the Bcl-2 family of proteins play a critical role in relaying signals to the mitochondrion where these signals are aggregated. Pro-apoptotic signals lead to loss of the mitochondrial membrane potential and release of cytochrome *c* which results in activation of the caspase-3/caspase-9 cascade of proteases. The precise mechanism of action of Bcl-2 proteins is not fully understood; however, it appears that certain members promote cell death whereas others inhibit cell death, and the balance of these tendencies is controlled by the distribution of heterodimers and homodimers of various family members. NMR studies and site-directed mutagenesis have implicated conserved protein domains known as BH3 domains as mediators of protein–protein

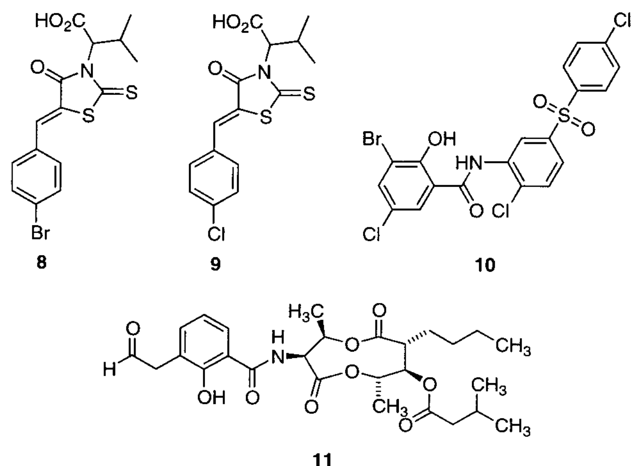

Figure 5. Compound **8**, BH3I-1; **9**, BH3I-1′; **10**, BH3I-2; **11**, Antimycin A.

association in the Bcl-2 family, and short peptides encompassing the BH3 domains of pro-apoptotic Bcl-2 family members can trigger apoptosis in cells.

Hypothesizing that small molecule mimics of BH3 domains might similarly induce apoptosis by inhibiting the association of anti-apoptotic Bcl-2 proteins with pro-apoptotic Bcl-2 proteins, Degterev and co-workers set out to identify such molecules by screening a library of 16 320 compounds.[25] A fluorescence polarization assay was employed in which a fluorescently labeled BH3 domain from Bak (a Bcl-2 family protein) was used as the reference ligand for binding to a recombinant GST-Bcl-x_L fusion protein. Three compounds with micromolar affinity for Bcl-x_L (BH3I-1, BH3I-1′, and BH3I-2; **8**–**10**, Figure 5) were selected from the screen for further analysis. To control for binding to the fluorescent tag, all three inhibitors were evaluated using *unlabeled* BH3 peptide to measure their association with immobilized Bcl-x_L on a surface-enhanced laser desorption/ionization (SELDI) chip. Association of the BH3 peptide and Bcl-x_L was observed by mass spectrometry. Binding was inhibited by all three BH3 inhibitor (BH3I) molecules. These compounds also inhibited the BH3 peptide binding to Bcl-2 (another Bcl-2 family protein) and prevented truncated Bid from associating with (His-tagged) Bcl-x_L immobilized on Ni^{2+} beads. Finally, K_i values for BH3Is binding to Bcl-x_L were determined by NMR titration. Thus four different techniques were used to demonstrate binding of these small molecules to various Bcl-2 family members. These same techniques were used to demonstrate the selectivity of these BH3Is for Bcl-2 family proteins versus a variety of other protein–protein interactions including splicing factors U2AF^{35}/U2AF^{65}, Apaf-1/CARD, and CIDE-B/DFF40. This last experiment underscores a particular obstacle to the discovery of specific inhibitors of protein–protein binding; it is generally difficult to define appropriate controls for nonspecific effects. More structural data on protein interfaces and physiologically relevant protein partnering will be required before sequence data, or better still, three-dimensional structural data, will be searchable for potential recognition sites that might compete with the intended target.

The described BH3I molecules induced apoptosis in JK cells as indicated by TUNEL staining, the appearance of annexin V, DNA fragmentation, and flow cytometry. Cytochrome *c* was released from the mitochondrion, although the mitochondrial membrane potential did not apparently decrease. Caspase activation occurred with caspase-9 activity increasing prior to caspase-8 activity, consistent with apoptosis triggered through the mitochondrial pathway (as distinct from the FAS pathway). The cytotoxicity of BH3Is followed the same order as their binding affinity for Bcl-x_L in vitro.

To examine whether BH3Is disrupt the heterodimerization of Bcl-2 family members in cells, the authors used an intracellular FRET assay. HEK-293 cells were cotransfected with yellow-fluorescent protein (YFP)-fused Bax and cyan fluorescent protein (CFP)-fused Bcl-x_L. The extent of FRET between the two proteins was determined as the ratio between the fluorescence at 527 nm and the fluorescence at 475 nm following excitation at 433 nm. Cotransfection of the two tagged proteins increased this ratio to 1.7 compared to 1.0 for the two proteins expressed separately, indicating that the proteins are in close proximity in the cell (probably associated). The BH3Is all reduced this ratio, with the extent of reduction correlating to the potency of the compounds in cytotoxicity and binding assays. A time course showed that loss of FRET preceded cell death.

Comparison of the BH3Is and Bak BH3 peptide binding to Bcl-x_L was performed using NMR. A 2-D $^{15}N/^{1}H$ heteronuclear single-quantum correlation (HSQC) spectrum of ^{15}N-labeled Bcl-x_L in the presence of BH3 peptide showed that peptide binding primarily affected residues at the BH1–BH3 hydrophobic cleft, consistent with the known structure of this complex. Similarly, all three BH3Is induced significant changes at the surface of Bcl-x_L in the hydrophobic cleft formed by the BH1, BH2, and BH3 domains, although different BH3Is affected slightly different residue sets on the Bcl-x_L protein. Nuclear Overhauser effects between BH3I-1 and residues Tyr-65 and Phe-107 of Bcl-x_L further pinpointed the binding locus for this inhibitor. Interestingly, Phe-107 is buried in the structure of free Bcl-x_L and is inaccessible to solvent; however, this residue becomes surface-exposed upon BH3 peptide binding, making contacts with a leucine residue in the BH3 peptide. The NMR data suggest that Bcl-x_L undergoes a similar conformational change upon binding of the small molecule inhibitor BH3I-1.

Overall, the evidence provided by Degterev et al. supports the hypothesis that their BH3Is act as BH3 mimics, inhibiting heterodimerization between Bcl-x_L or Bcl-2 and pro-apoptotic family members, thereby releasing these pro-apoptotic members of the Bcl-2 protein family to promote cell death.[26] In related work, Tzung and co-workers demonstrated that a similar mechanism of action may also hold for the inhibitor of electron transfer complex III known as antimycin A (**11**, Figure 5).[27] While it is difficult to know whether precisely this mechanism operates in cells, these results demonstrate the potential of small organic molecules to inhibit important protein–protein interactions in a cellular environment. In addition, they highlight the importance of Bcl-2 family protein–protein association events in the regulation of cell homeostasis. The balance that exists between a pro-apoptotic state and an anti-apoptotic state appears to be controlled by the relative levels of multiple heterodimeric complexes, and it may

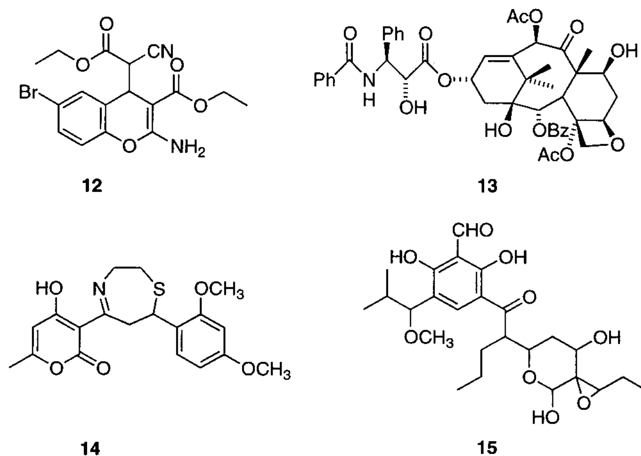

Figure 6. Compound **12**, HA14-1; **13**, Taxol; **14**, Synstab A; **15**, UCS15A.

be that *complete* (100%) inhibition of any one complex is unnecessary to shift the equilibrium significantly in one direction or the other.

Virtual Screening. Rapid developments in computing power and the increasing incorporation of computational methods into chemistry and biology made it inevitable that small molecule screening would become a virtual experiment. An illustrative example of this parsimonious approach to ligand identification is provided in the work of Wang and co-workers who also were concerned with inhibitors of Bcl-2 function.[28] Wang used an homology model for Bcl-2, derived from the solution structure of Bcl-x_L. A total of 193 383 compounds were screened in silico using the program DOCK 3.5 to score shape complementarity for each virtual compound bound to the Bcl-2 model in a variety of conformations. The 1000 best results were "optimized" using SYBYL 6.2 to assign an appropriate geometry and then the binding energy for each was calculated. The 53 best compounds then were selected manually using binding energy, shape complementarity, and hydrogen bond forming potential as criteria for the selection. A total of 28 of these compounds were available commercially, and these were obtained for testing. Binding to Bcl-2 was measured using a fluorescence polarization assay in which the reference ligand was a 5-carboxyfluorescein labeled peptide derived from Bak with $K_D \sim 0.20\ \mu M$ for binding to Bcl-2. A compound dubbed HA14-1 (**12**, Figure 6) proved to be the best ligand for Bcl-2 with $IC_{50} \sim 9\ \mu M$. At 50 μM, HA14-1 caused cell death in >90% of cultured HL60 human leukemia cells. Cell death was accompanied by DNA fragmentation, consistent with apoptosis, and this fragmentation was completely prevented by the caspase inhibitor zVADfmk. Additional evidence for apoptotic cell death included cleavage of caspase-9 and caspase-3, PARP cleavage, and decrease in the mitochondrial membrane potential. However, HA14-1-induced apoptosis was not inhibited by CrmA, indicting that HA14-1 signaled via the Bcl-2 pathway and not the FAS pathway. Bcl-2 inhibits Apaf-1 activation of procaspase-9 cleavage and cytochrome *c* release from the mitochondrion, whereas FAS-mediated apoptosis does not require Apaf-1. It was found that HA14-1 had little effect on Apaf-1 deficient cells but was a potent inducer of apoptosis in Apaf-1 positive cells. In aggregate these results support the hypothesis that HA14-1 induces apoptosis by inhibiting the function of Bcl-2 as would be expected for a compound that inhibited Bcl-2 heterodimerization with pro-apoptotic protein partners.

It is interesting to note that the antimitotic antitumor drug paclitaxel (Taxol; **13**, Figure 6) also has been demonstrated to bind to Bcl-2.[29] The significance of this observation is not clear, although paclitaxel is a potent inducer of apoptosis. This effect may be at least partially mediated by the inhibition of Bcl-2 binding to other Bcl-2 family members.

Phenotypic Assays. When a particular cellular behavior or phenotype is anticipated to result from the inhibition of a specific protein–protein interaction, then this phenotype can be used to provide the read-out in a screen for inhibitors. While a risk of false positives due to alternative mechanisms is inherent to this approach, these false positives can readily be removed by use of a more specific secondary assay. Meanwhile, the phenotype-based screen possesses the benefit of identifying as hits compounds that (a) are cell-permeable, (b) produce the desired effect on living cells, and (c) if they work via the intended mechanism are sufficiently potent and specific to work in the cellular milieu. Haggarty et al. used a cell-based screen to identify compounds that inhibit mitosis.[30] Reasoning that the majority of compounds that are known to inhibit mitosis do so by interfering with the polymerization of α and β tubulin, their secondary assay was for inhibition of in vitro tubulin polymerization. An antibody (TG-3) against the phosphorylated form of nucleolin that is expressed specifically by cells in mitosis was used to determine the extent to which A549 lung epithelial cells were blocked in mitosis by 16 320 diverse compounds from a commercially available library (Chembridge, Diverse E set). This screen identified 139 compounds that increased the amount of phosphorylated nucleolin by at least 2.5-fold. Of these 139 compounds, 52 destabilized microtubules but had no effect on the polymerization of actin (e.g., Synstab, **14**, Figure 6), similar to the known antimitotics colchicine and nocodazole. Although no detailed mechanistic studies were reported, these compounds were proposed to inhibit microtubule formation by preventing the association of α and β tubulin.

A form of phenotypic assay was used recently by Sharma et al.[31] The phenotype in this example was cell growth. Yeast cells overexpressing the tyrosine kinase v-src under control of the Gal-I promoter grow poorly in culture on glucose medium. Alleviation of this growth arrest by a compound provided in the form of a soaked filter-paper disk was visible in the form of a halo of growing yeast around the disk. A total of 20 000 compounds were screened using this technique which identified several known src-kinase inhibitors and a new compound (**15**, Figure 6), which was named UCS15A. This new compound decreased the levels of phosphorylated v-src substrates in transformed NIH cells, including cortactin and Sam68 even though the expression levels of these proteins were unaltered. However, the kinase activity of v-src against a Cdc2 peptide in vitro was unaffected by UCS15A. In addition, UCS15A was demonstrated not to inhibit the activation of v-src through phosphorylation at tyrosine-416 or to destablize the v-src protein. It was hypothesized that UCS15A may prevent the association of v-src with its substrates, and

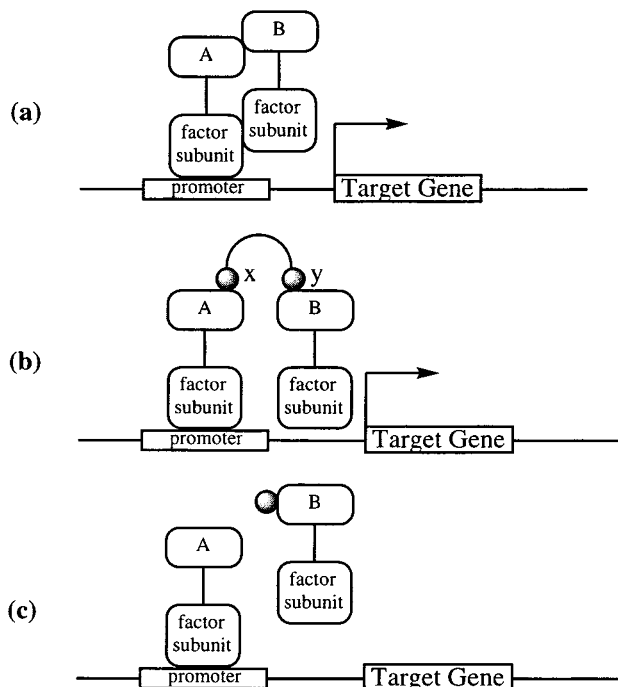

Figure 7. (a) Two-hybrid system: detects association between proteins A and B. (b) Three-hybrid system. The tethered dimer *x*–*y* bridges A and B, bringing the two subunits of the transcription factor into association. If *x* is a known ligand for A, the three-hybrid system can be used to identify protein targets for ligand *y*. (c) Reverse two-hybrid system: detects inhibitors of A–B association.

this hypothesis was supported by immunoprecipitation studies in which UCS15A prevented the coprecipitation of Sam68 and an unknown 64 KDa protein (pp64) in a dose-dependent manner. UCS15A inhibited bone resorption by osteoclast-like multinucleated cells and by the mouse calvaria organ in culture without significant cytotoxicity. The molecular mechanism of UCS15A has yet to be delineated; however, preliminary studies implicate the SH3 module as a potential target. Inspection of the molecular structure of UCS15A suggests that it could form a covalent attachment to its target through any of several potential alkylating groups, particularly the epoxide ring.

Another form of phenotypic assay is called the yeast two-hybrid assay (Figure 7a). This technique, originally described by Fields and Song,[32] detects protein–protein binding through the expression of a reporter gene whose transcription is activated by a heterodimeric transcription factor. Two fusion proteins are required, each comprising one of the transcription factor monomers fused to one of two partner proteins. When the partner proteins bind to each other, the active transcription factor heterodimer is formed. If the two partner proteins do not normally associate with each other, they may be induced to do so by a heterodimeric ligand comprising one ligand for each protein tethered to each other via a linker (Figure 7b). This latter technique has been referred to as a three-hybrid system and is an example of chemical-induced dimerization (CID, see below).[33,34] Alternatively, the partner proteins may be inhibited from associating by a PPBI (Figure 7c). If a growth critical gene is placed under the regulation of the transcription factor, then the PPBI will cause growth inhibition. For example, in one report, a yeast strain was used that requires histidine to be supplied in the growth medium unless the reporter gene *HIS3* is expressed.[35] Thus, protein–protein binding was indicated by the ability of the yeast to grow in the absence of exogenous histidine. In this case, an inhibitor of protein association resulted in the phenotype, inability to grow on histidine-deficient medium.

Huang and Schreiber turned this concept around and developed an assay for PPBIs in which the phenotype indicating successful inhibition was survival not death.[36] The DNA-binding protein lexA and activation domain B42 were separately fused to either FKBP12 or to the R1 subunit of transforming growth factor-β type I receptor. In the absence of a PPBI, LexA bound to its DNA binding site, and B42 was brought into close proximity as a result of the association between FKBP12 and R1 resulting in transcription of a *URA3* reporter gene. When the ura3 protein was expressed in cells, they were rendered sensitive to the pro-toxin 5-fluoroorotic acid (5-FOA). The FKBP12 ligand, FK506, inhibited FKBP binding to R1 and thus blocked the delivery of B42 to the LexA DNA complex. The protein ura3 was not expressed, and the cells were insensitive to 5-FOA.

In principle, with some attention to the molecular biology, this reverse two-hybrid system may be applied to any protein–protein binding event to discover new PPBIs. Indeed, by random generation and coexpression of fusion proteins in distinct yeast colonies, it may be possible to simultaneously select for protein–protein binding pairs and identify small molecules that inhibit them. As yet, the full potential of the reverse two-hybrid system does not appear to have been realized.

Inhibitors by Design

Peptide-Derived Ligands. A common approach to the design of PPBIs is to dissect the binding locus of one of the proteins and to prepare truncated peptides that contain the essential residues from the binding epitope. For a skilled molecular biologist or peptide chemist this approach may well provide the fastest route to a compound that can be used to test the result of interfering with a specific protein–protein binding event. From a pharmaceutical standpoint, however, this approach is generally unsatisfactory because the peptides themselves are unlikely to make useful drugs due to poor bioavailability, and the path from peptides to small molecule peptide mimetics is fraught with difficulties. Despite these caveats, there are many examples of effective peptide inhibitors of protein–protein binding.[5,6] For the purposes of this review, we will focus only on examples where the initial peptide inhibitor lead has been further adapted toward a non-peptide drug-like molecule.

Src-homology 2 (SH2) domains offer important targets for PPBIs due to their importance in relaying signals between cell surface receptors and cytosolic proteins. The SH2 domain mediates the association of src-family kinases with downstream signaling proteins by binding to phosphorylated tyrosine residues (pTyr) in these downstream proteins. Many peptides and peptide mimetic inhibitors of SH2 are known, and these may be used as a basis for the design of non-peptide ligands.[37] The protein p56^{lck} is a SH2 domain-containing tyrosine kinase, which is involved in T-cell activation. Inhibitors

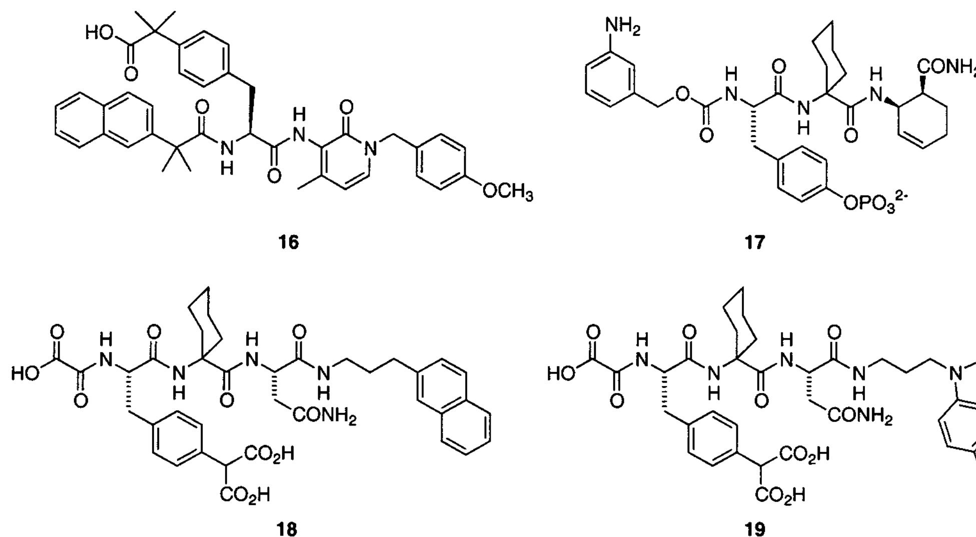

Figure 8. Peptide-derived SH2 ligands.

of p56^{lck} have the potential to block the interaction of this protein with the T-cell receptor and derail the signaling cascade that leads to cytokine production and cell proliferation. Starting with the peptide inhibitor Ac-pYEEI ($K_d = 0.1\ \mu$M), which bears five negative charges at physiological pH, Boehringer Ingelheim workers designed a singly charged peptide mimetic which is a weaker inhibitor of the p56^{lck} SH2 domain ($K_d = 1\ \mu$M) but is cell permeable (**16**, Figure 8).[38] The steps involved in this transformation included replacement of the two C-terminal amino acids with a *p*-methoxybenzyl group, effectively removing two charges at essentially no cost in terms of potency. The phosphate group was replaced by a carboxylic acid, removing one more charge, but also decreasing the binding affinity by 200- to 500-fold. The introduction of lipophilic substituents recovered some of this lost potency. Finally, removal of the last glutamate residue and introduction of a conformation-constraining bridge between two adjacent residues gave compound **16** which displayed some permeability across a Caco-2 membrane in vitro (21% permeated in 3 h) and bound to p56^{lck} with $K_d = 1 \pm 0.2\ \mu$M. Compound **16** inhibited calcium release in Jurkat cells following exposure to T-cell receptor cross-linking antibodies with $EC_{50} = 10\ \mu$M.

The SH2 domain bearing adapter protein Grb2 has been a target for several groups interested in disrupting mitogenic signaling.[39] In this case, the minimal sequence necessary for achieving micromolar binding affinity to the Grb2-SH2 domain is the relatively short tripeptide pTyr-Ile-Asn. Guided by structural information on the SH2 domain of Grb2, workers at Novartis incorporated several modifications into this simple ligand and arrived at 3-benzyloxycarbonylamino-pTyr-1-aminocyclohexane carboxylic acid-Asn-NH_2 which is a potent antagonist of the Grb2-SH2 domain with $IC_{50} = 1$ nM as measured in an ELISA.[40] Further structural modifications were made, less to improve potency[41] than to decrease the peptidic nature of this ligand. For example, replacement of the asparagine residue by (1*S*,2*R*)-2-amino-cyclohex-3-ene carboxylic acid provided a ligand (**17**, Figure 8) containing 3 nonproteinogenic amino acids and displaying an $IC_{50} = 1.6$ nM for inhibition of phospho-EGFr (intracellular domain) binding to Grb2-SH2.[42] Remarkably, the inhibitor was specific and did not bind to the related src-family kinase p56^{lck} at concentrations up to 10 μM.

Building on the work of Furet,[39-43] Burke and co-workers have used a similar tripeptide scaffold to evaluate bioisosteres of the phosphotyrosine residue.[44,45] Guided by molecular modeling, this group discovered that N^{α}-oxalyl *p*-(2-malonyl)phenylalanine is able to mimic both the charge state and the size of a phosphotyrosine residue. While the dicarboxylate mimics the parent phosphate group, the oxalate appears to confer additional potency for the Grb2-SH2 domain through interactions with Arg-67. This phosphatase-insensitive phosphotyrosine replacement possesses the added benefit that it does not significantly inhibit compounds from crossing cell membranes. Thus, compound **18** (MW = 763, Figure 8) is a potent inhibitor of Grb2-SH2 binding in vitro ($IC_{50} = 50$ nM) and blocks the association of c-Met (the hepatocyte growth factor receptor) with Grb2 in Okajima cells by 50% at a concentration of 30 nM.[46] Time course experiments indicated that optimal inhibition was achieved after approximately 8 h suggesting that the inhibitor enters cells by passive diffusion. Replacement of the naphthyl group in compound **18** by a methyl indole provided a compound that is 5-fold more potent (**19**, Figure 8).[45]

Peptide antagonists of the C5a receptor were first obtained by site-directed mutation of C5a, and the synthesis of peptide fragments containing the effector domain. Finch and co-workers have taken this effort one step further to produce cyclic peptides that mimic the structure of active peptide antagonists.[47] The most potent of these inhibitors (**20**, Figure 9) bound to the receptor with $IC_{50} = 0.3\ \mu$M as measured in a competition assay using ^{125}I-labeled C5a. This same peptide displayed antagonist potency in the presence of 100 nM C5a with $IC_{50} = 20$ nM as measured by myeloperoxidase release from cytochalasin B stimulated human polymorphonuclear cells. C5a is believed to be a pathogenic factor in a range of immunoinflammatory diseases including sepsis. C5a receptor antagonists therefore may be useful as antiinflammatory agents. Cyclic peptide **20** was dosed intravenously in anesthesized rats which then were challenged with either C5a or li-

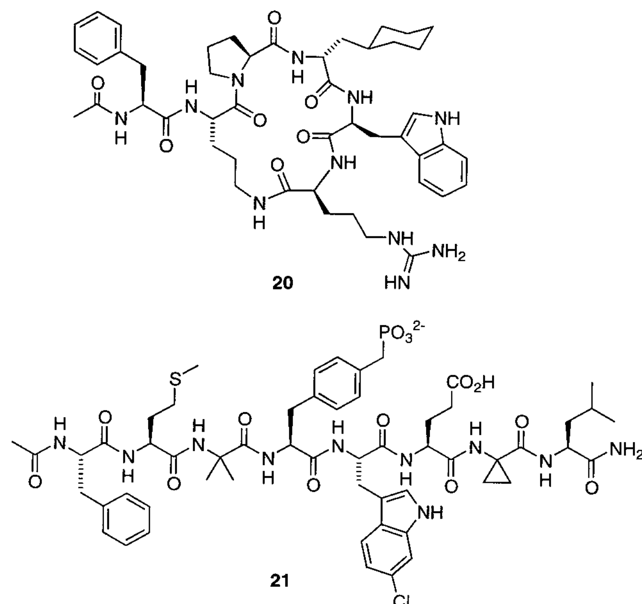

Figure 9. Compound **19**, AcF[OP-DCHa-WR]; **20**, antagonist of hdm2 binding to p53.

popolysaccharide (which stimulates an increase in endogenous C5a).[48] Compared to control animals, peptide **20** significantly reduced neutropenia (decrease in circulating PMNs) and blocked the elevation of serum TNF-α and IL6, two pro-inflammatory cytokines.

The work of García-Echeverría provides an excellent illustration of the variety of techniques that may be employed for the discovery of inhibitors of protein−protein binding.[49] Although the final inhibitor, with a peptidic backbone and a molecular weight of 1206, does not qualify as a small molecule, its derivation nicely illustrates the various tools that may be brought to bear on the discovery of PPBIs. The binding region between p53 and human double minute 2 (hdm2) was initially probed using antibodies to identify the binding region on each protein. Further mapping of the binding region on hdm2 employed synthetic peptides derived from the N-terminus of p53. A hexapeptide comprising residues 18−23 of p53 was found to bind to hdm2 with IC_{50} = 700 μM. With affinity this low, a hexapeptide was considered to be the minimal binding epitope for hdm2 recognition. A longer peptide comprising 12 residues from p53 displayed an IC_{50} = 8.7 μM, and this peptide was used as the starting point for further optimization. Increased binding affinity was pursued through the use of phage display. This technique provides a convenient method for screening peptide sequences for affinity toward any given molecular target. A 12-mer with 28-fold improved binding affinity was identified, and synthetic peptides representing truncates of this sequence were prepared to determine the minimum length required for micromolar affinity toward hdm2. This minimal length turned out to be eight amino acids. The availability of structural data from both X-ray crystallography[50] and NMR spectroscopy[51] for various p53-derived peptides bound to hdm2 proved valuable in guiding efforts to optimize binding of such a short peptide. For example, crystallography data revealed that a 15-mer p53-derived peptide bound in a deep hydrophobic cleft on hdm2, adopting a helical conformation. Both solid-phase and solution-based studies identified residues that formed contacts with the hdm2 protein as well as residues that may be structurally important but more appropriate for modification.

To bias their unbound p53-derived peptides toward a helical conformation and so decrease the entropic cost of binding, García-Echeverría elected to introduce α,α-disubstituted amino acids at positions shown to form no, or weak, direct interactions with hdm2. In addition, based on crystal structure data, a tyrosine residue was replaced by phosphonomethylphenylalanine to introduce an electrostatic interaction with Lys-94 in hdm2; substitution on the tryptophan residue was included to better complement a hydrophobic "hole" in the hdm2 protein. These final replacements led to a peptide (**21**, Figure 9) with IC_{50} = 5 nM for inhibition of p53 binding to hdm2-GST. This result represents a 1700-fold improvement in overall binding affinity.

Structure-Based Design. A long-standing goal of medicinal chemists is to develop techniques for the de novo design of compounds based on knowledge of a protein's three-dimensional structure. Not surprisingly, this general approach has been pursued also in the search for inhibitors of protein−protein association. One strategy that has proven useful is the selection of a scaffolding template upon which may be appended side chains specifically oriented to occupy the same relative regions of space as key side chains of a known ligand (where the ligand here is itself a peptide or protein). These side chains may be optimized in an iterative fashion either through multiple rounds of synthesis, assay, structure and design, or combinatorially. An early example of such an approach was described by Hirshmann and co-workers who used a glucose template to build simple but effective ligands for the somatostatin receptor.[52] Software has been written to help select scaffolding molecules, e.g., from known ring systems, that will position groups in a defined relative spatial and directional manner, notably the program CAVEAT.[53]

A group from Hoffmann-La Roche led by Olson employed benzene as a template in their design of interleukin-1 receptor antagonists.[54] Site-directed mutagenesis studies were used to map the binding epitopes of IL-1α and β to a common region in their crystalographically determined three-dimensional structures. Three IL-1β residues were identified as essential for receptor binding based on their relative contribution to binding affinity, namely Arg-4, Phe-46, and Lys-93. A 1,2,4-trisubstituted benzene ring was selected as the scaffold because it allowed appendage of the side chains in a manner that would allow for the potential interdigitation of IL-1 and IL-1 receptor residues upon binding. Since the location of the lysine δ-amine differed between the two IL-1 crystal structures, a tether was used that would permit access to either of the documented orientations.

One of the advantages of the structure-based design approach is that synthetic considerations can be built into the ligand design, facilitating access to compounds that can be easily assembled to test the binding models. The corresponding disadvantage is that it is easy to let synthetic ease subvert the original model such that the ligands produced are not optimal from a binding perspective. Olson's group prepared putative IL-1 antago-

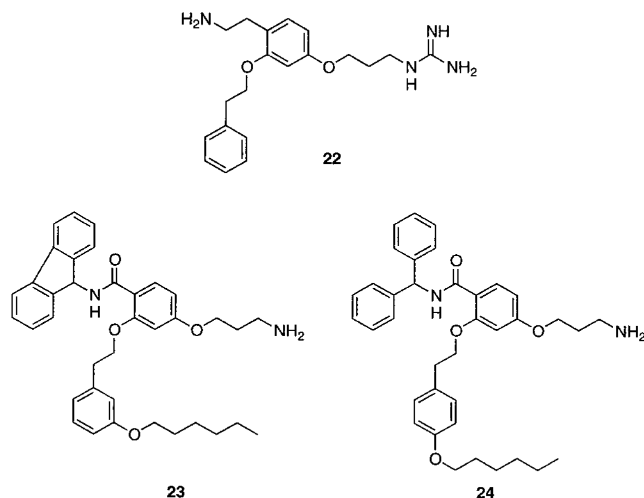

Figure 10. IL-1 inhibitors.

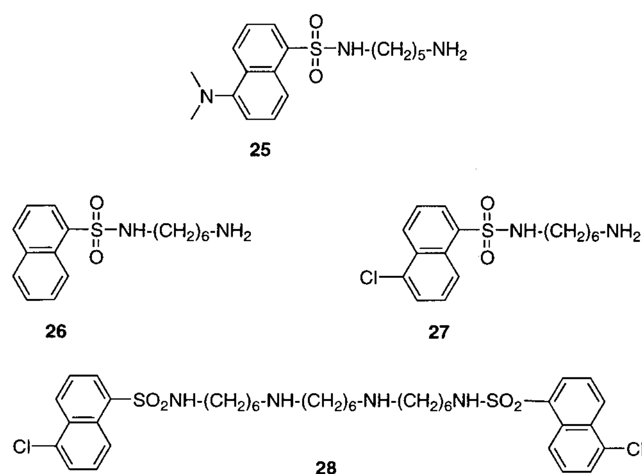

Figure 11. Compound **25**, Dansylcadaverine; **26**, W-5; **27**, W-7; **28**, (W-7)$_2$-5.

nists in eight steps from 2,4-dihydroxybenzene. While the initially designed ligand (**22**, Figure 10) antagonized IL1-α binding to the IL-1 receptor with IC_{50} = 770 μM as measured by ELISA, further optimization led to compounds **23** and **24** (Figure 10), which inhibited both IL-1α and IL-1β binding with IC_{50} values of <10 μM. The profile of compounds **23** and **24** differed considerably from the initial design, suggesting that the binding mode of these ligands was not the one originally intended. Pharmacophore modeling with a set of 30 diverse molecules that had been generated as part of the study led to a new binding hypothesis in which the benzene ring itself functions as part of the pharmacophore, mimicking Phe-46 in IL-1β.

As mentioned previously, trifluoroperazine (**5**, Figure 3) inhibits the activation of certain downstream enzymes by the calcium-sensor calmodulin. These calmodulin-dependent processes are similarly inhibited by a class of naphthylsulfonamides including dansylcadaverine (**25**, Figure 11) and two molecules known as W-5 (**26**) and W-7 (**27**).[55] *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) binds to Ca^{2+}/calmodulin (CaM) with $K_d = 1.1 \times 10^{-5}$ M. In contrast, Ca^{2+}/CaM binds to its target enzymes with $K_d = 10^{-9}$ M, necessitating the use of W-7 at high concentrations (5–50 μM) in both in vitro and in vivo studies requiring a CaM antagonist. The solution of a crystal structure for W-7 bound to CaM provided information on its binding mode and indicated that the chloronaphthalene ring resides in a hydrophobic pocket that is required for the recognition of target enzymes. The close proximity of two such hydrophobic pockets in the three-dimensional structure of CaM led Yokokura and co-workers to consider tethering two W-7 molecules together to increase the affinity of this inhibitor for its target.[56] Of the 11 tethered dimers that they prepared, several were more potent at inhibiting CaM kinase I than the parent inhibitor, including one called (W-7)$_2$-5 (**28**, Figure 11) that showed a 74-fold improvement. HSQC NMR spectra for the protein were collected as a function of ligand concentration and a comparison between W-7 and (W-7)$_2$-5 indicated that the tethered dimer bound to the protein with a 1:1 stoichiometry whereas W-7 adopted a 2:1 stoichiometry.

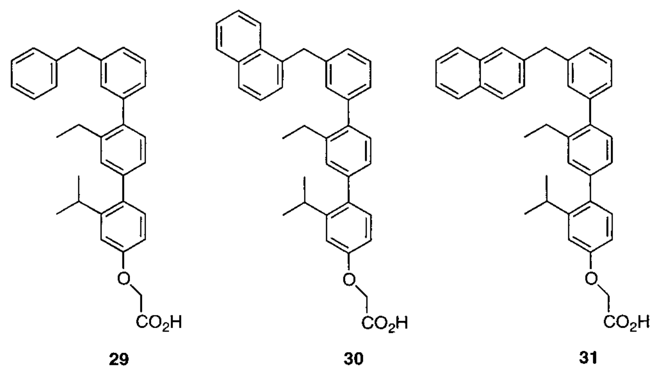

Figure 12. α-Helix minics.

For inhibiting protein–protein binding, general methods for mimicking protein surface structures would represent a significant advance. Protein binding interfaces comprise β-sheet, α-helix, turns, and loop secondary structures in an apparently unbiased manner,[1] thus small molecules that can mimic each of these structures will be required. In the field of proteomimetics, most work has focused on the development of compounds designed to imitate extended or β-turn conformations,[57] and there are relatively few examples of α-helix mimics. However, recent progress toward an α-helix mimic was reported by Orner and co-workers who designed and synthesized terphenyl derivatives as inhibitors of the interaction between CaM and small muscle myosin light chain kinase (smMLCK).[58] The prototype ligand (**29**, Figure 12) bound to CaM, as indicated by affinity chromatography, and inhibited the CaM-mediated activation of 3′,5′-phosphodiesterase (PDE), which is believed to bind CaM at the same site employed by smMLCK. Compound **29** was competitive with a peptide derived from smMLCK. Further optimization provided compounds **30** and **31** with IC_{50} values of 9 nM and 20 nM for activation of PDE. It is likely that **29**–**31** bind to CaM at the same site as W-5 and W-7 reported above.

The three-dimensional model of a protein–ligand complex was successfully exploited for the design of an inhibitor of the heat shock protein Hsp90. Hsp90 is a chaperone that stabilizes partially folded proteins, preventing their aggregation.[59] Interestingly, Hsp90 appears to be required for the correct folding of a subset of proteins that include steroid hormone receptors and some kinases including src, raf, and eIF-2α kinase. The ability of Hsp90 to associate with these target proteins

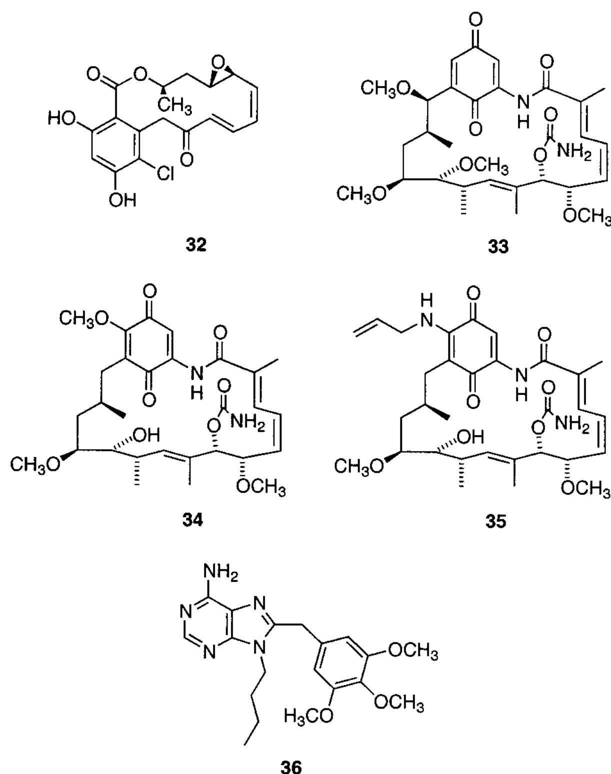

Figure 13. Compound **32**, radidicol; **33**, herbimycin A; **34**, geldanamycin; **35**, 17-AAG; **36**, PU3.

appears to depend on a co-chaperone protein called p23; however, the release of fully functional target protein has been proposed to require the hydrolysis of ATP. Indeed, the N-terminus of Hsp90 bears a weak affinity (K_d = 400 μM) ATP/ADP binding site. The discovery that the ansamycin antibiotics radidicol (**32**, Figure 13), herbimycin A (**33**), and geldanamycin (GA, **34**) bind to Hsp90 and reverse the transformed phenotype of v-src transformed cells identified Hsp90 as a potential target for antitumor therapy.[60] Indeed, the GA analogue 17-allylamino-geldanamycin (17-AAG) (**35**) is currently in phase I clinical trials for treating cancer. Although these natural products bind to the ATP/ADP site on Hsp90, their mechanism of action is proposed to involve the inhibition of association between Hsp90 and its co-chaperone p23.[61,62]

The crystal structures of the Hsp90 N-terminal domain bound to GA and to radidicol have been published.[63,64] By examination of these structures, Chiosis and co-workers identified key interactions between the ligand and Hsp90 that they believed contributed to protein inhibition, including contacts with Asp-93/Ser-52, hydrogen bonds to Lys-112/Lys-58, and interaction with a hydrophobic pocket comprising contributions from six hydrophobic amino acid side chains.[65] Selecting purine as a starting point, partly to favor good bioavailability and cell permeability, these workers designed compound PU3 (**36**, Figure 13) to take advantage of the key interactions noted. Computer docking of this molecule into the Hsp90 ADP/ATP binding site showed that it should satisfy all of the important binding interactions except the one with Lys-58. By using an affinity column of immobilized GA, the competition between PU3 and GA for binding to Hsp90 was examined to estimate the relative affinity of PU3 for Hsp90. An EC_{50} of 15−20 μM for inhibition of Hsp90 binding to immobilized GA was determined, compared to EC_{50} = 1 μM for 17-AAG (GA binds to Hsp90 with K_d = 1.2 μM).

As expected for an inhibitor of Hsp90, PU3 destabilized the estrogen receptor and promoted its proteasome-dependent degradation. Similarly, Her2 levels in MCF7 breast cancer cells decreased in response to treatment with 10 μM PU3. Proteins that are not sensitive to GM were similarly unaffected by PU3. PU3 showed antiproliferative activity against MCF-7 and two other cell lines in vitro at similar concentrations to those required to compete for Hsp90 binding and to induce protein degradation. In addition, the transformed phenotype of MCF7 cells, such as their round shape with indistinct cell margins and prominent nucleoli, were reversed by treatment with PU3.

Collectively, the data on Hsp90 inhibitors suggests that PU3 functions as an inhibitor of Hsp90 in cells and that it most likely works by preventing the association of Hsp90 with p23 or its target proteins. PU3 therefore represents one of the first examples of a PPBI designed de novo from a crystal structure of the target protein. As its inventors point out in their article, PU3 has a molecular weight of 371 and is 'rule-of-5' compliant, i.e., by current standards it would be defined as a drug-like molecule.

A group at Parke-Davis used molecular modeling and an X-ray crystal structure of a phosphopeptide-bound pp60^{c-src} protein to design a benzylamine-based SH2 ligand for the c-src tyrosine kinase.[66] Noticing that much of the binding of an 11-mer peptide to the c-src SH2 domain could be ascribed to two primary recognition elements, Lunney et al. set out to design a less highly charged, non-peptide ligand that incorporated these two essential contacts. From a search of the Cambridge Crystalographic Database, a benzoxazinone ring structure initally was selected as a potential template for positioning the key recognition elements in the protein binding site. Synthetic considerations led to the modification of this template to a "ring-opened" aminomethylbenzene carboxamide. The first generation of designed inhibitors displayed moderate affinity for c-src (IC_{50} = 6−10 μM), supporting the design strategy. The crystal structure of one of these designed inhibitors (**37**, Figure 14) confirmed that the two targeted contacts had been achieved; however, it also revealed a binding mode that differed from the original model as a consequence of reorientation of the key phenyl phosphate group in the protein binding site compared to the peptide ligand referenced in the design process. This study serves to underscore the flexibility of proteins in adapting to even similar recognition elements when presented in a different context. It reinforces the necessity for structure determination, modeling, and synthesis to be performed iteratively to compensate for the complexity of designing ligands for flexible proteins based on static structural data.

Other recent examples of structure-based design have been published by a group at ARIAD. This team was interested in inhibitors of the SH2 domain as antiresorption agents for possible use against osteoporosis. The ARIAD group employed crystal structure data for Src and the related kinase Lck in conjunction with molecular modeling to design two non-peptide ligands

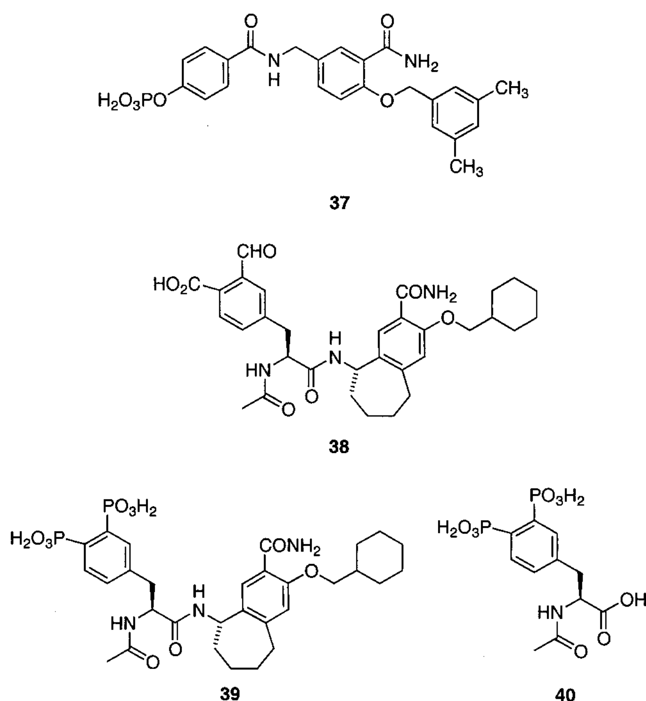

Figure 14. SH2 ligands. Compound **38**, AP22161; **39**, AP22408; **40**, DPP.

of the SH2 domain pTyr binding site (**38** and **39**, Figure 14).[67,68] Design considerations included (1) the incorporation of an edge to face contact between the ligand and Tyr-181; (2) the addition of two hydrogen bonds; (3) burial of hydrophobic surface (a cyclohexane ring) in the Ile (pY + 3) binding site; and (4) the inclusion of additional hydrophobic contacts by building off of the ligand's benzene ring to complement the SH2 domain hydrophobic architecture.

Two separate approaches were used to target the inibitor to Src vs related tyrosine kinases. The protein Src is unique among its class in possessing a cysteine residue (C188) in the SH2 domain that is not present in other Src tyrosine kinase family members (e.g., Lck, Fyn, Yes, Yrk, Hck, Fgr, Blk, Lyn, Frk/Rak, and Iyk/Bsk). The environment of the cysteine thiol, surrounded by positively charged residues that are present to facilitate phosphate binding, lowers the pK_a of this residue making it more a reactive nucleophile.[69] AP22161 (**38**) was designed to exploit this unique cysteine residue to form a covalent attachment to the protein through addition to a benzaldehyde, which was positioned to trap the cysteine thiol as a hemithioacetal. This compound inhibited Src binding to a fluorescein-tagged SH2 ligand peptide with IC_{50} = 5.5 μM.

The ability of AP22161 to penetrate cells was assessed using a sophisticated mammalian two-hybrid assay.[67] Briefly, expression of a secreted alkaline phosphatase reporter protein was engineered to be dependent on the appropriate recognition of a SH2 binding peptide by a Src family kinase. In the presence of a SH2 domain inhibitor, no alkaline phosphatase is produced. With appropriate controls, this assay was used to demonstrate that AP22161 selectively inhibits Src SH2 vs ZAP SH2 in cells, albeit at relatively high concentrations (IC_{50} = 60−80 μM). No cell toxicity was observed at these concentrations. Consistent with these results, AP22161 was a relatively weak inhibitor of bone resorption with IC_{50} = 42.9 μM.

An observation that the protein Src cocrystallized with a molecule of citrate bound in the SH2 domain led to a second approach for specifically targeting Src in bone.[68] The phosphonate-containing amino acid Dpp (**40**, Figure 14) was introduced as both a pTyr mimic and a potential bone-targeting residue. The incorporation of Dpp into the non-peptide SH2 ligand led to the synthesis of AP22408 (**39**) which inhibited Src with IC_{50} = 0.3 μM, as measured using a fluorescence polarization assay. The affinity of AP22408 for bone was confirmed by hydroxyapaptite chromatography and the association of tritium-labeled AP22408 with dentine (a bone substitute).[68] AP22408 inhibited the absorption of dentine slices by rabbit osteoclasts in vitro with an IC_{50} = 1.6 μM, providing the osteoclasts were preincubated with the inhibitor. Moreover, in an established animal model for the in vivo evaluation of antiresorptive compounds against thyroid hormone-induced bone resorption, AP22408 significantly decreased serum calcium (Ca^{2+}) compared to control consistent with a robust antiresorptive effect. This in vivo demonstration of a phenotypically observable outcome from the use of a small molecule SH2 inhibitor provides one of the few existing validations for the use of inhibitors of protein−protein interactions to modulate signaling in a biologically relevant setting. It should provide significant impetus to the field of SH2 inhibitor design as well as encourage related efforts toward the inhibition of other signaling pathways that depend on SH2-mediatied protein associated events.

Future Directions

Allosteric Regulation and Protein Stabilization. Although it may be conceptually simpler to imagine inhibiting protein−protein association by interfering directly at the binding interface, nature has taught us that remotely binding inhibitors may be equally effective.[70] This is the concept of allosteric control that is used so effectively by enzymes. The binding of a small molecule allosteric effector can result in large conformational changes in a protein which relay information to the active site with the result that either substrates no longer bind or the catalytic residues are no longer correctly aligned to perform chemistry.[71] In principle, this strategy may equally well apply to the inhibition of protein−protein binding. Indeed, we have already seen one example in the form of an NOS inhibitor that bound to the catalytic iron, inducing a conformational change that prevented dimerization of the NOS monomer.[19] Many members of the growing class of small molecule antagonists of G-protein-coupled receptors may work allosterically by binding to a transmembrane region of the receptor and thereby preventing binding of the natural ligand to an extracellular site.[9−13]

The feasibility of discovering small molecules that alter protein conformation was illustrated in recent work from a group at Pfizer who devised a method for stabilizing the protein p53. The p53 tumor suppressor protein is frequently mutated in cancer cells, diminishing its ability to respond to DNA damage. Consequently, the processes of cell cycle arrest or apoptosis that would normally be initiated by p53 fail to occur, and cells

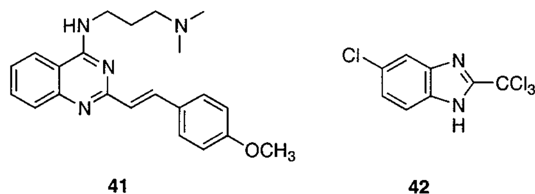

Figure 15. Compound **41**, CP-31398; **42**, E8.

containing damaged DNA are allowed to divide leading to tumor formation. Evidence suggests that the structural basis of this functional failure is a destabilization of the p53 DNA binding domain. Foster and co-workers discovered a small molecule, CP-31398 (**41**, Figure 15), from a collection of greater than 100 000 compounds, that stabilized mutant p53 in a wild-type structure capable of binding DNA as indicated in a gel-shift assay.[72] Mutant p53, when treated with CP-31398, was thermally more stable than untreated protein. Moreover, H1299 cells transfected with mutant p53 expressed a p53-regulated reporter gene in response to the addition of CP-31398 in a concentration-dependent manner. Similar effects were evident in vivo in mice with the growth of A375·S2 melanoma and DLD-1 colon carcinoma, both inhibited with daily or twice daily doses of CP-31398, indicating that the function of p53 could be restored even in an animal model.

The concept of protein stabilization has been successfully, albeit unwittingly, exploited through use of the class of antitumor agents known as antimitotics.[73] Microtubule stabilizing drugs such as Taxol appear to function by stabilizing a form of β-tubulin that would otherwise be unstable once GTP hydrolysis occurred.[74] Thus, Taxol allows β-tubulin·GDP complexes to participate in microtubule formation when they normally would not. In this example, the small molecule facilitates protein−protein binding rather than inhibiting it; however, it is easy to imagine how this outcome could be reversed. Indeed, the original antimitotic drugs, including colchicine and nocodazole, do function by destabilizing microtubules, not by stabilizing them. As seen above, the compounds identified by Haggarty and co-workers also are proposed to operate by this mechanism.[30] An excellent discussion of how various small molecules, including colchicines and *vinca* alkaloids, may affect microtubule dynamics is provided by Downing.[75]

The potential of small molecules to affect protein stability was further illustrated by Schultz and co-workers using a model system derived from human growth hormone (hGH).[76] A mutation was made in hGH to deliberately introduce a cavity that destabilized the protein so that it bound its receptor (hGHbp) 10^6-fold more weakly (K_d(wt) = 0.3 nM, K_d(mutant) > 1 mM). Selection from a small library of indole analogues identified 5-chloro-2-trichloromethylimidazole (E8; **42**, Figure 15) as a compound capable of restoring receptor binding affinity. In the presence of 100 μM ligand, hGH bound to hGHbp with K_d = 260 nM. In cells, E8 restored the agonist potential of mutant hGH, as indicated by cell proliferation, and facilitated mitogenic signaling as indicated by the phosphorylation of the downstream protein Jak2.

Several groups have been studying methods for dimerizing and "reverse-dimerizing" proteins to control macromolecular interactions in vivo. The ability of tethered ligands to bring their respective partner proteins together is now well established.[77] In one recent example, Lin et al. used a dexamethazone-methotrexate dimer to activate transcription of a *LacZ* reporter gene in yeast via a LexA-DHFR fusion protein and a B42-GR fusion protein.[78] An extension of this idea proposes the use of chemical-induced dimerization to hijack a protein that is not a natural partner protein into blocking a targeted protein−protein binding interaction. For example, a tethered dimer of FK506 and a peptide ligand for the SH2 domain of the src kinase Fyn promotes the formation of a complex between FKBP, the ligand dimer, and Fyn, which might be expected to interfere with Fyn binding to its natural downstream proteins.[79] A similar approach was used by Chiosis and co-workers to inhibit PI-3-kinase.[80] Other workers have tethered geldanamycin (GA) to steroid molecules such as testeoterone and estradiol to target specific cell types.[81,82] In addition, a four-carbon tethered GA dimer exhibits, as yet unexplained, selectivity for promoting the degradation of HER-2 kinase over other kinases.[83]

From studies on the prototypical dimerizer, FK506, and its target protein FKBP, a point mutant of FKBP (F_M) was discovered that rendered this normally monomeric protein a weakly stable dimer (K_d = 30 μM).[84] In this case, the dimer could be dissociated by the addition of an FKBP ligand such as **43** or **44** (Figure 16). This phenomenon was subsequently exploited in vivo to regulate protein secretion through the endoplasmic reticulum.[85] Fusion proteins of F_M (four copies) joined to either hGH or human proinsulin via a furin cleavage sequence were shown to aggregate in the ER of HT1080 cells (a human fibrosarcoma line) in culture. Addition of a tight binding ligand (K_d = 1 nM) that is selective for F_M vs FKBP caused disaggregation of the complexes and subsequent release of the correctly processed and folded hGH or insulin proteins which were secreted from the cells and could be detected in the supernatant. Regulated secretion of hGH and insulin was also achieved in mice, demonstrating that these small molecule inhibitors of F_M aggregation were also fully functional in vivo.

Challenges Ahead. The growing number of examples of small molecules that have been demonstrated to inhibit protein−protein binding should provide encouragement to those who believe that there are opportunities for exploiting this approach in drug discovery. Clearly, high molecular weight is not a prerequisite for a PPBI to be effective, even in vivo. Moreover, there does not appear to be an intrinsic limit on the level of affinity achievable (witness the 9 nM inhibitor of CaM-dependent PDE activation **30** and the 1.6 nM inhibitor of SH2, **17**). It would appear that we are on the verge of seeing the first examples of designed PPBIs approaching preclinical development. Yet, it is premature to claim that the difficulties accompanying the discovery of effective PPBIs have been overcome. For sure, this approach will work only in well-chosen situations, and better methods for characterizing protein−protein interfaces will play an important role in helping to select the most pliable cases for drug discovery. Theoretical and experimental approaches to this problem are already being developed. For example, Ringe and Mattos

have advocated the crystallization of proteins in various organic solvents to identify surface regions that might provide binding sites for small organic molecules by the location of bound solvent molecules.[86] Weiss and co-workers have developed a combinatorial approach to alanine scanning using phage-display technology and have used this approach to identify key side chains involved in binding between hGH and its receptor.[87] Massova and Kollman have described a computational version of alanine scanning that also may be used to probe the contribution of individual residues to protein–protein binding energies.[88]

Currently, there are no general techniques or approaches that will reliably illuminate the path toward the synthesis of potent and effective drug-like PPBIs. However, these techniques are being developed and with increased use and experience should provide some guidelines for inhibitor design. As in other areas of drug discovery, the judicious employment of combinatorial chemistry has the potential to speed up the discovery of PPBIs.[89] Also, NMR methods such as SAR by NMR[90] and related techniques may be used to identify weak binding ligands as lead structures for inhibitor optimization. Covalent linkage of two or more weak binding ligands is anticipated, under optimal circumstances, to produce a compound of substantially improved affinity.[91] Alternatively, specific noncovalent ligands may be converted to covalent ligands by the incorporation of a reactive functional group.[92]

In conclusion, a combination of screening and structure-based design will provide starting points for the discovery of novel PPBIs, and structural data from protein–ligand complexes should be able to guide further optimization. Success will likely hinge on being able to bury sufficient hydrophobic surface without inordinately increasing the size of the small molecule. If this is not possible at the protein interface, then allosteric sites may be considered. It is to be hoped that over time some themes may emerge, highlighting for example particular generic structures that may form a basis for PPBIs. Compound libraries then can be populated with compounds exemplifying these structures, increasing the chances of lead discovery through high throughput screening. Perhaps then the trepidation that protein–protein binding currently imbues in many medicinal chemists will be overcome, and the rich opportunities available for drug discovery finally will be recognized.

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Note Added in Proof

For a complementary review of this field, see: Ockey, D. A.; Gadek, T. R. Inhibitors of Protein–Protein Interactions. *Expert Opin. Ther. Pat.* **2002**, *12*, 393–400.

Biography

Peter L. Toogood was born and educated in England. He obtained his B.Sc. and Ph.D. at Imperial College, London, where he performed research on the synthesis and antifeedant properties of azadirachtin under the supervision of Professor Steven V. Ley. Subsequently, he held a NATO postdoctoral fellowship at Harvard University, where he worked with Professor Jeremy R. Knowles to prepare and study bivalent inhibitors of influenza hemagglutinin. In 1992, Dr. Toogood moved to the University of Michigan as an Assistant Professor, where he initiated a program of research in organic synthesis and biochemistry that led to a formal synthesis of althiomycin, a total synthesis of motuporin, and a proposed revision of the structure of keramamide F. His research on the mechanism of action of didemnin B provided new insights into the inhibition of protein synthesis by this natural product and led to a reinterpretation of the role of elongation factor-1α during polypeptide elongation. In 1998, Dr. Toogood joined the medicinal chemistry department of the Parke-Davis Pharmaceutical Company to work on cancer drug discovery. He currently holds the position of Assistant Director for Oncology at Pfizer Global Research and Development, Ann Arbor.

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