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REVIEWARTICLE

State-of-the-art Strategies of Targeting Protein-Protein Interactions by Small-molecule Inhibitors

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Targeting protein-protein interactions (PPIs) has emerged as viable approaches in modern drug discovery. However, the identification of small molecules enabling to effectively interrupt their interactions presents significant challenges. In the recent past, significant advances have been made in the development of new biological and chemical strategies to facilitate the discovery process of small-molecule PPI inhibitors. This review aims to highlight the state-of-the-art technologies and the achievements made recently in this field. The "hot spots" of PPIs has been proved to be critical for small molecules to bind. Three strategies including screening, designing, and synthetic approaches have been explored for discovering PPI inhibitors by targeting the "hot spots." Although the classic high throughput screening approach can be used, fragment screening, fragment-based drug design and newly improved virtual screening are demonstrated to be more effective in the discovery of PPI inhibitors. In addition to screening approaches, designing strategy including anchor-based and small molecule mimetics of secondary structure involved in PPI becomes powerful tools as well. Finally, constructing new chemically spaced libraries with high diversity and complexity is becoming an important area of interests for PPI inhibitors. The successful cases from the recent five year studies are used to illustrate how these approaches are implemented to uncover and optimize small molecule PPI inhibitors and notably some of them have become promising therapeutics.

1. Introduction

A number of biological processes are mediated by protein-protein interactions (PPIs).[1] It is estimated that there are about 130,000 to 650,000 types of PPIs in human interactome.[2, 3] PPIs, therefore, offer a rich source of novel drug targets. Toward this end, significant efforts have been made on the development of new generation therapeutics by targeting PPIs.[4] However, the design of selective and potent small-molecule inhibitors for PPIs is more difficult than that of traditional targets (*e.g.* kinases, G-protein-coupled receptors, ion channels, etc.) because of the nature of the PPI interfaces.[5-7] Unlike traditional drug targets having well-defined pockets for small molecules to bind,[8] the PPI interface is generally large and flat, and is often exposed to solvent.[9] The contact surfaces involved interactions are about 1,500–3,000 Å^2 and are often dominated with hydrophobic and charged characteristics.[10, 11] These features make small molecules difficult to effectively interrupt their interactions. A potent PPI inhibitor should cover a large surface area and make a large number of hydrophobic contacts. Such a binder may face pharmacokinetic issues because it usually has large molecular weight (MW) and poor solubility. It is also highly challenging to find a good starting point for the design and identification of small molecule PPI inhibitors. The natural binders of PPI interfaces are generally not available. Moreover, amino acids involved in PPIs are not contiguous in the polymer chain and thus the protein counterpart itself cannot be used as a lead compound for the design of small-molecule peptidomimetics. On the other hand, distinguishing a real PPI inhibitor from artifactual binding requires a number of biological experiments, while validating ligands of traditional targets is relatively simple.

Despite the above challenges, remarkable progress has been made in discovery, characterization and development of small-molecule PPI inhibitors as results of increasing understanding of action of mechanisms of PPIs in recent years.[12-14] First, critical "hot spots" responsible for the majority of the binding free energy have been identified in many PPI interfaces.[15, 16] They represent a few key residues and their surface area is significantly smaller than the entire interface.[17] The opening of so-called transient pockets has been observed when binding of small-molecules in protein-protein interfaces.[18, 19] Thus, it is feasible to design drug-like small molecules by targeting the "hot spots." Second, dynamic flexibility is observed for PPIs interface.[20] Well-defined binding pockets cannot be found in free proteins or protein-protein complexes, but are available for small molecule binding in PPIs interface.[21] Third, screening techniques, particularly fragment screening, have been rapidly developed for identification and validation of PPI inhibitors.[22] As a result, the discovery of PPIs inhibitors has been greatly accelerated with the advent of these technologies and a great number of highly potent binders have been identified.[23] Finally,

the druggability of PPI inhibitors has been validated by a series of clinical candidates. They have comparable affinity to natural protein binders and among them, ABT263 (**1**),[24]I-BET762 (**2**),[25]SAR 1118 (**3**),[26] and RG7112 (**4**),[27] RG7388 (**5**),[28] MI-77301 (**6**)[28] and AMG232 (**7**)[28] have shown promising drug-like properties in clinical trials (**Figure 1**).

Due to the success in the discovery and development of small molecule PPI inhibitors, it is highly desirable to summarize the recent advances and guide future endeavors in this emerging field. Although some of the methodologies and examples have been covered in several excellent reviews,[22, 29-41] they more focused on the specific topics. Herein we intend to more comprehensively cover state-of-the-art strategies of PPI-based drug discovery with focusing on new developments of the methodologies. Moreover, successful stories in drug development of PPI inhibitors as well as a number of newly reported studies will be highlighted. In addition, we will provide in-depth discussion about merits and limitations of different strategies and give perspectives to guide future research.

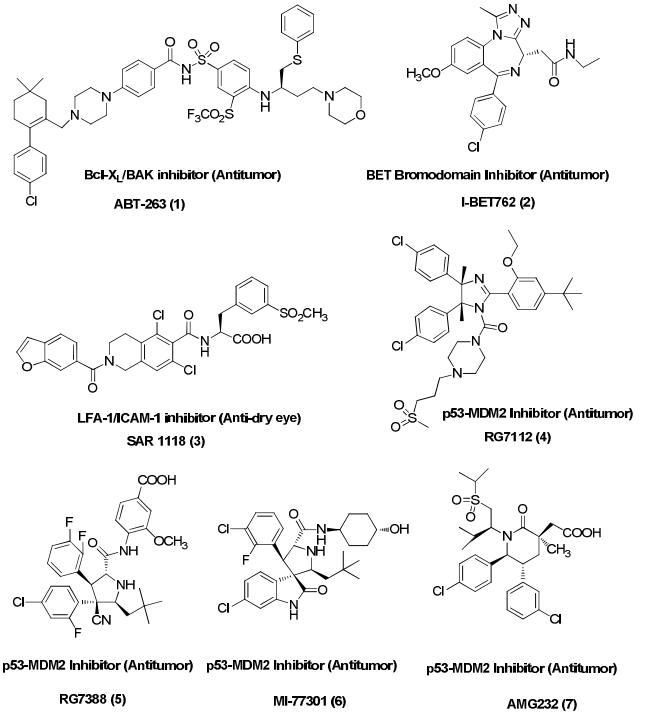


Figure 1. Small-molecule PPI inhibitors under clinical development

2. "Hot Spots" as Structural Basis for the Design of Small-molecule PPI Inhibitors

The understanding of PPIs interface features is critically important for the design and identification of effective inhibitors. Generally, PPI interface consists of a core region and a rim region.[15] According to the PPI contact area and binding affinity, PPI interfaces can be classified into narrow (surface area < 2500 Å^2)/wide (surface area > 2500 Å^2) surface and tight (K_d < 200 nM)/loose (K_d > 200 nM) affinity.[42] Among them, the "narrow and tight" PPIs are more amenable to design small-molecule inhibitors.

In 1995, Clackson and Wells introduced the concept of "hot spots" into this field for the first time.[16] They found only a small set of residues in the interface between human growth hormone (hGH) and its bound receptor (hGHbp) critical for their interactions. The existence of such "hot spots" was observed to be general in protein-protein interfaces by a number of subsequent studies.[15] "Hot spots" account for an average of 9.5% of the interfacial residues and most of them are located in the core regions.[43] Moreover, the "hot spots" have an interesting functional and structural adaptive feature upon binding small molecules.[20, 44] Arkin *et al.* compared the crystal structures of free-state and small-molecule bound cytokine IL-2.[18] It was found that the small molecule bound to the same site that binds the natural IL-2 receptor α (IL-2Rα). Interestingly, a groove that was not seen in the free structure of IL-2 was observed for small-molecule binding. This suggests that the "hot spots" of IL-2 are highly adaptive and the energy barriers for conformational changes are low. The plasticity or flexibility of "hot spots" for small molecules to bind has been generalized to other druggable PPI targets such as Bcl-X$_L$,[45] HDM2,[46] and HPV-18-E2.[47] Systematic analysis of "hot spots" revealed that they are often enriched in tryptophan (21%), arginine (13.3%), and tyrosine (12.3%) residues.[43] These residues allow adaptive conformational change to accommodate small molecules. Furthermore, they are able to form various forces with ligands including hydrophobic, hydrogen bonding, π-π stacking, and electrostatic interactions. The dynamic features of PPI interfaces underscore the challenge of using structure-based drug design (SBDD) strategies to identify small-molecule inhibitors. The straightforward solution to this challenging issue is to obtain crystal structures of the target protein in complex with different ligands or under different conditions. The information of the differences between the conformations in unbound protein or between the unbound and bound structures is highly valuable for inhibitor design. Alternatively, computational tools, such as flexible molecular docking and molecular dynamics (MD) simulations, are also effective tools to study the dynamic features of the PPI interface. The conformational changes can be reproduced by considering side-chain rotamers[48, 49] or MD simulation.[50, 51] For example, Eyrisch and colleagues performed a set of MD simulations on IL-2, MDM2 and Bcl-X$_L$ to understand the dynamics of the opening of transient pockets on protein surfaces.[19] Their results indicated that the transient pockets for ligand binding were reproducible and observable at the native binding site during MD simulation studies.

Alanine scanning mutagenesis[16, 43, 52] in combination with X-ray crystallography are well-established methods to identify the "hot spots" of PPIs. Alanine scanning mutagenesis is able to measure the impact of each residue on the affinity for binding to the partner protein by serially mutating each interface residue to alanine. Moreover, structural biology enables to offer the distribution and orientation of these hot spot residues in PPI interfaces and thus provides key structural information for rational inhibitor design. However, "hot spots" identified by alanine scanning mutagenesis only reflect contributions to the mutual interaction energy within a protein−protein complex. It is important to assure whether they are really the binding sites for small-molecules. It is noteworthy that a hot spot residue identified by alanine scanning mutagenesis does not imply the existence of a druggable site at that region. Only a subset of "hot spots" residues have the potential to bind small molecules because the hot spots identified by alanine scanning were based on energetic contributions. Only when they have additional topological features that are appropriate to binding a small ligand, they can be regarded as druggable sites. Moreover, a recent analysis by London *et al.* revealed that hot segments, a

continuous binding epitope, are good predictors of PPI druggability.[38]

The druggable site of a protein can be determined by finding consensus sites that are characterized by their ability to bind a variety of fragments or smaller organic "probes." Such consensus sites can be identified by MSCS[53] (multiple solvent crystal structures) approach and fragment screening[54] techniques (such as "SAR by NMR"[55]). Very recently, Zerbe *et al.* investigated the relationship between "hot spots" and consensus ligand-binding sites of PPIs.[56] The results revealed that the two types of sites are largely complementary and strong correlation between them is observed. Besides the experimental approaches, a number of computational tools have been developed to identify the druggable sites of PPIs, which have been summarized in two recent reviews.[22, 57] Herein only two newly reported approaches are highlighted. Kozakov *et al.* described a novel algorithm to identify probable druggable sites of PPI interface by combining computational solvent mapping (finding energetically accessible hot spots) with conformational sampling (considering flexibility to accommodate drug-size molecules).[48] They proposed that druggable sites at PPI interface should comprise a cluster of binding hot spots that possess a general tendency to bind organic molecules with some polar groups decorated on largely hydrophobic scaffolds. More recently, Metz *et al*. developed a computational strategy which enables to identify the hot spots, transient pockets and determinants of small-molecule binding in PPI interface by simultaneously considering aspects of energetics and plasticity.[58] This method is applicable to SBDD of PPI inhibitors in terms of pocket identification, binding mode prediction and virtual screening. Notably, the energetics and interface plasticity are both taken into account in the two above mentioned methodologies and the importance of MD simulations are highlighted in identifying binding "hot spots" in PPI interface.[59]

3. Overview of Current Strategies for the Design of Small-molecule PPI Inhibitors

Determining the structure of PPI interface is the first step for small-molecule inhibitor design (**Figure 2**).[60] Due to the dynamic feature of PPI interface, the availability of structures from different status (unbound protein, protein-protein complex, protein-ligand complex) can significantly facilitate the drug design process. The success in designing PPI inhibitors largely depends on the target type. Thus, druggability assessment is necessary to know whether the target has well-defined binding sites or grooves to accommodate small molecules.[61] The next step is to identify the "hot spots" on the PPI interface, which is central for inhibitor design.[62] Good targets often have small "hot spots" that can be covered by a drug-sized molecule. Then, it is important to select a suitable method to identify initial hits according to the properties of PPI hot spots. Currently, three major strategies are available for small-molecule PPI inhibitor design, namely screening strategies, designing strategies and synthetic strategies. Screening strategies aim to discover PPI inhibitors from known compounds. High throughput screening, fragment screening, and virtual screening have all been successfully used to identify hits. Particularly, fragment screening and fragment-based drug design (FBDD) have shown advantages in terms of higher hit rate and ligand efficiency (LE). In contrast, designing strategies build novel chemotypes to mimic the key interactions of the hot spot residues. The hot spots residue(s) can be used as starting point for analogue design by sub-structure search, bioisostere design and *de novo* design. On the other hand, small molecules can also be designed to mimic key secondary structure motif (*i.e.*α-helix, β-turn, and β-strand) involved in PPI interface. A new scaffold should be designed to decorate the side chains of hot spot residues whose spatial orientation is similar to that of original secondary structure. Synthetic strategies mainly focus on exploring and expanding the chemical space for PPI inhibitor screening. Efficient synthetic methods are powerful tools to construct new libraries with improved chemical diversity and complexity. When the initial hits are identified, secondary assays are necessary to validate whether these hit compounds are real PPI inhibitors. The hits can be validated by determining kinetic and thermodynamic parameters (*e.g.* association and dissociation rates) and structures of protein-hit complexes. After hit validation, following optimization study is to improve the binding affinity and drug-likeness. The strategies are similar to those for traditional targets. Finally, candidates can be selected for preclinical and clinical trials if the optimized molecule meets the requirements for new drug development.

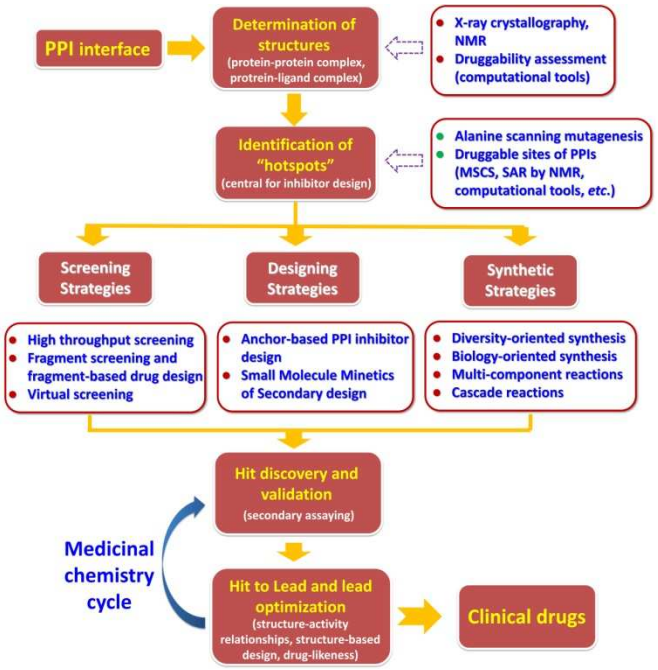


Figure 2. Current strategies for the design and development of small-molecule PPI inhibitors

4. Screening Strategies to Discover Small Molecule PPI inhibitors

4.1 High Throughput Screening

High throughput screening (HTS) is a well-established method used for discovering hits in traditional drug targets. Nonetheless, it is difficult to efficiently generate PPI inhibitors by HTS because compound libraries for screening are collected or designed for historical medicinal chemistry efforts that mainly act on conventional drug targets. The chemical space of PPI inhibitors may not be significantly covered by these libraries and thus the hit rates of HTS are generally low. However, HTS is still useful to identify starting points targeting PPIs, particularly in big pharmaceutical companies.

Recent examples of HTS-derived small molecule PPI inhibitors (**8**-**23**) are listed in **Table 1** and **Figure 3**. Most of the HTS hits are active in sub-micromolar or low-micromolar concentrations. Several of them have complex chemical structures with large MW but lack the potential for further optimization. From limited reports about optimization of HTS hits **(Table 2)**, it can be found that minor change of the chemical structures could lead to substantial improvement of the binding affinity. Such modifications might result in more favorable interactions with the "hot spots" and thus SBDD can efficiently accelerate the process of hit-to-lead. On the other hand, increasing attention on the programs targeting PPIs has been received from big pharms, such as Roche, Bristol-Myers Squibb, Pfizer and Abbott.[4] So far, the most successful cases of application of HTS in identification of PPI inhibitors are the discovery of nutlins[63] and benzodiazepinediones[46] as p53-MDM2 (mouse double minute 2) inhibitors and the development of nutlin derivative RG7112 (**4**) as a clinical candidate for the treatment of leukemias and solid tumors.[27, 64, 65] To improve the hit rates and quality of HTS, big pharmaceutical companies are trying to expand their screening libraries by adding diversity and complexity of the molecules. In this context, natural product libraries may offer the advantage to meet the demand for screening of PPI inhibitors. For example, Majmudar *et al*. performed parallel HTS studies on commercial compound collections and natural product extracts for identifying coactivator CBP/p300 inhibitors. No hit was discovered in the 5000 member conventional compound library. In contrast, three hits were successfully identified from the 15,000 natural product extracts.[66]

Table 1. Recent examples of small molecule PPI inhibitors identified by HTS[a]

Compound	Target	Activity	Hit Rate	Ref
8	ZipA/FtsZ	K_i: 12 μM	0.01%	67
9	CBP/β-Catenin	IC_{50}: 3.0 μM	0.06%	68
10	EGFR/p85	IC_{50}: 5 μM	0.005%	69
11	JIP/JNK	IC_{50}: 5.7 μM	0.0001%	70
12	JNK/JIP	IC_{50}: 0.28 μM	Not reported	71
13	JIP/JNK	IC_{50}: 5.7 μM	0.0001%	70
14	p53/GST	IC_{50}: 10 μM	0.3%	72
15	Atg8/Atg3	IC_{50}: 18.16μM	1.5%	73
16	Keap1/Nrf2	IC_{50}: 3μM	0.002 %	74
17	Keap1/Nrf2	IC_{50}: 118 μM	0.007 %	75

[a]Abbreviations: ZipA/FtsZ, cell division protein ZipA /filamenting temperature-sensitive mutant Z; CBP/β-catenin, β-catenin/CREB-binding protein; EGFR/p85, epidermal growth factor receptor/p85 protein; JIP/JNK, JNK-interacting protein/c-Jun *N*-terminal kinase; Plk1/PBD, polo-like kinase 1/polo-box domain; p53/GST, p53 protein/ glutathione s-transferase; Atg8/Atg3, Atg8 is a ubiquitin-like protein and Atg3 is a E2-like conjugating enzyme. Keap1/Nrf2, Kelch-like ECH-associated protein 1/nuclear factor erythroid 2-related factor 2.

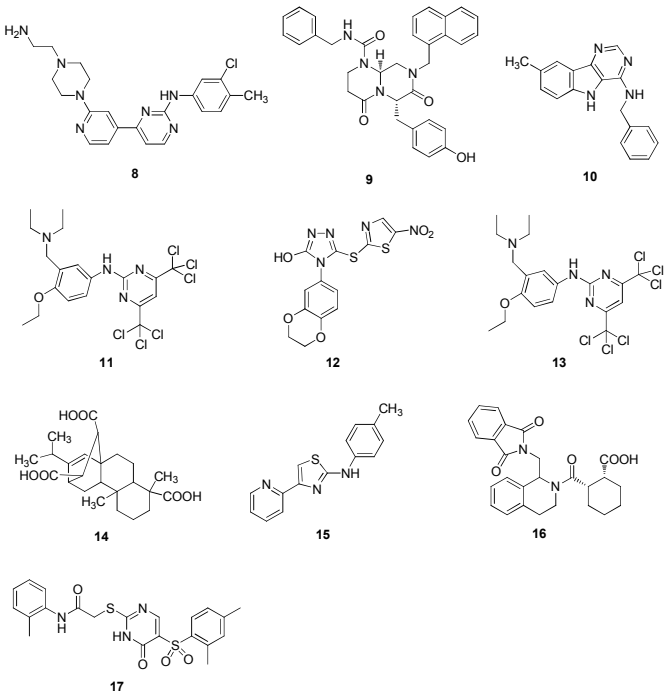
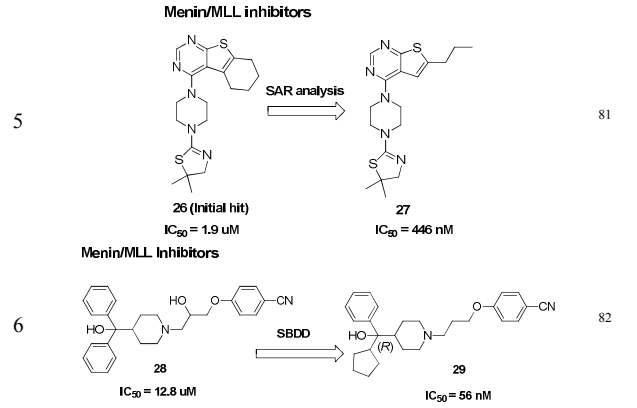


Figure 3. Chemical structures of small molecule PPI inhibitors identified by HTS

Table 2. Selected examples of optimizing HTS hits as PPI inhibitors[a]

Entry	Optimization process and biological activity	Ref
1	**LRH-1/SF-1 inhibitors** SAR analysis of the side chain **18 (initial hit)** LRH-1 EC_{50} = 0.43 μM SF-1 EC_{50} = 0.054 μM **19** LRH-1 EC_{50} = 0.012 μM SF-1 EC_{50} = 0.010 μM	76
2	**TLR1/TLR2 inhibitors** Introducing substitutions on the benzotropolone scaffold **20 (initial hit)** IC_{50} = 2.45 uM **21** IC_{50} = 0.58 uM	77
3	**HPV E2/E1 inhibitors** A combined medicinal chemistry, NMR and computational approach **22 (initial hit)** IC_{50} = 11 uM **23** IC_{50} = 6 nM	78, 79
4	**TR/CoR inhibitors** SAR analysis **24 (initial hit)** IC_{50} = 2 uM **25** IC_{50} = 0.58 uM	80



[a] Abbreviations:TLR1/TLR2, toll-like receptor 1/toll-like receptor 2; HPV E2/E1, human papilloma virus transcription factor E2/helicase E1; TR/CoR, thyroid hormone receptor/coregulator; LRH-1/SF-1, liver receptor homolog-1/steroidogenic factor-1; Menin/MLL, menin/mixed lineage leukemia.

Case study 1: Discovery and structure-based optimization of Keap1−Nrf2 inhibitors

Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nrf2), and antioxidant response elements (ARE) are three major cellular components involved in the regulation of cytoprotective responses to oxidative and/or electrophilic stresses.[83] Discovery of small molecule inhibitors directly interrupting the Keap1-Nrf2 interaction is an attractive strategy for the prevention of cancer and other chronic diseases, such as Alzheimer's, Parkinson's, and inflammatory bowel diseases.[84, 85] On the basis of the crystal structure Keap1 binding interface (PDB code: 1X2R),[86] Sun and You's group performed MD simulations and MM-GBSA free energy calculations to investigate the molecular determinants of binding between Keap1 and Nrf2.[87] As shown in Figure 5B, the binding pocket of Keap1 can be divided into five sub-pockets (P1-P5). Among them, the P1 and P2 pockets were identified as the "hot spots," which made key contributions to the total binding free energy. Several small molecule Keap1-Nrf2 inhibitors were discovered by HTS (**16**-**17**, Table 1).[74, 75] As shown in **Figure 4A**, inhibitor **30** (EC_{50} = 2.7 μM) mainly occupies the P3, P4, and P5 pockets (PDB code: 4IQK) and only forms a hydrogen bond with Ser508 at the edge of P1 pocket. On the basis of the binding mode of **30**, compound **31** was rationally designed using a SBDD approach (**Figure 4B**). Considering the the NH of the two sulfamides in compound **30** are pointed to the P1 and P2 pockets, two carboxyl groups were attached through a methylene linker, which formed extra strong polar interactions with Arg415 and Arg483 (**Figure 4C**). These additional interactions with hot spots P1 and P2 led to significant improvement of Keap1 inhibitory activity. Compound **31** showed a K_D value of 3.59 nM and an EC_{50} value of 28.6 nM in the FP assay, which was the most potent small-molecule inhibitor of Nrf2−Keap1 up to date.

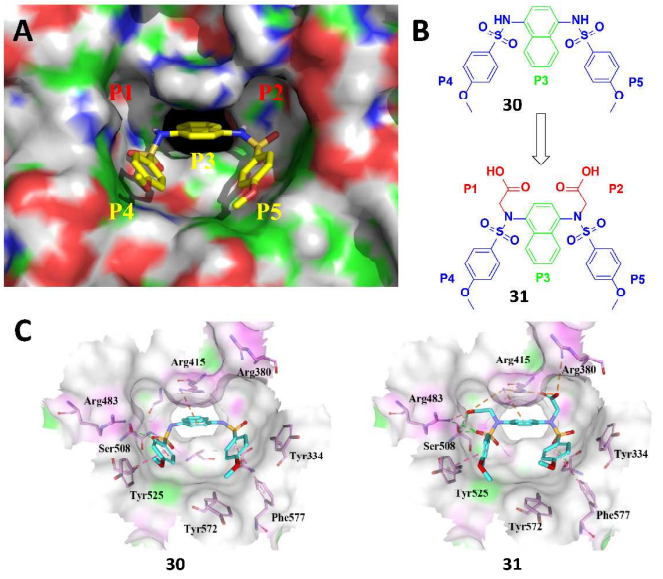


Figure 4. Discovery and optimization of Keap1−Nrf2 inhibitors. (A) Binding pockets of Keap1−Nrf2 interface (PDB code: 4IQK); (B) Chemical structures of Keap1 inhibitors **30** and **31**; (C) Binding mode of compounds **30** and **31** with Keap1.

Case study 2: Discovery and structure-based optimization of PDEδ–KRAS inhibitors

Oncogenic RAS signaling is an important antitumor target. Inhibition of the binding of mammalian PDEδ to KRAS by small molecules provides a novel opportunity to impair Ras localization and signaling, and discover novel antitumor agents.[88] Waldmann's group identified benzimidazole fragment **32** as a novel PDEδ-KRAS inhibitor by HTS of about 150,000 in-house compounds (**Figure 5A**).[89] Compound **32** binds to the farnesyl-binding pocket of PDEδ and has a K_D value of 165 nM. Cocrystallization of PDEδ with compound **32** reveals that two molecules of **32** bind to the different sites of the protein, which form two hydrogen bonds with Arg61 and Tyr149, respectively (**Figure 5B**). Structure-based approaches were used to optimize hit **32**.[90] First, the two benzimidazole hits were covalently linked by an ether bond and the resulting dimeric compound **33** showed significantly increased activity (K_D = 39 nM). Crystal structure of PDEδ in complex with linked bis-benzimidazole **33** indicates that the original orientation and hydrogen binding interaction of the two benzimidazoles are retained (**Figure 5C**). Also, the crystal complex reveals that the allyl group can be replaced by a larger moiety. Guided by the binding mode, compound **34** with more steric cyclohexyl substitution showed improved activity (K_D = 16 nM). Another chiral piperidine derivative **34** (named deltarasin, K_D = 38 nM) was only comparable to **33**, but it has favourable solubility and membrane permeability. Further optimization of the phenyl ether linker discovered piperidine 4-carboxylic acid ester **35** with increased activity (K_D = 10 nM). Crystal structure of **35** in complex with PDEδ confirms the hydrogen bonding interaction between the piperidine nitrogen and the backbone carbonyl of Cys56 (**Figure 5D**). Considering the hydrolytic stability and physicochemical properties, deltarasin was further evaluated in a

number of assays. Deltarasin showed potent *in vitro* and *in vivo* anti-proliferative activity against human pancreatic ductal adenocarcinoma cells by inhibiting oncogenic RAS signalling. At the dose of 10 mg/kg (twice per day), deltarasin could almost completely inhibited *in vivo* growth of xenografted pancreatic carcinoma in nude mice.

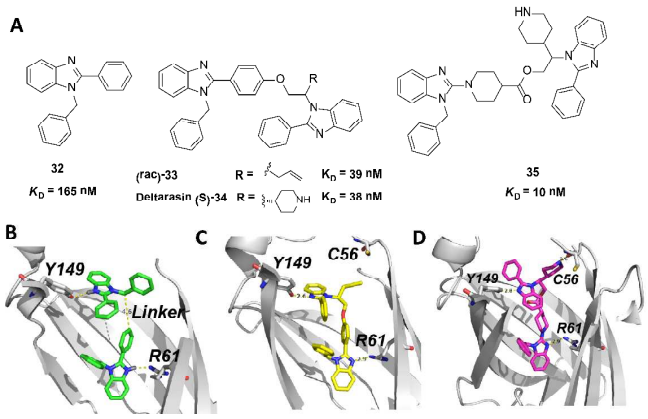


Figure 5. Discovery and optimization of PDEδ–KRAS inhibitors. (A) Structure and binding affinities of benzimidazole PDEδ inhibitors; The binding modes of inhibitors **32** (**B**), **33** (**C**) an **35** (**D**) with PDEδ were generated from the crystal structures in PDB database (PDB codes: 4JV6, 4JVB and 4JVF).

4.2 Fragment Screening and Fragment-based Drug Design (FBDD)

Compared to HTS, fragment screening and FBDD seems to be a more effective approach to discovering PPI inhibitors.[14] FBDD constructs novel lead structures from small molecular fragments by taking advantages of both random screening and structure-based drug design.[91] FBDD leads to weak to moderate binders (5 mM to 1 μM) of the desired target by screening fragment libraries that contain hundreds to thousands of small and low MW fragments.[92] Highly sensitive biophysical techniques have been developed to detect weak fragment binders.[93] Nuclear magnetic resonance (NMR),[94] mass spectroscopy (MS),[95, 96] X-ray crystallography,[97] surface plasmon resonance (SPR) spectroscopy,[98, 99] tethering, differential scanning fluorimetry (DSF) and isothermal calorimetry (ITC) are well-established methods employed to discover and validate fragment hits. In particular, X-Ray crystallography and NMR can also provide important structural information for further hit optimization and are the most frequently used methods for discovery and optimization of PPI inhibitors.[100] After identifying the fragment hits, structure-based optimization strategies including fragment linking, fragment evolution, fragment optimization and fragment self-assembly can be used to increase the affinity and drug-likeness.[101] For example, weak fragment hits bound to different PPI "hot spots" can be linked to generate a highly active lead compound. Also, a fragment hit in a specific hot spot can be extended to interact with other "hot spots" and thus the binding affinity of the resulting molecule can be significantly improved. Impressive success has been achieved in drug discovery and development for traditional druggable targets with FBDD.[91, 102-104] Therefore, in the field of PPIs, applications of FBDD are emerging rapidly (**Table 3**, **Figure 6**).[36, 105, 106] In comparison with HTS, fragment screening has several advantages including higher hit rates, higher LE (LE = 1.37 pIC_{50}/number of heavy atoms) and sampling a larger chemical space.[107] Furthermore, FBDD is more suitable for the design of PPI inhibitors because PPI interface often consists of multiple distinctive hot spots. Individual fragments may occupy different "hot spots" and they can be joined later into complete molecules. In principle, it is more feasible to identify a fragment that binds to a specific PPI hot spot instead of finding a ligand targeting several hot spots simultaneously.

Table 3. Recent examples of low molecule weight PPI inhibitors identified by fragment screening[a]

Compound	Target	Activity	Hit Rate	Ref
37	HIF/TACC3	IC_{50}: 25 μM	2.3%	[108]
38	VWF/GPIbα	IC_{50}: 25 μM	30%	[109]
39	ZipA/FtsZ	62.1% inhibition at 50 μg/mL	0.8 %	[110]
40	AF6 PDZ domain	IC_{50}: 460 μM	0.6%	[111]
41	CBP /p53	K_d: 19 μM	0.3 %	[112]
42	CDC25B-CDK2/Cyclin A	IC_{50} > 5 mM	0.7 %	[113]
43	Ras/SOS1	IC_{50}: 342 μM	0.7 %	[114]

[a] Abbreviations: HIF/TACC3, ARNT subunit of the hypoxia inducible factor activator/transforming acidic coiled coil containing protein 3 coactivator; VWF/GPIbα, von Willebrand factor/glycoprotein Iba receptor; ZipA/FtsZ, cell division protein ZipA/filamenting temperature-sensitive mutant Z; AF6 PDZ domain , postsynaptic density/discs large/zona occludens-1 domain of ALL-1 fusion partner on chromosome 6; CBP/ p53, histone acetyltransferase p300CREB binding protein/p53; CDC25B-CDK2/Cyclin A, CDC25B-cyclin-dependent kinase 2/Cyclin A; Ras/SOS1, Ras is a small GTP-binding protein and SOS1 is a guanine nucleotide exchange factor.

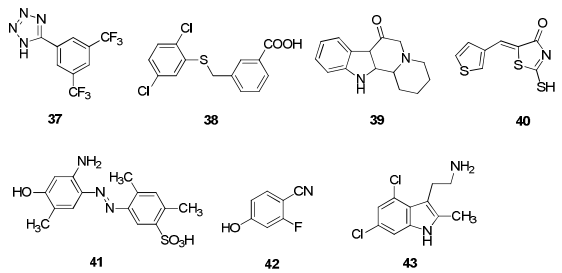


Figure 6. Chemical structures of PPI inhibitors identified by fragment screening

However, current FBDD approaches also face some obstacles. First, a significant amount of pure, soluble and suitable target proteins is needed for labeling or crystallization. Moreover, the biophysical techniques or fragment screening often require expensive detection equipment and specific expertise.[115] Such high demands for FBDD studies limit the broad application. Second, structural information of the ligand-target interaction is important for fragment optimization. As a result, FBDD is difficult to be applied to targets whose structures are unknown. Third, deviation of original orientations and key interactions for the fragment hits may be

occurred when they are evolved into leads.[116] New methods remain to be developed to select proper linkers to bridge or extend the initial fragment hits. Lastly, the criteria for constructing fragment library and the detecting techniques for fragment hits should be improved to make FBDD more powerful in discovering PPI inhibitors. Very recently, Ciulli's group performed a deconstructive study of known PPI inhibitors into fragments using the model of pVHL (von hippel-lindau protein)-HIF 1α (hypoxia inducible factor 1α) interface.[117] Their comprehensive analysis indicated that fragments contributing largely to the binding free energy could not be readily detected by techniques routinely used for fragment screening. When fragments became more complex and began targeting at least two "hot spots" at the pVHL/HIF 1α interface, they can be detected using current screening techniques. The results revealed fragments binding at PPI interface have inherently low LE and promising fragment hits may be missed using current screening methods. Therefore, it is highly important to develop sufficiently sensitive and specific biophysical techniques to reliably detect weak binders as hits. Moreover, most of the reported FBDD examples used NMR-based screening to identify hits.[105] Other detecting methods, such as tethering and SPR, have been routinely used for the study of PPI inhibitors. On the other hand, "rule of three" (RO3)[118] has been widely used to build fragment library. However, Ciulli's findings in combination of Klebe's work[119] suggested that fragments suitable for PPIs should be somewhat larger and reducing some of these strict criteria could lead to higher hit rates.[117]

The discovery of Bcl-X_L inhibitor ABT-263 (Navitoclax, **1**)[24, 120] as a novel antitumor agent represents one of the most successful examples of FBDD-based PPI drug discovery.[121, 12242, 124, 125] Because the drug discovery process of ABT-263 has been extensively reviewed, it was only briefly introduced in **Figure 7**. The following case studies were selected from recent successful examples.

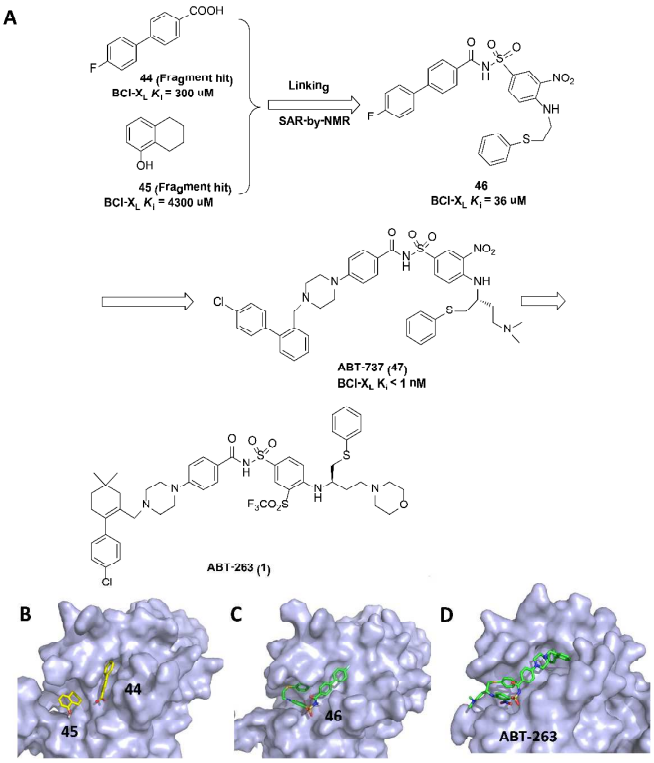


Figure 7. Fragment-based design of Bcl-X_L/BAK inhibitor ABT-263 as a phase II clinical candidate (A). The binding modes of the Bcl-X_L inhibitors (B-D) were generated from the crystal structures in PDB database (PDB codes: 1YSG, 1YSI and 2YXJ).

Case study 1: Fragment-based discovery of Mcl-1/BH3 inhibitors

Recent studies revealed that the over-expression of Mcl-1 conferred resistance to ABT-263.[123] Using a NMR-based FBDD approach, Fesik's group successfully identified selective small molecule inhibitors of Mcl-1/BH3-containing peptides.[124] Initially, 132 hits belonging to 11 distinct chemotypes (hit rate: 0.95%) were identified by NMR-based screening. Then, two classes of compounds were selected for follow-up validation and SAR studies on the basis of their affinity and structural features. The resulting fragments **48** and **49** were found to bind to different sites on Mcl-1 (**Figure 8**). After fragment merging and linker optimization, the binding affinity of compound **50** (K_i = 0.32 μM) was improved more than 100-fold over each original fragment. Moreover, compound **50** also showed selectivity for Mcl-1 over Bcl-x_L and Bcl-2 and could serve as a starting point for the development of Mcl-1 based antitumor therapies.

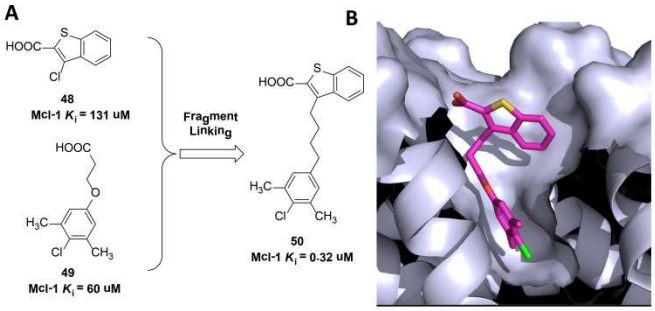


Figure 8. Fragment-based design and the binding mode of Mcl-1 inhibitor **50** (A). The binding mode of inhibitor **50** (B) was generated from the crystal structure in PDB database (PDB code: 4HW4).

Case study 2: Fragment-based discovery of bromodomain-containing protein inhibitors

The bromodomain-containing proteins (BCPs) is an important class of reader proteins of post-translational modifications that recognize acetylated lysine (KAc) residues within histone proteins.[125] The development of small molecule inhibitors to disrupt the protein-protein interaction between BCPs and KAc containing proteins (peptides) represents a new strategy to treat multiple diseases including cancer and inflammation.[126] Fragment screening using NMR-based assays[112, 127-129] and fluorescence anisotropy (FA)[130, 131] assays led to a variety of low molecular weight hits. Chung *et al.* screened a focused set of fragments to discover novel inhibitors of the BET family (*e.g.* BRD2, BRD3, BRD4) of BCPs.[131] 132 initial hits out of 1,376 compounds were identified (hit rate: 9.6%) and 40 hits were co-crystallized with BRD2 to provide important structural information for inhibitor design. One hit, 4-phenyl 3,5-dimethyl isoxazole (**51**), was rapidly optimized by structure-based design.[130] The extension of the hit by substituted sulfonamides could form additional hydrophobic interactions with an adjacent pocket (WPF shelf). As compared to the initial hit **51**, sulfonamide compound **52** (BRD2, IC_{50} = 0.5 μM, LE = 0.38) was at least 100-fold more potent (**Figure**

9). Further optimization was focused on improving solubility and led to more soluble compound **53** (BRD2, IC_{50} = 1.5 μM) while managing to retain the similar affinity. Notably, compound **53** also showed micromolar anti-inflammatory activity in the cellular assays.

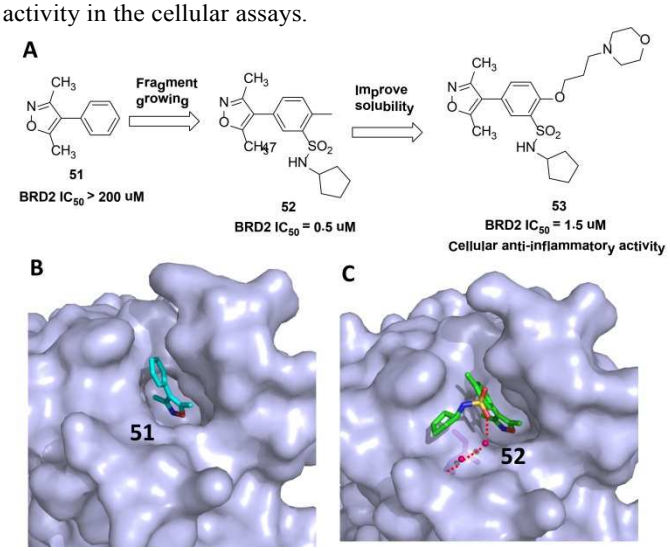


Figure 9. Fragment-based discovery and optimization of inhibitors of bromodomain-containing proteins (A). The binding modes of inhibitors **62** (B) and **63**(C) were generated from the crystal structures in PDB database (PDB codes: 4ALH and 4A9M)

Shen's group discovered novel inhibitors of the first bromodomain of BRD4 (BRD4(I)) by the combination of experimental and computational FBDD approaches.[132] First, a ZINC-based fragment library was docked into the BRD4(I) binding site using the Glide program. A total of 41 fragment hits were obtained at this stage. Second, an X-ray crystallography approach was used to screen the 41 hits and 9 fragment hits were finally identified. The crystallization experiments also obtained the binding conformation of the fragments to BRD4 (I), which provided important information for hit-to-lead optimization. The fragment hit **54** (**Figure 10A**) interacted with BRD4 (I) mainly through direct and water bridged hydrogen bonding interactions between the 2-thiazolidinone core and Asn140 and Tyr97 (**Figure 10B**). The comparison of bind mode of hit **54** and JQ1 (a highly active BRD4 inhibitor) revealed that introducing substitutions at the *meta*- or *para*- position of the phenyl ring of **54** might form additional interactions with BRD4 (I). After several rounds of structure guided optimizations, compound **55** was identified as a better inhibitor (IC_{50} = 4.1 μM). Co-crystallization studied confirmed that the thiophenesulfonamide group in **55** indeed formed new VDW interactions with Trp81 and Ile146 (**Figure 10C**). In order to further improve the BRD4 (I) inhibitory activity and antitumor activity, the reversed sulphonamide derivatives were designed.[133] SAR analysis led to the discovery of **56** as a potent BRD4 (I) inhibitor (IC_{50} = 0.14 μM). Moreover, compound **56** showed good *in vitro* antitumor activity (GI_{50} = 0.18 μM, leukemia MV4; 11 cancer cell lines) as well as favorable pharmacokinetic and selectivity profile, which represents a good lead compound for further optimization. This example also confirmed that the combination of computational docking, X-ray crystallography and structure-based lead optimization is an effective strategy to discover and optimize PPI inhibitors.

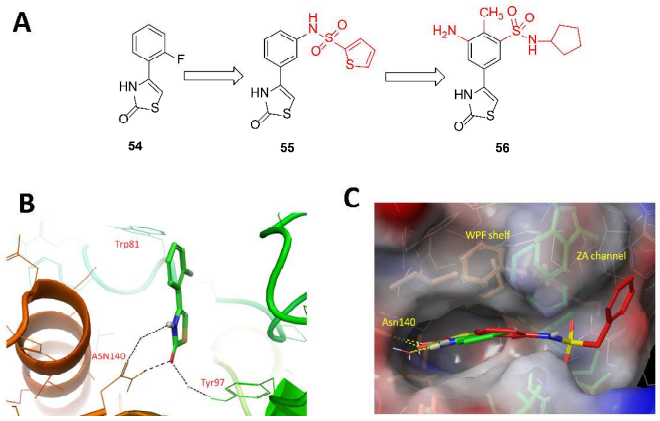


Figure 10. Fragment-based discovery and optimization of BRD4 inhibitors. (A) Chemical structures of BRD4 inhibitors; The binding mode of inhibitor **54** (**B**) and **55** (**C**) with BRD4.

4.3 Virtual Screening

Virtual screening is another complementary approach to HTS because screening of commercially available or in-house chemical libraries creates significant working burden. Computational screening methods can be classified into structure-based[134] and ligand-based approaches.[135] Structure-based virtual screening uses docking and scoring to select molecules that have good binding affinity with the target proteins. Ligand-based approaches prioritize molecules according to the fitness with the pharmacophore or chemical similarity. The cost-effective and high-throughput feature of virtual screening has made it appealing for initial hit identification of druggable targets. However, the application of virtual screening to target PPIs is more challenging compared with the enzymatic cavities. Recent examples of PPI inhibitors identified by virtual screening are summarized in **Table 4** and **Figure 11**. On the basis of the reported examples, virtual screening is more successful for PPI targets with well-defined hot spots.[136] For example, Betzi *et al*. preformed parallel virtual screening and HTS studies leading to discovery of inhibitors of HIV-1 Nef–SH3 binding surface.[137]

Table 4 Recent examples of small molecule PPI inhibitors identified by virtual screening.[a]

Compound	Target	Activity	Hit Rate	Ref
57	TLR4/MD-2	K_d < 10μM	Not reported	138
58	A2/S100A10	IC_{50}: 14 μM	7.4%	139
59	Nef/SH3	K_d: 1.8 μM	30.3%	137
60	trypsin/trypsin	IC_{50}: 14 μM	10%	140
61	IFN-α/IFNAR	K_d: 4.0 μM	16.7%	141
62	S100B/p53	0.1-0.5 mM	1.8%	142
63	c-Abl/14-3-3	Active at 5 μM	7.14%	143
64	IN−LEDGF/p75	IC_{50}: 400 nM	Not reported	144
65	Frataxin/Ub	IC_{50}: 45 μM	2.5%	145
66	BRD4	IC_{50}: 4.7 μM	2.5%	146
67	Keap1/Nrf2	IC_{50}: 4.7 μM	2.5%	147
68	Arf1/GEF Sec7 domain	IC_{50}: 4.7 μM	5.5%	148

69	PKCε/RACK2	IC_{50}: 5.9 μM	4%	[149]

[a] Abbreviations: TLR4/MD-2, Toll-like receptor 4/myeloid differentiation factor 2; A2/S100A10, annexin A2/S100A10 protein; Nef/SH3, HIV type I Nef protein/Src homology 3; IFN-α/IFNAR, interferon-α/interferon-α receptor; S100B/p53, S100B protein/p53; uPAR/uPA, urokinase receptor/urokinase-type plasminogen activator; c-Abl/14-3-3, c-Abl protein/14-3-3 protein; IN−LEDGF/p75, HIV‐1 integrase-lens epithelium-derived growth factor/p75; Frataxin/Ub, Frataxin/Ubiquitin; BRD4, bromodomain 4; Keap1/Nrf2, Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2); Arf1/GEF Sec7 domain, ADP ribosylation factor 1/guanine nucleotide exchange factor Sec7 domain; PKCε/RACK2, protein kinase C ε isoform/receptor for activated C-kinase 2.

Besides virtual screening using well-accepted protocols, new methods have been specifically developed to target PPIs. Schneider's group reported a novel strategy that combines ligand-based virtual screening with PPI hot-spot prediction to discover epitope mimetics.[140] In this method, PPI hot spots are computationally predicted and then used to generate a pharmacophore model, which is subsequently served as a query for virtual screening. This approach has been successfully used to identify interface-derived peptide mimetics of trypsin-trypsin interaction[140] (**Table 4**) and small-molecule inhibitor of IFN-a/IFNAR interface.[141] More recently, Li's group reported a new computational strategy for PPI drug design by combing multiple ligand simultaneous docking (MLSD) and drug repositioning.[150] Multiple fragments are simultaneously docked into the "hot spots" of PPI interface and then the hit fragments can be linked to generate templates. After similarity search of the hit fragments or virtual template compounds on drug databases, existing drugs can be repositioned as novel PPI inhibitors. Using this approach, the human estrogen receptor (ER) inhibitors raloxifene and bazedoxifene were identified as novel inhibitors of IL-6/GP130 interface. On the other hand, the highly flexible nature of PPI interface underscores the importance of using multi-conformations in virtual screening. For instance, Agamennonec *et al*. used an ensemble of S100B conformations for virtual screening of S100B-p53 inhibitors.[142] In particular, MD simulation is a powerful tool to select representative conformations of PPI interface for multi-conformation virtual screening. Recently, Meroueh's group discovered small-molecule inhibitors of uPAR-uPA PPI by virtual screening against multiple conformations of uPAR sampled from MD simulations.[151] The initial hit showed excellent uPAR binding affinity with a K_d value of 310 nM. It is believed that the successful studies will trigger significant interest in exploration of MD simulation methods to improve the hit rate and hit quality of virtual screening of PPI inhibitors.

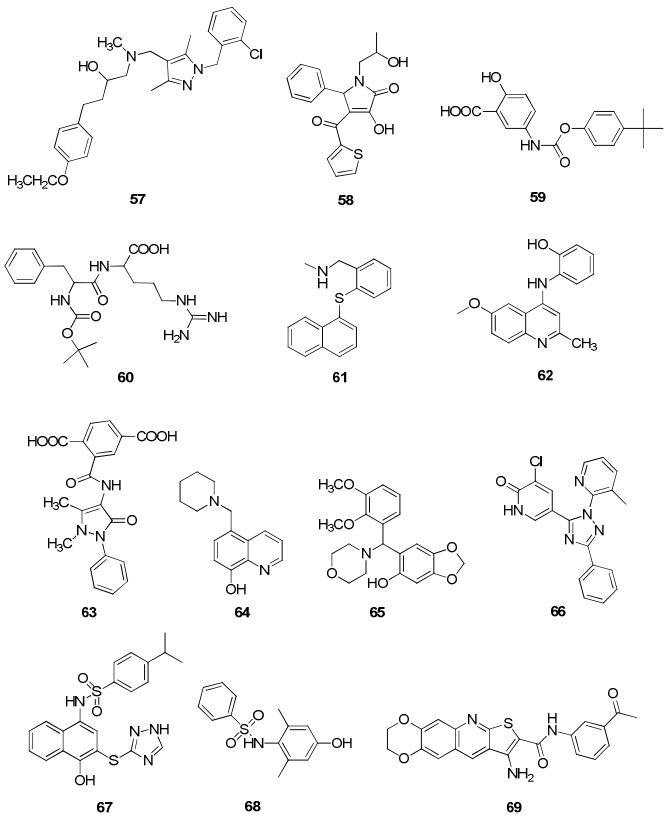


Figure 11. Chemical structures of small molecule PPI inhibitors identified by virtual screening.

Case study: Discovery of small molecule p53-MDM2/MDMX inhibitors by virtual screening

P53-MDM2 PPI represents a promising target for the development of new generation of antitumor agents. The crystal structure of MDM2 in complex with the α-helix of p53 (PDB code: 1T4F) reveals that there are three "hot spots" on MDM2 (namely Phe19, Trp23, and Leu26 pocket) suitable for small-molecule binding.[152] In addition to HTS explored to identify small molecule hits for p53-MDM2, structure-based virtual screening was also employed for the process and showed a higher hit rate. Wang's group identified quinolinol inhibitors of p53-MDM2 interaction by pharmacophore and structure-based screening of the NCI 3D database with hit rate of 14.9%.[153] Inspired by the impressive results, our group performed a docking-based virtual screening study to search the Specs database.[154] Nine hits out of 25 selected compounds (hit rate: 36%) were identified and most of them have a pyrrolidone scaffold. The nanomolar inhibitor **70** (K_i = 780 nM) has three aromatic substituents on the scaffolds, which mimic the three "hot spot" residues (Phe19, Trp23 and Leu26) of p53 (**Figure 12**). Further hit optimization resulted in compounds **71** (K_i = 260.0 nM) and **72** (K_i = 150.0 nM) with improved p53-MDM2 inhibitory activity. In addition, they also could effectively and selectively inhibit the growth of tumor cells with deleted p53. Importantly, they were orally active at a dose of 200 mg/Kg in the A549 lung cancer xenograft model and had little effect on the body weight of the nude mice. Based on the pyrrolidone scaffold, our group rationally designed pyrrolo[3,4-*c*]pyrazole derivatives as the first dual inhibitors of p53-MDM2 interaction and nuclear factor-κB (NF-κB) pathway. Compound **73** had a K_i value of 83 nM toward MDM2 and suppressed NF-κB activation through inhibition of IκBα phosphorylation and

elevation of the cytoplasmic levels of p65 and phosphorylated IKKα/β.[155] Interestingly, further mechanism study revealed that (*R*)-**73** and (*S*)-**73** targeted MDM2 and NF-κB pathways, respectively, and had synergistic antitumor effects both *in vitro* and *in vivo*.[156]

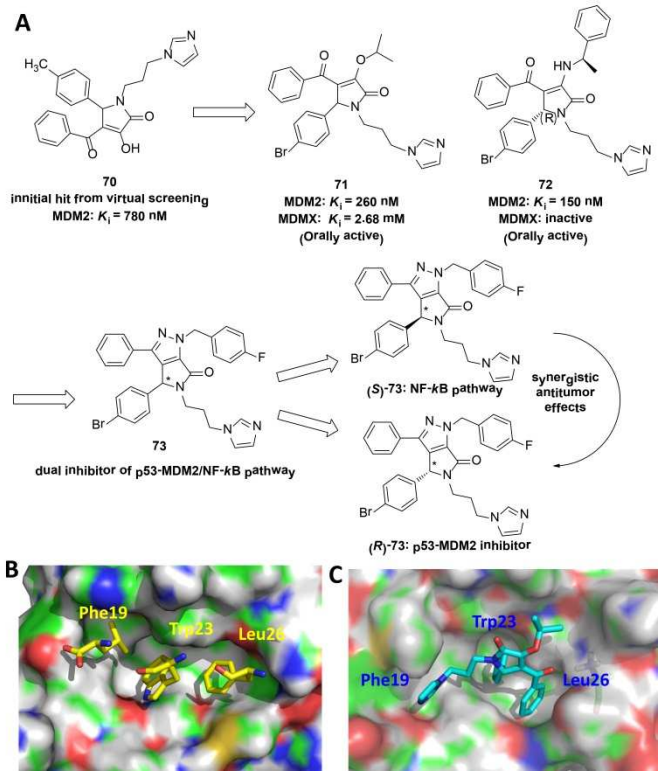


Figure 12. (A) Chemical structures and optimization process ofpyrrolidone p53-MDM2 inhibitors; (B) The hot spots of p53-MDM2 interaction; (C) The binding mode of compound **71** with MDM2.

5. Designing Strategies to Discover Small Molecule PPI inhibitors

5.1 Anchor-based PPI inhibitor design

An anchor refers to a hot-spot residue from a donor protein that plays an important role in molecular recognition and binding energy with the acceptor protein.[157] The anchor residues are always deeply buried in the acceptor protein and often bind relatively stable pockets on the surface of the acceptor protein. Such hot spot residue(s) can serve as a reasonable starting point for analogy design and derivatization. There are generally three kinds of strategies for anchor-based PPI inhibitor design. The first approach is the substructure search of the anchor side chain to find a new scaffold and then improve its binding affinity by growing the scaffold to interact with other hot spots. The second method employs the *de novo* design software or virtual library building tool to extend the anchor itself to form more interactions with the acceptor protein. The third technology aims to search bioisosteres of the anchor that are capable of mimicking the critical interactions with the acceptor protein. On the basis of the bioisosteres, structure-based methods can be used to design small molecule inhibitors whose structures are drastically different from the anchor. The application of these strategies is discussed in the following case studies.

Case study 1: Anchor-based discovery and optimization of p53-MDM2 inhibitors

Trp23 is the most critical "hot spot" residue for binding of p53 to MDM2 with its indole ring buried deeply inside a hydrophobic cavity in MDM2 and forming hydrogen bonding network. Wang's group used Trp23 as an anchor to substructure search and the spiro-oxindole scaffold (**74**) was chosen as the core structure by structure-based selection (**Figure 13**).[158] After structure-based design and optimization, a highly potent spiro-oxindole p53-MDM2 inhibitor (**75**) was discovered with a K_i value of 0.086 μM. Systemic optimization of the substitutions on the spiro-oxindole core (**76**)[159, 160] gave rise to MI-319 (**77**) as a potent, specific, cell-permeable and orally active small-molecule p53-MDM2 inhibitor.[161] Optimization of MI-319 afforded MI-888 (**78**) and finally its derivative MI-77301 (**6**) has entered phase I clinical trial as an anticancer agent.[28]

In another study of Dömling's group, Trp23 was also used as a starting point to construct virtual libraries.[162] In order to improve the synthetic efficiency, multi-component reactions (MCRs) were applied to create virtual compound libraries. Initial anchors (indole and its bioistosteric 4-chlorophenyl derivatives) were decorated with functional groups that were implemented for MCRs (**Figure 13**). The MCR scaffolds containing the anchor were generated automatically and screened by molecular docking. From the highest-ranking compounds, eleven scaffolds from different MCR series were selected for synthesis on the basis of their binding mode, chemical diversity and synthetic feasibility. As a result, seven scaffolds showed binding activities less than 60 μM and two representative p53-MDM2 inhibitors **79** and **80** are depicted in **Figure 13**. More recently, the strategy of anchor-based virtual MCRs was evolved into a web-based screening technology named *AnchorQuery*.[157] *AnchorQuery* designs synthetically accessible virtual compounds and takes advantage of similarity, docking and pharmacophore-based tools to improve the screening efficiency. The method can be used to target a broad set of PPIs with known structure and hot spots. Importantly, *AnchorQuery* is an open-access tool, which is freely available to researchers (http://anchorquery.ccbb.pitt.edu).

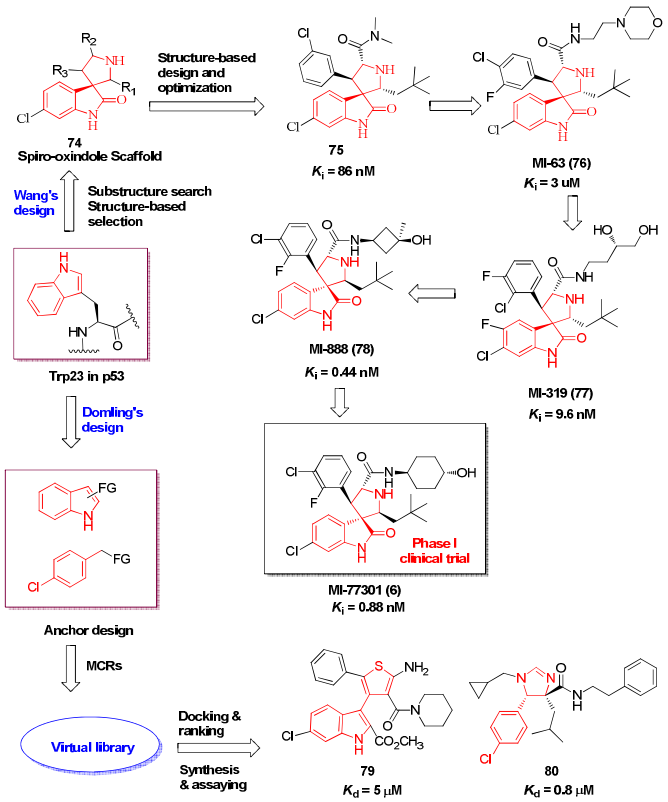


Figure 13. The anchor-based design process of small molecule p53-MDM2 inhibitors.

Case study 2: Discovery of VHL/HIF1α inhibitors by anchor-based *de novo* design

VHL/HIF1α is a promising target for the treatment of anemia. Hydroxyproline 564 (Hyp564, **81**) in HIF-1α is crucial for binding with VHL.[163] Crews's group used Hyp as a starting point to rationally design small-molecule VHL/HIF1α inhibitors (**Figure 14**).[164, 165] Initially, *de novo* design software BOMB[166] was used to design the Hyp analogues. The hit compound **82** showed moderate binding affinity to VHL with an IC_{50} value of 117 μM. Further SAR studies of molecule **83** were guided by the crystal structures of ligand-VHL complexes, which led to the discovery of the first small molecule sub-micromolar inhibitor **84**.

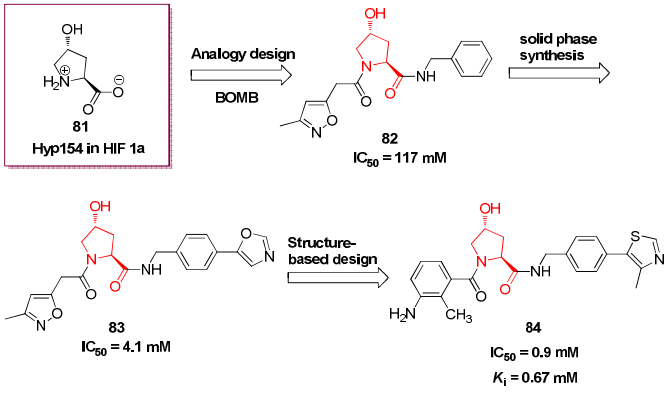


Figure 14. Anchor-based *de novo* design of hydroxyproline derivatives as VHL/HIF 1α inhibitors.

Case study 3: Discovery of β-catenin/Tcf inhibitors by anchor-based bioisostere replacement

Very recently, our group reported a new bioisostere replacement strategy to rationally design hot spot-directed PPI inhibitors (**Figure 15**).[167] As a proof-of-concept study, their innovative strategy was successfully used to identify small molecule β-catenin/Tcf PPI inhibitors. Firstly, the critical residues of β-catenin for binding to Tcf were derived by systematic alanine scanning and SPR assays. The carboxylic acid groups of Tcf Asp16 and Glu17 were found to be critical binding elements that formed important charge−charge and H-bond interactions with β-catenin. Then, the carboxylic acids of Asp16 and Glu17 were used as an anchor for inhibitor design. By searching the bioisostere and basic fragment libraries,[168] indazol-1-ol and tetrazole (5-thioxo-1,2,4-oxadiazole) were selected as the carboxylic acid bioisosteres, which were evolved into inhibitors after fragment docking and linking. The most active compound **85** and **86** inhibited the β-catenin/Tcf interaction with a K_i value of 3.1 μM and 7.2μM, respectively. Molecular docking and mutagenesis experiments validated the design rationale and the indazol and tetrazole were able to mimic the key charge-charge and H-bond interactions with β-catenin. Notably, the bioisostere strategy should be more generally applicable to the hot spot-oriented PPI inhibitor design.

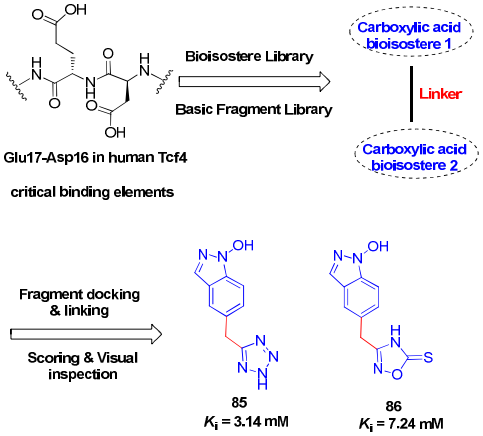


Figure 15. Discovery of small molecule β-catenin/Tcf inhibitors by bioisostere replacement of anchor residues.

5.2 Design of Small Molecule Mimetics of Secondary Structure Involved in PPI

Because there are no endogenous small-molecule PPI binders, peptide-based and peptidomimetic-based approaches can be effective strategies to disrupt PPIs. Although a number of successful applications have been reported,[169] their clinical application is hampered by low bioavailability and poor cell permeability. As an alternative, the design of non-peptidic secondary structure mimetics has received considerable attention.[170] Most of the PPIs are mediated by three kinds of secondary structure elements (*i.e.*α-helix, β-turn, and β-strand). Thus, the discovery of small molecules to mimic the key interactions of these elements represents an attractive approach to modulate PPIs. α-Helix is the most common secondary structure element observed in PPIs, which accounts for approximately 62% of the PPI complexes in the Protein Data Bank (PDB).[171] A number of small molecule α-helix mimetics

have been designed to target PPIs,[37, 172] and the following sections will mainly focus on recent progress.

Classical α-helix mimetics as PPI inhibitors mainly include terphenyl (**87**), terpyridine (**87**), benzamide (**88**), trispyridylamide (**88**) and terephthalamide scaffolds (**89**).[173] Several compounds derived from these scaffolds have showed potent p53/MDM2[174, 175] and Bcl-x_L/Bax[176] inhibitory activities. However, such terphenyl-related structures have inherent drawbacks such as low water solubility, long synthetic routes and limited flexibility. Current research interests in α-helix mimetics focus on designing cell-permeable and synthetically easily accessible scaffolds. A number of new α-helix mimetics with improved aqueous solubility have been reported (**90**-**96**, **Figure 16**). Most of the studies used the strategy of heterocycle-benzene replacement to improve the solubility (*e.g.* imidazole-phenyl-thiazole scaffold **91**,[177] pyridazine scaffold **92**,[178] oxazole-pyrrole-piperazine scaffold **94**[179] and oxopiperazine scaffold **95**[180, 181]). The conformational flexibility can be restricted by intra-molecular hydrogen bonds (*e.g.* terephthalamide **89**[182] and benzoylurea scaffold **90**[183]). The synthetic difficulty can be reduced by solid-phase synthesis (*e.g.* pyrrolopyrimidine scaffold **96**[184]) and MCRs (*e.g.* phenyl-imidazole-phenyl scaffold **93**[185]). Representative PPI inhibitors (**97**-**102**)[174, 175, 177, 183-185] derived from these scaffolds are summarized in **Table 5** and **Figure 17**. In particular, several of them (*e.g.* imidazole Bcl-x_L/Bax inhibitor **100**[184] and pyrrolopyrimidine p53/MDM2 inhibitor **102**[184]) were proved to be active in cellular level.

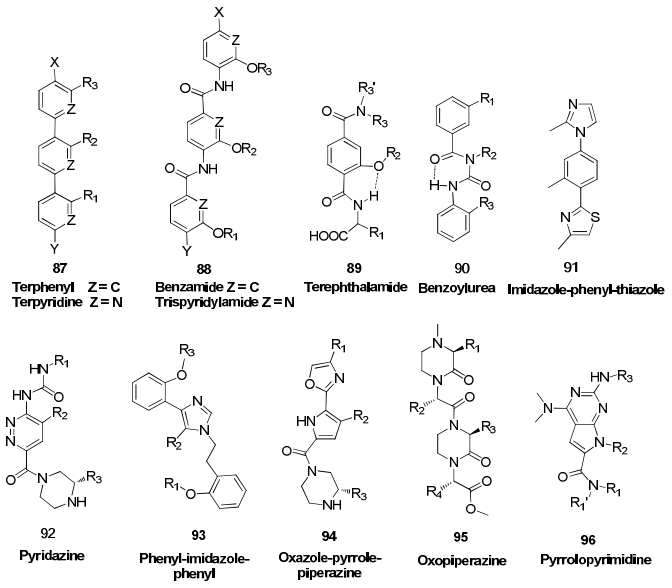


Figure 16. Classical terphenyl-based small molecule α-helix mimetics and new scaffolds with improved solubility.

The design of small-molecule secondary structure mimetics is likely to be the most useful strategy for PPI targets whose structures are unknown. However, current research is also faced with several challenges. First, it is important to construct libraries of secondary structural mimetics for HTS against diverse PPI targets. Diverse space that corresponds to PPI "hot-spots" is necessary for the discovery of small-molecule inhibitors. Second, there are few studies about small-molecule secondary structure mimetics that possess *in vivo* therapeutic potency. Further medicinal efforts are necessary to optimize them to become leads instead of just finding structurally similar scaffolds. Another question of secondary structure mimetics is how to improve the selectivity toward a number of PPIs with conserved motif. To address the problem, it is important to explore the subtle differences in the binding clefts and perform in-depth SAR studies.[173] For example, the terphenyl scaffold with only difference in the position of a naphthyl substituent can show selectivity between HDM2 and Bcl-x_L.[174, 176] In 2015, Wilson's group synthesized a library of *N*-alkylated aromatic oligoamide helix mimetics and discovered selective PPI inhibitors.[186]

Table 5. Representative small molecule α-helix mimetics as PPI inhibitors

Compound	Target	Activity	Ref
97	p53/MDM2	K_i: 0.18 μM	174
98	p53/MDM2	IC_{50}: 1 μM	175
99	Bcl-xL/Bak	K_i: 2.5 μM	183
100	Bcl-xL/Bak	IC_{50}: 8.09 μM	185
101	Cdc42/Dbs	IC_{50}: 67 μM	177
102	p53-MDM2/MDMX	K_i: 0.62 μM	184

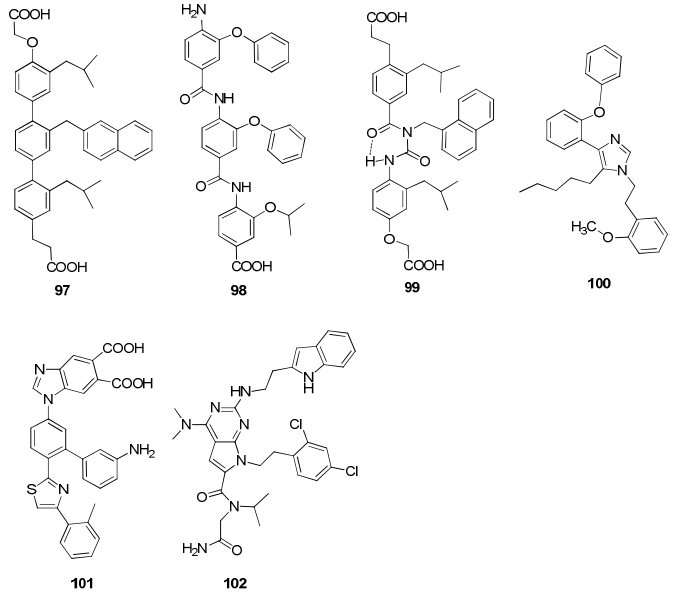


Figure 17. Chemical structures of bioactive small molecule α-helix mimetics as PPI inhibitors

Case study 1: Comprehensive small molecule library of secondary structure mimetics as PPI inhibitors

Boger's group reported a comprehensive small molecule library that contained secondary structure mimetics of three major recognition motifs in PPIs (**Figure 18**).[172] The library was designed in a combinatorial manner by substitution of 20 natural amino acid side chains on the given templates. Their α-helix mimetic library (8,000 molecules) and β-turn mimetic library (4,200 molecules) were subjected for biological screening. In a p53/MDM2 assay, new MDM2 inhibitors were successfully identified.[187] The most active compound **103** had an IC_{50} value of 15 μM by mimicking the key residues of the p53 peptide. The α-helix mimetic library was also screened for inhibitors of the interaction between HIV-1 envelope glycoprotein gp41 and C-heptad repeat (CHR), a promising anti-HIV target in viral cell entry.[188] Three series of novel HIV-

1 gp41 inhibitors were identified and the most active compound **104** (K_i = 1.3 μM) mimicked the interactions of the CHR α-helix. Moreover, compound **104** was also proved to be active in a functional cell-cell fusion assay (IC_{50} = 5 μM). The β-turn mimetic library has also been screened for inhibitors of the opioid receptors.[172] Due to the limitations of current libraries for HTS of PPI inhibitors, such comprehensive libraries specifically designed for interrogating PPIs may be powerful for PPI target validation and lead compound discovery.

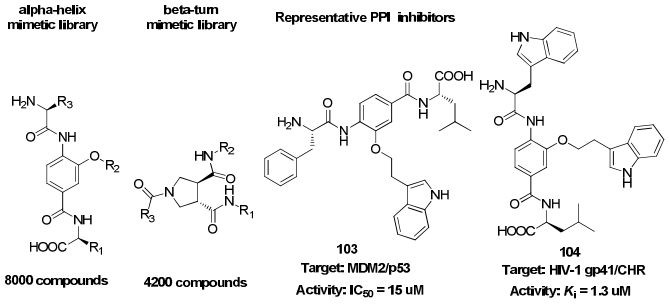
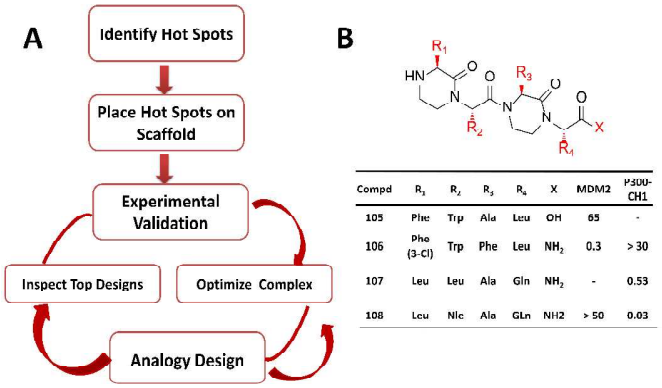


Figure 18. The comprehensive small molecule library of α-helix and β-turn mimetic library and representative PPI inhibitors.

Case Study 2: Computational design of α-helix mimics as potent p53-MDM2 and p300-HIF1α inhibitors

Oxopiperazine helix mimetics (OHMs) is a key α-helix mimic that have attracted broad interests.[189] In 2014, Arora's group reported an efficient computational approach named Rosetta to rational design and optimize OHM PPI inhibitors.[181] The key steps in Rosetta[190] (https://www.rosettacommons.org/) to design PPI inhibitors include (1) identification of positions on the scaffold to mimic "hot spot" residues; (2) experimental validation of the scaffold featuring the "hot spot" mimics; (3) computational optimization of lead compound and design of new "hot spot" analogues; (4) experimental validation of top ranked molecules (**Figure 19A**). Rosetta has been successfully used to identify highly potent p53-MDM2 and p300-HIF1α inhibitors. For the design of p53-MDM2 inhibitors, computational modelling indicated that R_1, R_2, and R_4 on the oxopiperazine scaffold overlay well on the three hot spot residues Phe19, Trp23, and Leu26, respectively. The R_3 group did not directly interact with MDM2 and solubilizing or hydrophobic groups were preferred at this position. Initial experimental validation led to the design of compound **105** (K_d = 65 μM) with wild-type hot spot residues at the equivalent positions and alanine at the R_3 position. Then, computational methods were used to investigate the binding mode and predict high-affinity molecules. The top designs were selected by binding energy, binding mode, synthetic possibility and physicochemical properties. Experimental validation results revealed that the Rosetta algorithm generated a library dramatically enriched for high-affinity binders. For example, compound **106** showed significantly improved activity with a K_d value of 0.3 μM. Using a similar approach, OHMp300-HIF1α inhibitors were rationally designed and optimized. The R_1, R_2, and R_4 groups on the oxopiperazine scaffold were predicted to mimic the three hot spot residues Leu818, Leu822, and Gln824 on HIF1α helix, respectively. The R_3 group was not predicted to make contacts with the CH1 domain of p300. The resulting lead compound **107** containing wild-type residues showed a K_d value of 533 nM to the CH1 domain of p300. Further computational predictions by Rosetta suggested the replacement of leucine with norleucine (Nle) would lead to better contacts with the hydrophobic pocket. As a result, compound **108** providing a 13-fold improvement in binding affinity (K_d = 30.2 nM). These results highlighted the advantages of the combination of computational design and experimental SAR validation in designing small molecule helix mimetics. Moreover, the Rosetta program is freely available to academic researchers via the Rosetta Commons (rosettacommons.org).



Compd	R_1	R_2	R_3	R_4	X	MDM2	P300-CH1
105	Phe	Trp	Ala	Leu	OH	65	-
106	Pho (3-Cl)	Trp	Phe	Leu	NH_2	0.3	> 30
107	Leu	Leu	Ala	Gln	NH_2	-	0.53
108	Leu	Nle	Ala	Gln	NH2	> 50	0.03

Figure 19. (A) Computational protocols for rational design of α-helix mimics; (B) Chemical structures and binding affinities (K_d, μM) of the designed oxopiperazine helix mimetics.

Case Study 2: Rational design of novel α-helix mimetics as potent c-Myc-Max inhibitors

The discovery of small-molecules that interfere with c-Myc-Max hetero-dimerization is expected to inhibit the oncogenic activity of Myc and has high therapeutic value in multiple types of cancer.[191] The rational design of direct c-Myc inhibitors is hampered by the intrinsic disorder of the PPI binding domain and most of the inhibitors were identified by random screening. Considering high percentage (~70%) of α-helical content in the c-Myc-Max heterodimer,[192] the design of synthetic α-helix mimetics is a promising approach. Starting from lead compound **109**[193] that targets c-$Myc_{363–381}$ peptide, the α-helix mimetic **110** was rationally designed (**Figure 20**).[194] Oligoamide was used as the backbone of the α-helix mimetics with the hydrophobic R_1 and R_2 groups recognize Phe374 (i) and Leu377 (i+3), respectively. Inspired by the lead compound **109**, the electron poor nitro group was selected to recognize Arg366, Arg367 and/or Arg372. Moreover, a carboxylic acid was use to recognize Arg378 at the *C*-terminal end and improve the solubility. Thus, a focused library of derivatives of **110** was synthesized and screened. Compound **111** can completely disrupt the c-Myc−Max/DNA complex at the concentration of 100 μM. NMR experiments confirmed that compound **111** recognized the α-helical structure of c-Myc in the c-Myc−Max(S) heterodimer (K_d =~ 13 μM) with selectivity over Max(S)−Max(S) homodimers or c-Myc monomers.

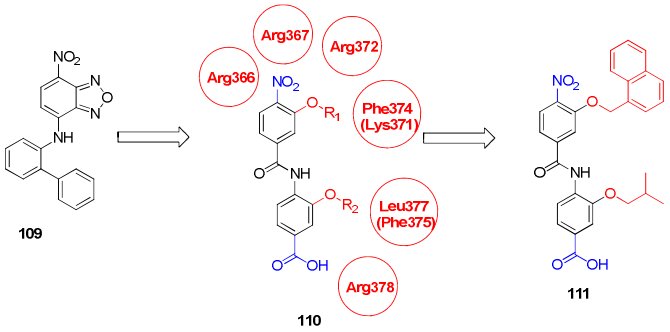


Figure 20. Structure-based design of novel oligoamide α-helix mimetics as c-Myc-Max inhibitors.

6. Synthetic Strategies to Discover Small Molecule PPI inhibitors

Current screening libraries are mostly collected or designed for traditional drug-like targets, which often result in low hit rates for PPI inhibitors. Therefore, compound libraries need to be expanded into new chemical space. It is estimated that the chemical space for drug-like molecules is about 10^{60} compounds, which makes it difficult to target the whole space. The development and application of new synthetic approaches for constructing new and biological relevant libraries with diversity and complexity is becoming an important area of interest for screening PPI inhibitors. Highly efficient synthetic methods such as diversity-oriented synthesis (DOS), biology-oriented synthesis (BIOS), domino (or cascade) reactions and multi component reactions (MCRs) were powerful tools to provide chemically diverse compound collections for targeting PPIs.

DOS is a new synthetic strategy to investigate drug-like chemical space in an efficient manner, which focuses on generating a high degree of molecular diversity.[195, 196] The build/couple/pair (B/C/P) strategy[197] is one of the most commonly used approaches in DOS and the resulting libraries are featured as scaffold diversity, structural complexity and a high fraction of sp^3-hybridized carbon atoms with generating more stereogenic centers.[198] Such properties are proven to be fruitful in enhancing the hit rate, selectivity and potency for a given target and a number of bioactive small molecules have been identified from such DOS library.[199] More recently, Young's Group merged the concept of DOS with FBDD by creating 3D fragments for screening.[200]

Currently, DOS contributes to PPI-based drug discovery mainly by highly efficient construction of macrocycle libraries.[201, 202] Macrocycles have several unique features as PPI inhibitors.[203] First, they are conformational pre-organized and can bind to topologically defined surfaces without major entropic loss. Second, macrocycles have restricted conformational flexibility and hence can serve as potential mimics for interactions with hot spots. Third, macrocycles have better cellular penetration as compared with peptidic PPI inhibitors. Some examples are described in the following case study (**Figure 21**).

The libraries inspired by natural products (NPs) are considered to be rich source of PPI inhibitors. The "NP–like" libraries by developing new synthetic chemistries have successfully yielded several classes of small-molecule PPI inhibitors (**Figure 21**). BIOS is a hypothesis-driven approach that uses biological relevance and prevalidation as the key criteria to design the synthesis of compound libraries enriched in bioactivity.[204] Structural information and underlying scaffolds in complex NPs are embedded in the BIOS-derived compound collections and thus efficient synthetic methods are required to be developed.[205] A typical BIOS library generally contains 200–500 compounds and the hits rate is about 0.2–1.5%.[204] Although BIOS have been widely applied in medicinal chemistry and chemical biology,[206-208] successful examples in discovery of PPI inhibitors are rare. Even though, the BIOS libraries provide the basis for screening PPI inhibitors.

MCRs is an efficient synthetic procedure where three or more reactants react to form a single product in a 'one-step, one pot' manner. MCRs has become an important tool for the rapid generation of drug-like compound libraries.[209] MCR-derived compounds have been frequently reported as PPIs inhibitors including p53/MDM2,[185] Bcl2,[185] and HIV-1/gp414[210] inhibitors and so on. Notably, most of them are designed as α-helix mimetic or hot spot mimetic, and some examples are described in Figures 13 and 21. Despite highly efficiency of MCR in synthesis, molecular and stereo diversity of MCR library is limited.[209] Thus, future efforts should be focused on designing novel reactions to improve scaffold diversity and stereocontrol.

Alternatively, organocatalytic cascade reactions are powerful approaches to facile assembly of diverse and complex frameworks in one-pot operations with high enanotio- and diastereoselectivity. In this context, our group has developed a divergent organocatalytic cascade approach (DOCA) by merging the power of divergent synthesis and cascade organocatalysis.[211] It has been shown that the DOCA strategy has been successfully used to create "privileged substructure"-based compound library and led to novel bioactive molecules[211, 212] and the screening of PPI inhibitors for new biological studies is in progress (to be published).

Case study: Small molecule PPI inhibitors derived from new synthetic chemistry

The interaction between sonic hedgehog (Shh) and Patched (Ptch1) is important in the hedgehog signaling pathway, playing an important role in regulating cell proliferation and differentiation.[213] Schreiber's group screened a DOS library for binding affinity to ShhN (*N*-terminal construct of Shh) and identified macrocycle hit **112** (**Figure 21**).[214] Further optimization effort led to the 12-membered macrocycle robotnikinin (**113**) with increased ShhN binding affinity (K_d = 3.1 μM). It represented the first example of small molecule ShhN inhibitors.[214] Marcaurelle *et al*. designed and synthesized a diverse library of 15,000 compounds containing four types of chiral cytisine-inspired scaffolds.[215] The NP-inspired DOS library was screened for binding affinity against Bcl-2 (hit rate: 1.1%) and Bcl-xL (hit rate: 0.2%). The studies led to the discovery of novel Bcl-2 and Bcl-xL inhibitors with low micromolar activity. However, the bridged bicyclic pyridone scaffold **114** showed no selectivity between Bcl-2 (K_i = 2.0 μM) and Bcl-xL (K_i = 5.7 μM). Another screen of a DOS library also identified sub-micromolar Bcl-2 inhibitor **115** (K_i < 0.8 μM) with good selectivity over Bcl-xL (K_i > 100 μM).[196] Notably, compound **115** also showed potent *in vitro* and *in vivo* antitumor activities. Very recently, Arai *et al*. reported a new method to synthesize NP-like chiral 3,3'-bisindoles by efficient catalytic asymmetric coupling reaction of indoles with isatin-derived nitroalkenes.[216] Interestingly, these 3,3'-bisindole derivatives (*e.g.* compound **116**) showed potent TCF/β-catenin transcription signaling inhibitory activity.

Besides DOS, Dömling's group also reported several classes of PPI inhibitors by screening of MCR-derived compound libraries. For example, MCR derived imidazoles were designed as α-helix mimetic. Compound **117** is a selective inhibitor to disrupt the interaction between Bcl-w and the BH3 peptide (IC_{50} = 8.09 μM).[185] Notably, this compound showed micromolar inhibitory activity (GI_{50} = 9.43 μM) against a HL-60 leukaemia cancer cell line and could induce apoptosis in a dose-dependent manner. The same group also discovered several classes of inhibitors of Bir3 domain of XIAP (X-linked inhibitor of apoptosis) by anchor-based generation of MCR virtual libraries, virtual screening and biological screening.[217] Compound **118** is the best inhibitor with an IC_{50} value of 4.9 μM in this series. More recently, the MCR approach was used to design selective p53-Mdm4 inhibitors.[218] On the basis of the Mdm4-inhibitor cocrystal structure, Ugi four-component reaction containing an indole fragment was specially designed to mimic the hot spot residue Trp23. The best compound **119** showed good selectivity for MDM4 (K_i = 5 μM) over MDM2 (K_i = 54 μM).

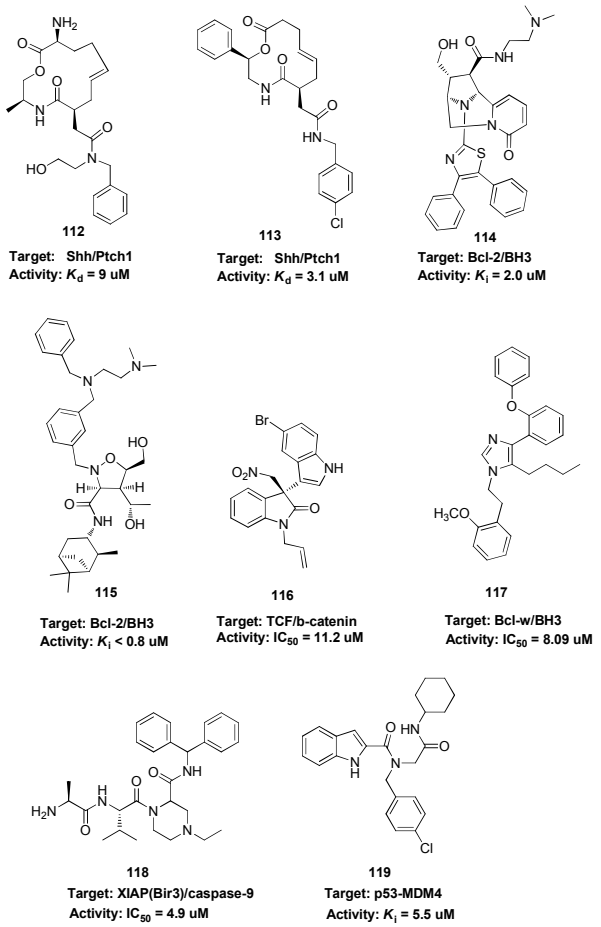


Figure 21. Representative PPI inhibitors identified from new synthetic chemistry.

7. Future Challenges in PPI-based Drug Discovery: Flexibility, Druggability, Selectivity and Difficult Targets

Despite important progress has been made in PPI-based drug discovery, there are still significant challenges to be addressed. First, the flexibility of PPI interface plays important roles in druggability of the target. The flat nature of PPI surface requires a relatively exposed inhibitor. It is estimated that LE of PPI inhibitors (median value: 0.29 kcal/mol·atom) is lower than that of inhibitors of traditional targets (range: 0.36 to 0.41 kcal/mol·atom).[219, 220] Upon binding to inhibitors, the protein surface may undergo conformational changes to form alternate pocket shapes and make inhibitors bound in a sufficiently buried manner. In this case, LE of the inhibitors may be improved. However, such protein flexibility is unknown, which causes difficulty for virtual screening and structure-based design. Thus, the generation of ensembles of low-energy conformations and detection of suitable surface pockets for inhibitor binding is important to design drug-like molecules. Moreover, the identification of so called "distinct" pockets that are unique among the protein family members can facilitate the discovery of highly selective PPI inhibitors. Recently, several computational methods, such as the above mentioned Rosetta software suite,[190] have been developed to efficiently sample the alternative conformations (pockets) on protein surfaces that are suitable for inhibitor binding.[48, 221, 222] Second, new approaches and new inhibitors remain to be developed for difficult PPI targets, which involve weak interactions (K_d > 200 nM) over large contact surfaces (>2,500 $Å^2$). It is a challenging task to develop robust HTS assays to detect binders. New methods, such as fragment-based screening, high content screening (HCS), and HTS of multiprotein complexes, were established to expand the number of "druggable" PPIs.[223, 224] Third, selective disruption of PPIs by small molecules becomes a major challenge for probing the structure and dynamic aspects of protein networks and developing drug leads. In the PPI networks, the same interface of a protein tends to bind with the various other proteins, and results in unwanted side effects. The discovery of selective PPI inhibitors can be facilitated by establishing new assays to quantify inhibitor selectivities between different protein−protein complexes and identifying selective binding site. Although limited success has been achieved, several reports opened an window to rational design of selective PPI inhibitors.[186] For example, Ji's group reported two robust and high throughput functional assays to quantify inhibitor selectivity between β-catenin/Tcf, β-catenin/E-cadherin, and β-catenin/APC PPIs.[225] Moreover, the same group used two computational approaches, Site Map[226] and MCSS,[227] to identify selective binding site for β-catenin/Tcf and successfully discovered small-molecule inhibitors with dual selectivity for β-catenin/Tcf over β-catenin/cadherin and β-catenin/APC interactions.[228]

8. Conclusions and Perspectives

With the advancement of our understanding of the structural biology of PPIs, scientists are able to identify "hot spots" as critical interaction components for the design and development of small molecule PPI inhibitors. As demonstrated, PPI inhibitors as a new class of promising therapeutics has come into reality with several drug candidates undergoing clinical studies. No doubt, the encouraging success has significantly attracted growing interests and activities in this exciting field. However, there are still several important road blocks to overcome. The drug design principles used for traditional druggable targets are needed to further modify and improve according to the nature of PPI large and multi interaction face

and "hot spots." Furthermore, the PPI interfaces are different from one another and there is no such 'privileged' structures for PPI inhibitors. Compared with the classic clinically used drugs, small molecule PPI inhibitors tend to have larger molecular weight (size), more hydrophobic feature, and more rigid and complex structures.[229] Therefore, the criteria used for the evaluation of the drug-likeness of PPI inhibitors need to be further studied because the Lipinski's "rule-of-five" appears to be unsuitable for PPI inhibitors.[12] Moreover, to enhance the screening hit rate for PPIs, the collection of small molecule structures generally possesses hydrophobic features, and rigid and complex molecular architectures. Accordingly, exploration of new chemical space becomes essential. NPs and NP-inspired libraries covering an important area of biologically relevant chemical space in terms of their structural diversity and complexity represent good sources of potent PPI inhibitors. Rapid access to novel complex compound libraries with improved structural, functional and topological diversity demand new powerful synthetic strategies.

In summary, PPI-based drug discovery is moving from infancy to mature phase. With increased knowledge and experience gained for PPIs and their inhibitor design, the challenging class of targets will become more accessible to drug discovery. It is expected that the golden age of PPIs is coming.

Acknowledgements

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