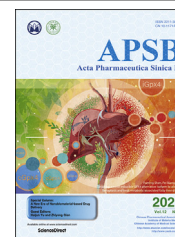




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REVIEW

Molecular glues modulate protein functions by inducing protein aggregation: A promising therapeutic strategy of small molecules for disease treatment

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Abstract Molecular glues can specifically induce aggregation between two or more proteins to modulate biological functions. In recent years, molecular glues have been widely used as protein degraders. In addition, however, molecular glues play a variety of vital roles, such as complex stabilization, interactome modulation and transporter inhibition, enabling challenging therapeutic targets to be druggable and offering an exciting novel approach for drug discovery. Since most molecular glues are identified serendipitously, exploration of their systematic discovery and rational design are important. In this review, representative examples of molecular glues with various physiological functions are divided into those mediating homo-dimerization, homo-polymerization and hetero-dimerization according to their aggregation modes, and we attempt to elucidate their mechanisms of action. In particular, we aim to highlight some biochemical techniques typically exploited within these representative studies and classify them in terms of three stages of molecular glue development: starting point, optimization and identification.

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1. Introduction

Since the publication of the first successful cases in the early 1990s, the last three decades have witnessed rapid advances of protein structure-based drug design^{1–3}. With the current advances in proteomics, molecular biology and computational engineering, numerous excellent protein targets have been identified, leading to a boom in structure-based drug design and the success of many novel drug discoveries⁴. In the human body, target proteins commonly exert biological activities by binding to endogenous ligands, such as small molecules, proteins, or nucleic acids. An orthosteric site is defined as the target protein's surface that directly interacts with endogenous ligands, and an allosteric site is defined as the target protein's region that can affect the conformation of the target protein to modulate functions (Fig. 1A)⁵. The strategy to design small molecules that occupy orthosteric or allosteric sites of the target protein has dominated the discovery of small-molecular drugs over the past thirty years. However, on account of the lack of suitable binding pockets and appropriate chemical entities, more than 80 percent of the human proteome is undruggable⁶. To break through this bottleneck, in recent years, researchers have attempted to regulate the functions of targets by developing small molecules that can recruit other proteins (Fig. 1B and C)^{7–9}. Classified by their characteristics, there are two types of small molecules that induce the target proteins to bind to "recruited proteins": bivalent molecules and molecular

glues. A bivalent molecule possesses three components, including two functional groups and a linker (Fig. 1B). Functional group A is used to bind the target protein, and functional group B is used to bind the "recruited protein". The strategy to develop bivalent molecules has recently focused on discovering small molecules of target protein degraders, such as proteolysis targeting chimeras (PROTACs)¹⁰, lysosome targeting chimeras (LYTACs)^{11,12}, chaperone-mediated autophagy (CMA)¹³, and autophagy targeting chimeras (AUTACs)^{14,15}. There are a vast number of systematic reviews on those studies. Even though bivalent molecules have achieved superior bioactivities, a major drawback of bivalent molecules is their sheer size¹⁶. Not only may those bivalent molecules be orally unavailable due to solubility problems, but they also tend to have poor membrane permeability, resulting in adverse pharmacokinetics. Moreover, most important therapeutic proteins such as transcription factors lack ligand binding sites, and thus using PROTAC approach is challenging^{17–19}.

In contrast, molecular glues refer to monovalent small molecules that rely on the secondary structural characteristics of target protein to mediate protein–protein interactions (PPIs, Fig. 1C). When a molecular glue binds the target protein, the molecule itself and some residues of the target protein can form a binding region together, which mediates the bond between the target protein and the "recruited protein(s)". Subsequently, such polynary complexes cause changes in a series of biological functions, such as protein degradation, complex stabilization, interactome modulation or

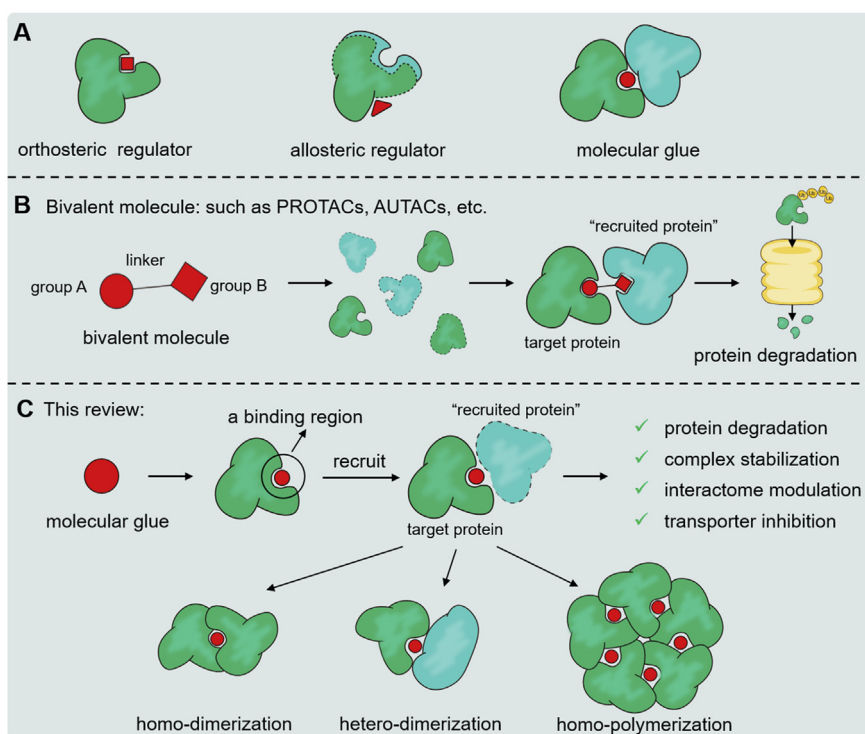


Figure 1 (A) Difference of orthosteric regulator, allosteric regulator and molecular glue. (B) Schematic diagram of divalent molecular interaction mode. (C) Schematic diagram of molecular interaction mode and classification of protein polymerization patterns induced by molecular glues.

transporter inhibition. In contrast with bivalent molecules, a molecular glue behaves as an indivisible structure, rather than two protein-binding fragments linked by a connectome with an appropriate length. Therefore, molecular glues are much smaller, possess good medicinal properties, have high atomic availability, and more readily adhere to “Lipinski’s rule of five”²⁰. In recent years, due to their fascinating pharmaceutical properties, the discovery of molecular glues has received increasing research interest. However, a number of challenges for drug discovery remain, including the fact that the design of molecular glues still depends on retrospective elucidation of modes of action and serendipitous discovery, and to date, only a few have been successfully discovered^{21,22}.

This review discusses representative cases of molecular glues and analyzes the binding mode of the molecular glue–protein complex. Based on the aggregation mode of molecular glues mediating protein–protein interactions, three mediating modes including homo-dimerization, homo-polymerization and hetero-dimerization are reviewed in detail (Fig. 1C). Overall, we hope to explain the general law of molecular glue mediating mechanisms, exploit the strategies and approaches of their design, optimization and identification, and finally summarize the potentials and prospects from the perspective of pharmaceutical chemistry.

2. Mediating homo-dimerization

2.1. PD-L1 dimerization

During the immune response, immune checkpoints can modulate antigen recognizing of the T-cell receptor (TCR) and repress the immune system from targeting self-antigens under normal conditions²³. Unfortunately, cancer cells suppress T-cell activation by hijacking the checkpoint pathway, and thus avoiding immune attack²⁴. The crucial checkpoint molecule, programmed cell death-1 (PD-1, express on the surface of activated T lymphocytes) binds to programmed cell death-ligand 1 (PD-L1, overexpression on the surface of cancer cells), causing tumor immune escape and promoting tumor progression^{25–27}. Therefore, regulation of the PD-1/PD-L1 interaction represents a considerable strategy for tumor immunotherapy.

To date, U.S. Food and Drug Administration (FDA) have approved seven PD-1/PD-L1 monoclonal antibodies for clinical use^{28,29}. However, due to the drawbacks of antibodies, such as high manufacturing cost, lack of oral activity and immune-related side effects, it is necessary to design PD-1/PD-L1 small molecule inhibitors as alternatives for monoclonal antibodies. Nevertheless,

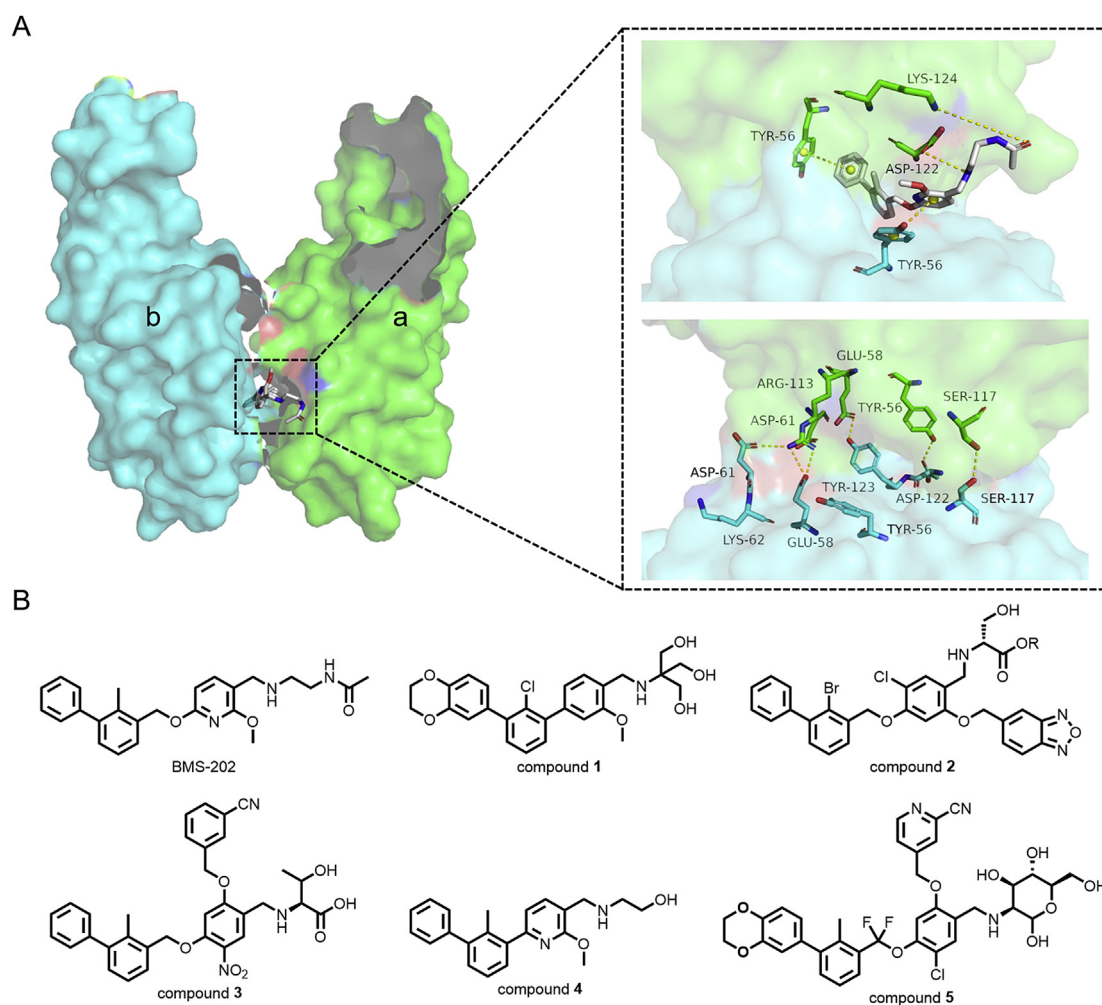


Figure 2 (A) The molecular glue BMS-202 mediates the homo-dimerization of PD-L1 (monomer a: green, monomer b: cyan, PDB:5J89). (B) Compounds 1–5 were the best compounds reported by Muszak³³, Liu³⁴, Ouyang³⁵, Wang³⁶ and Song³⁷, respectively.

the flat and large PPI interface between PD-1 and PD-L1 makes the design of small molecular inhibitors difficult.

In 2015, several compounds possessing hydrophobic biphenyl scaffolds were disclosed by Bristol-Myers-Squibb (BMS) as the first PD-1/PD-L1 nonpeptidic PPI inhibitors in their patents^{30,31}. Through a homogeneous time-resolved fluorescence (HTRF) assay, these compounds were identified to inhibit the formation of PD-1/PD-L1 complex, while the detailed mechanism remained ambiguous. In 2017, Zak et al.³² performed NMR experiments and found that BMS-202 directly binds to its target protein PD-L1, but not to PD-1. Moreover, the results also indicated that BMS-202 induces formation of an approximately 30 kDa complex, which is consistent with the mass of the 2:1 complex of PD-L1 (2) and BMS-202 (1). Further crystal structural analysis confirmed that molecular glue BMS-202 mediated the dimerization of PD-L1³². As shown in Fig. 2A, ^aAsp122 and ^aLys124 form hydrogen bonds with BMS-202, the ^bGlu58, ^bASP61, ^bAsp122 and ^bTyr123 residues form hydrogen bonds with ^aTyr56, ^aGlu58, ^aAsp61 and ^aArg113. There is a π - π stacking interaction between the biphenyl ring of BMS-202 and ^aTyr56 residue, which locks the BMS-202 molecule and PD-L1 dimer in place to generate a stable ternary complex. These findings contributed to the recent discoveries of PD-L1 dimer molecular glues reported by Muszak³³, Liu³⁴, Ouyang³⁵, Wang³⁶ and Song³⁷ (compounds 1–5, Fig. 2B).

2.2. REV1 dimerization

Mutagenic translesion synthesis (TLS) can lead to chemotherapy resistance; therefore targeting TLS is a powerful way to sensitize tumors to chemotherapy drugs³⁸. TLS occurs in two steps in mammalian cells, first by inserting a TLS DNA polymerase (like REV1) to introduce the nucleotide opposite the lesion, and then

extending TLS DNA polymerase (such as REV7) to extend the 3' terminus³⁹. The REV1 C-terminal domain (CTD) recruits POL- ζ by interacting with REV7 constituents, and the complex plays an essential role in harmonizing TLS (Fig. 3A)³⁸. Inhibition of REV1–REV7 interactions has received much attention, while the shallow binding interface limits the development of small molecules.

In 2019, Wojtaszek et al.^{40,41} screened approximately 10,000 structurally diverse compounds using a special enzyme linked immunosorbent assay (ELISA), and found that compound JH-RE-06 acts as a molecular glue to potently hinder the REV1–REV7 PPI interaction. Further cocrystal structure studies revealed that JH-RE-06 induces REV1 dimerization. In the chimeric REV1 CTD (cREV1 CTD) dimer, two REV7-binding pockets face each other to form a deep cavity for JH-RE-06 binding (Fig. 3B). The CTD of monomer A is led out by the acyl chain of JH-RE-06 and a large 1,4-dihydroquinolinone group, and there are a lot of hydrophobic interactions between cREV1 CTD and JH-RE-06 (e.g., ^bIle66, ^aLeu71, ^aLeu74, ^aLeu108, ^bTyr112, ^aLeu116 and ^aVal118; Fig. 3B). The sidechains of ^aGln105 and ^bSer114 form direct polar interactions with the hydrogen bond receptor groups of the 1,4-dihydroquinolinone moiety of JH-RE-06. The C-terminal tails of the two monomers pair with each other to form an antiparallel β sheet for the binding of JH-RE-06. Specifically, ^bLys69, ^bGlu72 and ^bAsp75 residues form hydrogen bonds with ^aGlu75, ^aTyr112 and ^aThr115 to stabilize the cREV1 CTD dimer.

The formation of JH-RE-06 induced dimer shields the REV7-binding surface, thus blocking the interaction between REV7 and REV1 CTD. Further *in vitro* and *in vivo* pharmacodynamics studies showed that JH-RE-06 combined with cisplatin enhanced the toxicity of cisplatin to various cell lines, and reduced the volume of the xenograft human melanoma in mice⁴².

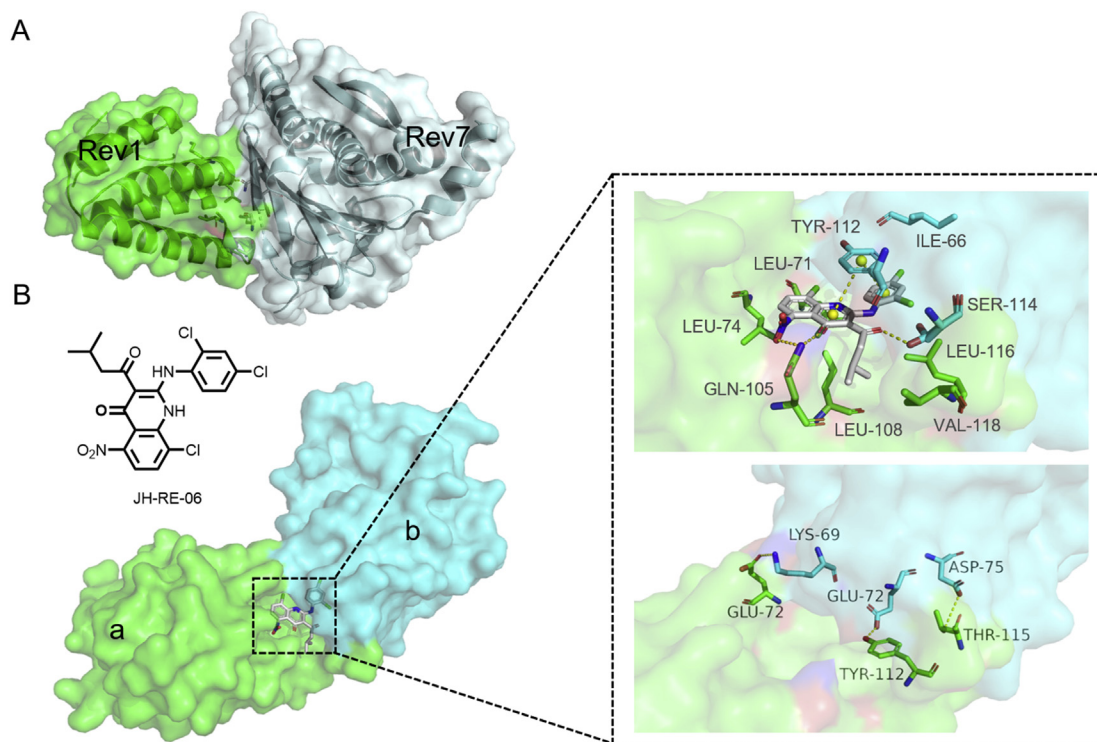


Figure 3 (A) REV1 CTD–REV7 complex (REV7: gray, REV1: green, PDB:4FJO). (B) The molecular glue JH-RE-06 mediates homo-dimerization of cREV1 CTD (monomer a: green, monomer b: cyan, PDB:6C8C).

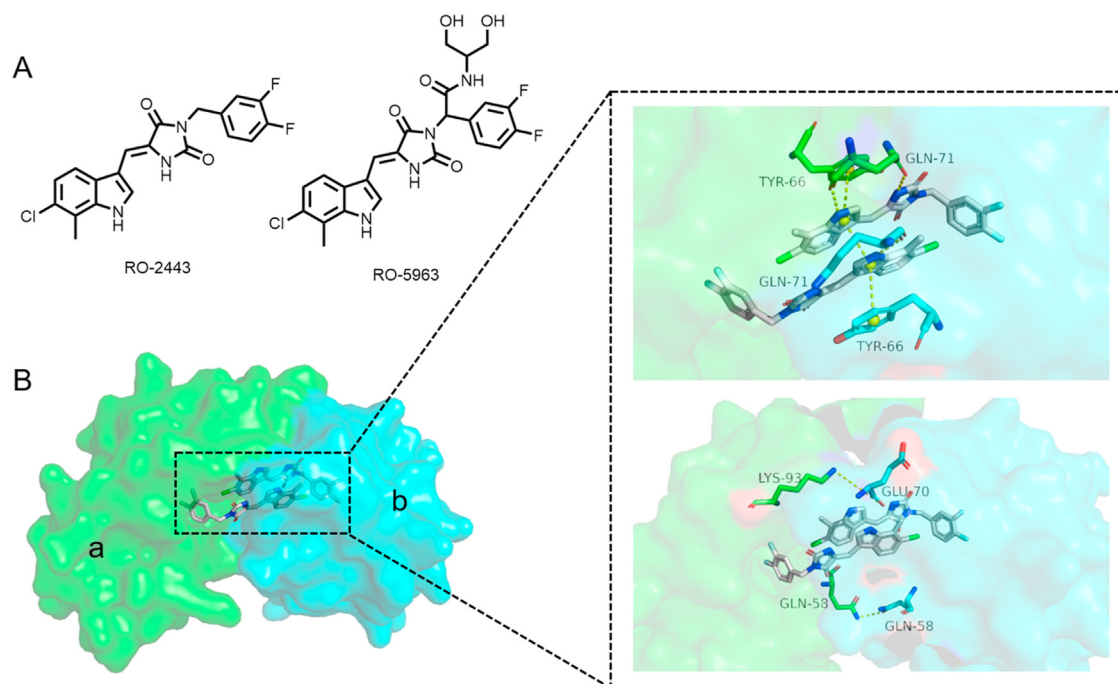


Figure 4 (A) Structures of the reported molecular glues mediating MDMX dimerization. (B) The molecular glue RO-2443 mediates the homodimerization of MDMX (monomer a: green, monomer b: cyan, PDB:3U15).

2.3. MDMX dimerization

Murine double minute 2 (MDM2) and MDMX, the negative regulator of the P53 tumor suppressor, are promising targets for cancer therapy⁴³. MDM2 antagonists alone may cause tumor cells

to overexpress MDMX to inhibit the P53 pathway, therefore, the identification of MDM2/MDMX dual antagonists has been intensively studied recently^{44,45}.

Through small molecules library screening, RO-2443 was found to exhibit similar inhibitory activity against both P53–

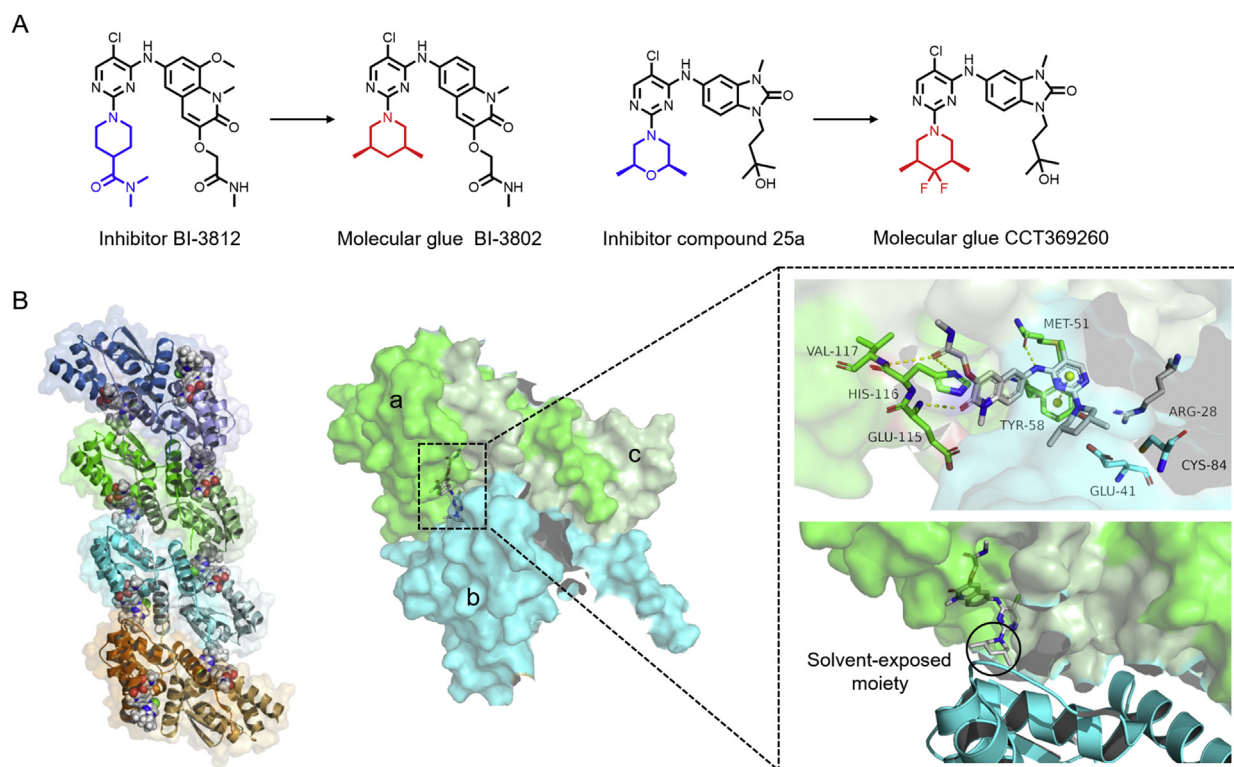


Figure 5 (A) Structures of molecular glues BI-3802, CCT369260 and their corresponding inhibitors. (B) BI-3802 mediates the homopolymerization of BCL6 (monomer a: green, monomer b: cyan, monomer c: palegreen, PDB: 6XMX).

MDMX and P53–MDM2 interactions (Fig. 4A)⁴⁶. In the mechanistic study examining the interaction between RO-2443 and MDMX, the results from NMR spectroscopy, static light scattering (SEC-SLS) and kinetic analysis revealed that RO-2443 may act as a molecular glue to induce MDMX dimerization. Co-crystals of MDMX/RO-2443 with high resolution at 1.8 Å confirmed this prediction (Fig. 4B), RO-2443 occupies the P53 binding pocket of MDMX to form a dimer and be incorporated into a central cavity, in which the two RO-2443 molecules are surrounded by the interaction of the two proteins, and the four monomers are arranged in a pair of dimers. The four-level π -sandwich interaction between the two chloroindole groups of RO-2443 and Tyr66 of the corresponding protein monomer forms an extensive aromatic stacking interaction, which is the key interaction of polymer formation. In addition, RO-2443 forms two hydrogen bond interactions with the Gln71 residue. Because the molecular glue pulls the two MDMX dimers closer, the ^aLys93 residue interacts with the ^bGlu70 residue, and ^aGlu58 and ^bGlu58 form hydrogen bond interactions that stabilize the dimer.

The molecular glue RO-2443 and its derivative RO-5963 may provide an effective treatment for MDMX-overexpressing cancers by mediating the activation of P53 apoptotic activity, while their poor water solubility and pharmacological properties require further optimization.

3. Mediating homo-polymerization

3.1. BCL6 polymerization

B-cell lymphoma 6 (BCL6), a transcriptional repressor protein, is a potential drug target for non-Hodgkin's lymphoma^{47,48}. Several

small molecule inhibitors and peptides of BCL6 with *in vivo* efficacy can only work at high concentrations, limiting their clinical application^{49–51}.

Kerres et al.⁵² showed that the broad-complex, tramtrack and bric-à-brac (BTB) domain of BCL6 is highly druggable, the compounds targeting this region may lead to development of potential BCL6 inhibitors. They screened a library of 1.7 million compounds using a fluorescence polarization (FP) assay, and identified a series of 4-amino-5-chloropyrimidine compounds. Among them, BI-3802, a close analogue of the BCL6 inhibitor BI-3812 (Fig. 5A), was unexpectedly found to mediate protein aggregation. Cryo-EM structural analysis showed that the key residues ^aTyr58, ^cArg28, ^bGlu41 and ^bCys84 between BCL6 dimers form a groove for BI-3802 to bind⁵³. The ^aMet51, ^aVal117, ^aHis116 and ^aGlu115 residues also form hydrogen bonds with BI-3802 (Fig. 5B). This complex structure is modeled as a polymer by ordering dimers in the same binding pattern, which can be identified by the non-cullin E3 ubiquitin ligase seven in absentia homolog 1 (SIAH1) for ubiquitination and degradation (Fig. 5B).

A large part of the degrading compound BI-3802 locates at the BTB domain, and the dimethylpiperidine ring remains solvent exposed that can be used to bind with another monomer, which provides an idea for the design of small molecule protein aggregation inducers by modifying the different substituted groups of small molecules when they are exposed to solvent. In 2020, a series of BCL6 inhibitors structurally similar to BI-3802 were reported, the researchers speculated that a similar molecular glue might be obtained by modifying the structure of the solvent exposed region⁵⁴. Ultimately, a subset of these inhibitors, such as CCT369260 (Fig. 5A), was found to form BCL6 polymers, which can be recognized and degraded by SIAH1.

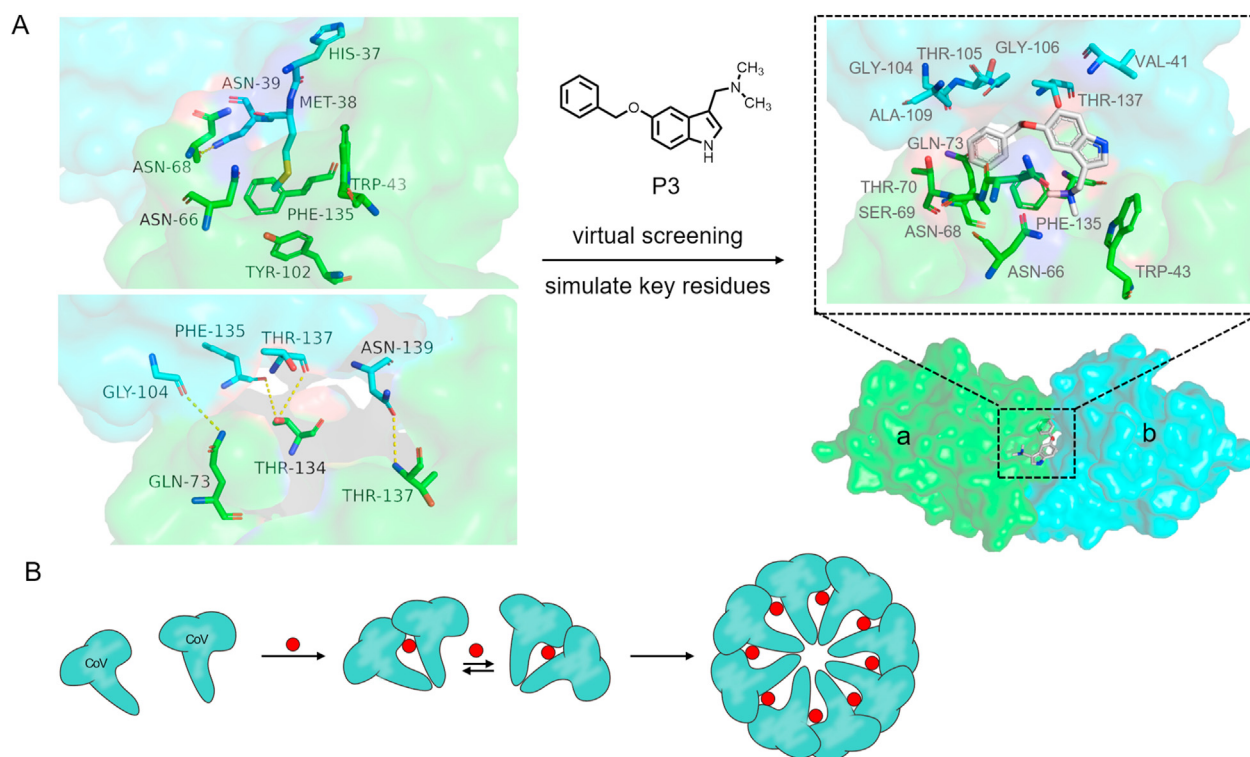


Figure 6 (A) The conservative hydrophobic interactions of CoV N-NTD dimers are shown on the upper left and hydrogen bonds on the lower left (monomer a: green, monomer b: cyan, PDB:4UD1). P3 mediates the homo-polymerization of MERS-CoV N-NTD (right, PDB:6KL6). (B) Diagram of molecular glue P3-mediated polymerization of viral proteins.

3.2. MERS-CoV polymerization

Middle East respiratory syndrome coronavirus (MERS-CoV) can cause severe acute respiratory syndrome (SARS) with a high fatality rate^{55,56}. The strongly related novel coronavirus corona virus disease 2019 (COVID-19) is ravaging the world, further highlighting the global public health risks posed by coronaviruses^{57,58}. Development of small molecules inducing the polymerization of CoV nucleocapsid (N) protein is a viable strategy for the exploitation of antiviral drugs⁵⁹. The CoV N protein consists of N-terminal domains (NTDs) and CTDs, and CoV N-CTD is responsible for the oligomerization of the N protein through protein–protein interactions⁶⁰. Structural studies have shown that His37, Met38 and Trp43 residues play important roles in the formation of hydrophobic vesicles containing carrier fusion residues and mediate the formation of CoV N-NTD dimers (Fig. 6A)⁶¹. As shown in

Fig. 6, the side chain of ^BMet38 interacts with a conserved hydrophobic pocket composed of ^aTrp43, ^aAsn66, ^aAsn68, ^aTyr102, and ^aPhe135. ^bHis37 and ^bAsn39 are packed against ^aTrp43 and ^aPhe135, and contribute to this hydrophobic interaction. The primary chain oxygens of ^bGly104, ^bPhe135, ^bThr137 and ^aThr137 and the side chains of ^aGln73, ^aThr134 and ^bAsn139 form hydrogen bonds, respectively.

It is possible to substitute small molecules for carrier fusion residues to stabilize the PPIs of CoV N-NTD dimers. Using structure-based screening to target the key Trp43 residue in the template where His37 and Met38 were removed, Lin et al.⁶¹ attempted to search compounds that could replace these residues and thus act as molecular glues. They identified 5-benzoxylglycine P3 as a novel positive N protein PPI stabilizer with N-NTD protein stabilizing and antiviral activity, which can be used in drug discovery for anti-CoV diseases. X-ray crystallography revealed that

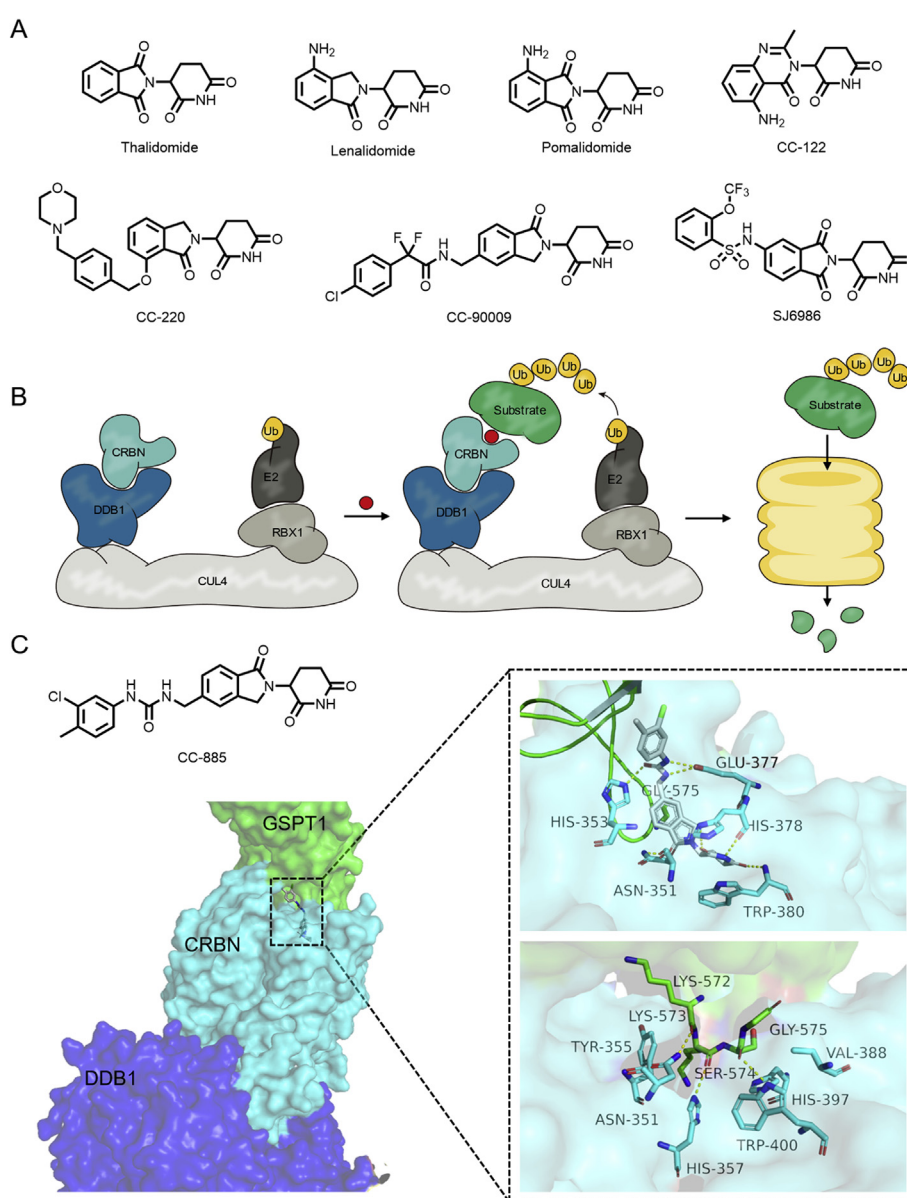


Figure 7 (A) Structures of CRBN-binding drugs. (B) Diagram of molecular glues mediated hetero-dimerization of DDB1^{CRBN} and substrate. (C) CC-885 mediates the hetero-dimerization of CRBN–GSPT1 (DDB1 colored in blue, CRBN colored in cyan, GSPT1 colored in green; PDB: 5HXB). Hydrogen bonds are shown as yellow dotted lines.

P3 along with residues of two monomers generates a large hydrophobic driving force simultaneously, which stabilizes the dimeric conformation of N-NTD, and the residues composition is ^aTrp43, ^aAsn66, ^aAsn68, ^aSer69, ^aThr70, ^aGln73, ^aPhe135, ^bVal41, ^bGly104, ^bThr105, ^bGly106, ^bAla109 and ^bThr137. Small-angle X-ray scattering (SAXS) indicated that P3 mediates the polymerization of N proteins to form an eight-membered circular structure (Fig. 6B), which blocks the CTD associated with viral transcriptional assembly, producing an antiviral effect.

4. Mediating hetero-dimerization

Molecular glues can not only regulate polymerization of proteins themselves, but also mediate target proteins by hijacking the functions of foreign proteins. For example, by inducing interactions with E3 ubiquitin ligases or enhancing pre-existing PPIs, molecular glues can trigger the ubiquitination and degradation of target proteins, and thus those molecules are commonly referenced as “molecular glue degraders”. Although the design of

molecular glue degraders still depends on accidental discovery, some systems have been well studied and created tremendous sensation in the field of chemical biology. In the next sections, we focus on the molecular glues that mediate the dimerization of heterologous proteins, most of which are molecular glue degraders.

4.1. *DDB1*^{CRBN}-substrate

The immunomodulatory drugs (IMiDs) thalidomide and its analogs bind to the substrate receptor cereblon (CRBN) of E3 ligase complex and modulate the binding region of CRBN to recruit the “neo-substrate” protein, causing degradation of the “neo-substrate” protein and exerting immunosuppressant activity (Fig. 7A,B)^{62,63}.

The transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) are the primary neo-substrate of lenalidomide-bound CRL4^{CRBN}^{64,65}. Compared with lenalidomide or pomalidomide, CC-122 and CC-220 have a higher affinity for CRBN and stronger

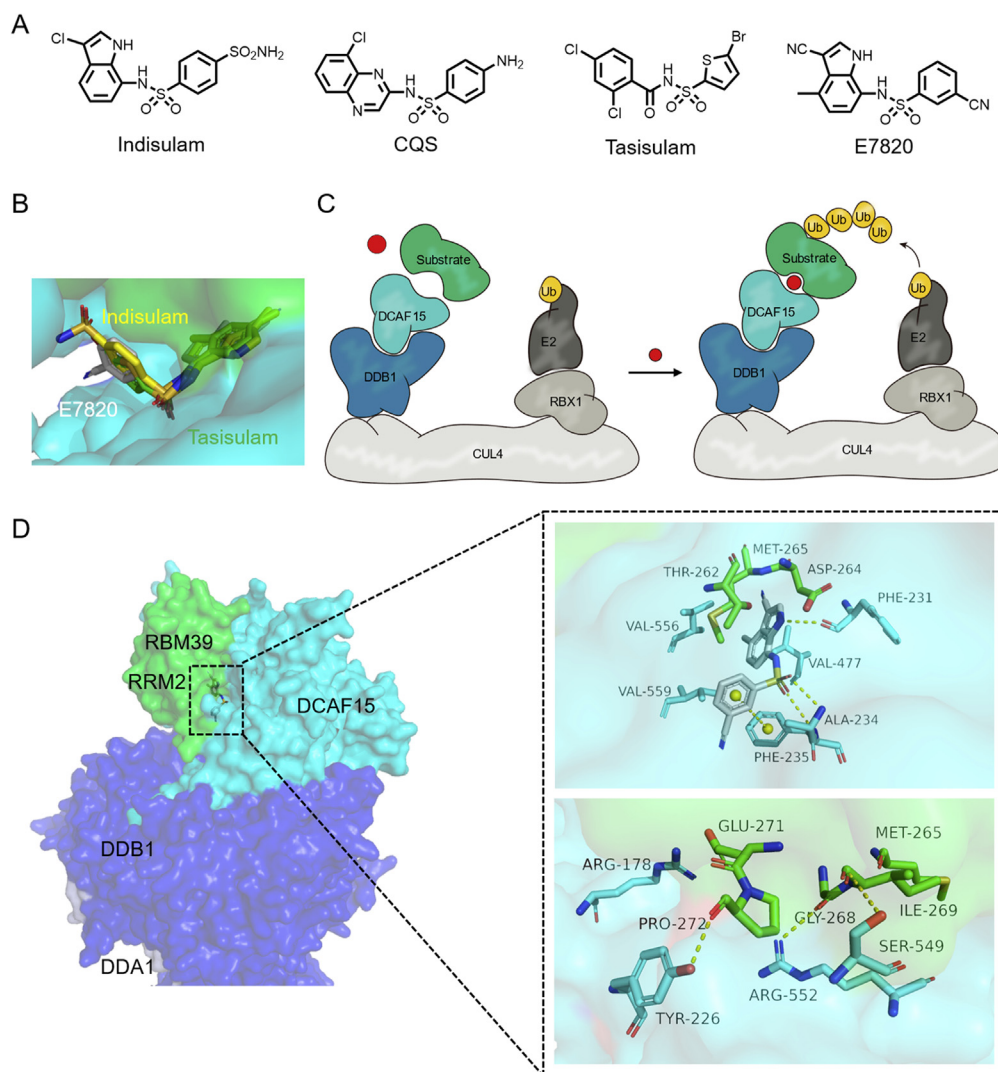


Figure 8 (A) Chemical structures of aryl-sulfonamides molecular glues. (B) A combination of E7820 and indisulam on the surface of DCAF15 and RBM39 (indisulam PDB:6Q0W). (C) Schematic diagram of the action mechanism of aryl sulfonamides molecular glues. (D) DDB1-DCAF15-E7820-RBM39 co-crystal structure (DDB1 colored in blue, DCAF15 colored in cyan, RBM39 colored in green, DDA1 colored in lightblue; PDB:6Q0R). Hydrogen bonds are shown as yellow dotted lines.

ability to induce IKZF1/3 degradation, and the therapeutic effect of CC-220 on systemic lupus erythematosus has been studied in 2018 as well. Matysiela et al.⁶⁶ synthesized a series of compounds that are bound to CRBN, among which CC-885 exhibits particularly significant anti-proliferative activity against acute myeloid leukemia (AML) (Fig. 7C). Subsequent immunoprecipitation experiments revealed that CC-885 recruits GSPT1 (G1 to S phase transition 1/eukaryotic peptide chain release factor GTP-binding subunit ERF3A) to form dimers⁶⁷. To date, molecular glues CC-90009 and SJ6986 with a similar mechanism have also been reported, and CC-90009 has entered clinical development^{68,69}. Other neo-substrates reported include the protein kinase CK1 α , the transcription factor ZBTB16⁷⁰, the putative ubiquitin ligase ZFP91⁷¹, the kinase CSNK1A1⁷², and the P63 isoforms Δ Np63 α and TAp63 α (related to teratogenicity)⁷³. Related studies are ongoing.

Although each IMiD displays distinct patterns of substrate specificity, a comparison of IMiDs and closely related analogs revealed that most of these compounds possess a common C₂H₂ zinc finger recognition degron motif. These reports also indicated that a β -hairpin loop with a critical glycine is found within this motif. In addition, CRBN-ligand-GSPT1 binding is mainly mediated by hydrogen bonds formed by the neo-substrate

backbone⁷⁰, which is crucial to understand the principle and basis of how ligand modifies the interaction interface of the neo-substrate and CRBN. Taking CC-885 as an example, we will introduce the mechanism of IMiDs-mediated hetero-dimerization of CRBN and their substrate. CC-885 forms hydrogen bonds with Trp380, His378, Asn351, Glu377 and His353 residues, while the binding pattern of isoindolinone ring to CRBN is similar to lenalidomide (Fig. 7C). The terminal substituted benzene ring of CC-885 is located near the β -hairpin loop of GSPT1, and CC-885 works as an interaction hotspot in CRBN through planer hydrophobic group. GSPT1 forms protein-protein interactions with CRBN Asn351, His357 and Trp400 residues, all directly contributing to forming the hydrogen bonds with oxygen atoms from GSPT1 Lys572, Lys573, and Ser574. In addition, GSPT1 has a special folding structure around Gly575 that binds CC-885 and CRBN. The side chains of CRBN residues Tyr355, His397 and Val388 provide further van der Waals interactions.

4.2. DDB1^{DCAF15}-RBM39

Indisulam⁷⁴ and E7820⁷⁵ have significant anti-proliferative effects on many hematopoietic and lymphoid cancer cell lines (Fig. 8A). In 2017, Han et al.⁷⁶ found that RNA binding motif protein 39

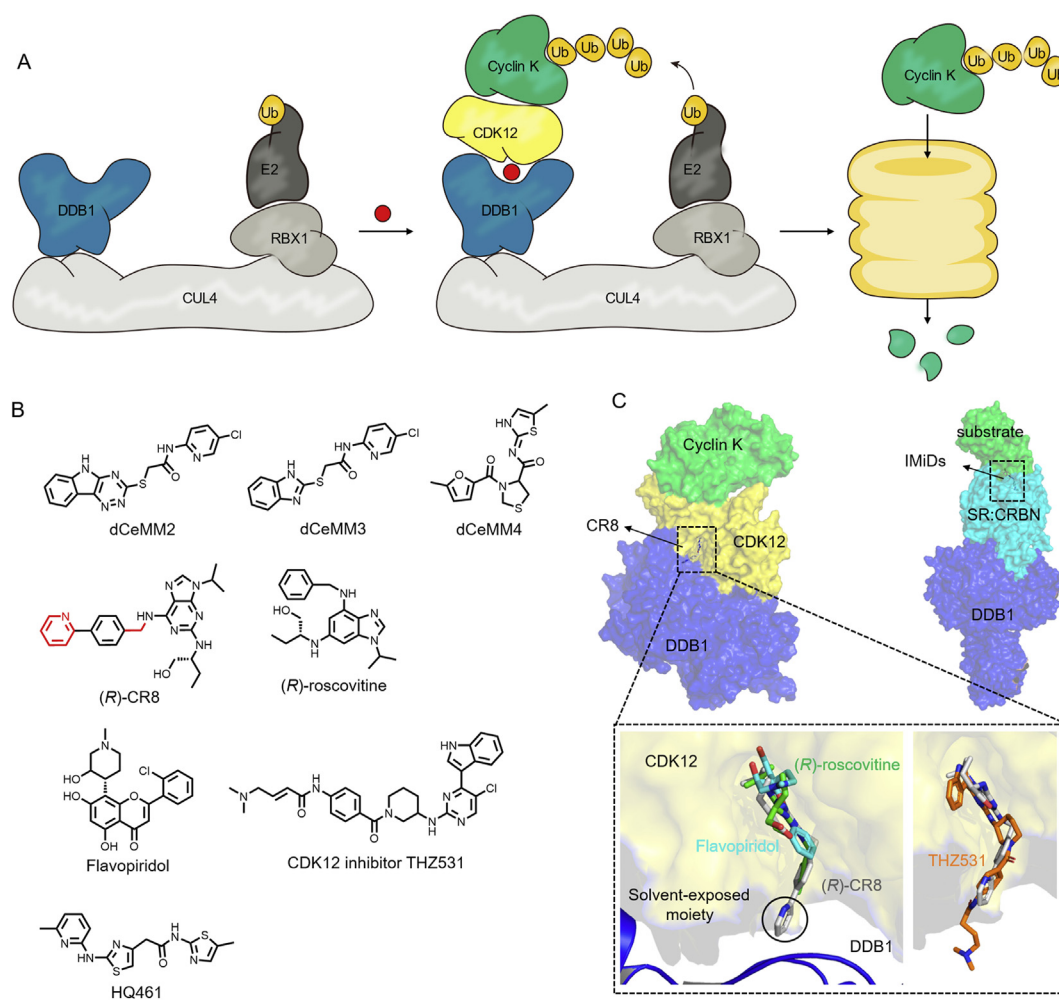


Figure 9 (A) Schematic diagram of the action mechanism of molecular glue CR8. (B) Structures of three types of DDB1 and CDK12/cyclinK molecular glues. (C) Comparison of protein complex structures of CR8 (PDB:6TD3) and IMiDs molecular glues. A partial enlargement of the CR8 solvent exposure area compared with (R)-roscovitine (PDB:2A4L), flavopiridol (PDB:3BLR) and THZ531 (PDB:5ACB).

(RBM39) is the target of these anticancer molecules. The indisulam-induced inactivation of RBM39 leads to abnormal pre-mRNA splicing, contributing to its lethal effect on cancer cells. Using CRISPR/Cas9 engineering, they subsequently demonstrated that indisulam may function as a molecular glue that induces the recruitment of RBM39 to the CUL4-DCAF15 E3 ubiquitin ligase, ultimately resulting in RBM39 ubiquitination and degradation (Fig. 8B and C)⁷⁶.

Du et al.⁷⁷ solved the crystal structure of the DCAF15–E7820–RBM39 complex in 2019, Faust et al.⁷⁸ presented a more detailed cryo-EM structure at a resolution of 4.4 Å in 2020. They both showed that E7820 binds to the shallow pocket on DCAF15 surface, and forms hydrogen bonds with the Ala234,

Phe235 and Phe231 residues with good geometry (Fig. 8D). A key π - π stacking interaction is formed between the Phe235 residue of DCAF15 and the benzonitrile ring of E7820. The E7820-modified DCAF15 interface interacts with RBM39 through the α -helix of the second RNA recognition motif (RRM2) domain. The side chains of Arg552, Ser549, and Tyr226 of DCAF15 form hydrogen bonds with Gly268, Ile269, and Pro272 of the RRM2 domain, respectively. There are also some salt bridges and van der Waals interactions.

Kinetic studies revealed that DCAF15 has a certain affinity with RBM39 itself, and the combination of aryl sulfonamides results in a formation of the ternary complex with higher affinity⁷⁷. While IMiDs have nanomolar affinity for CRBN, there

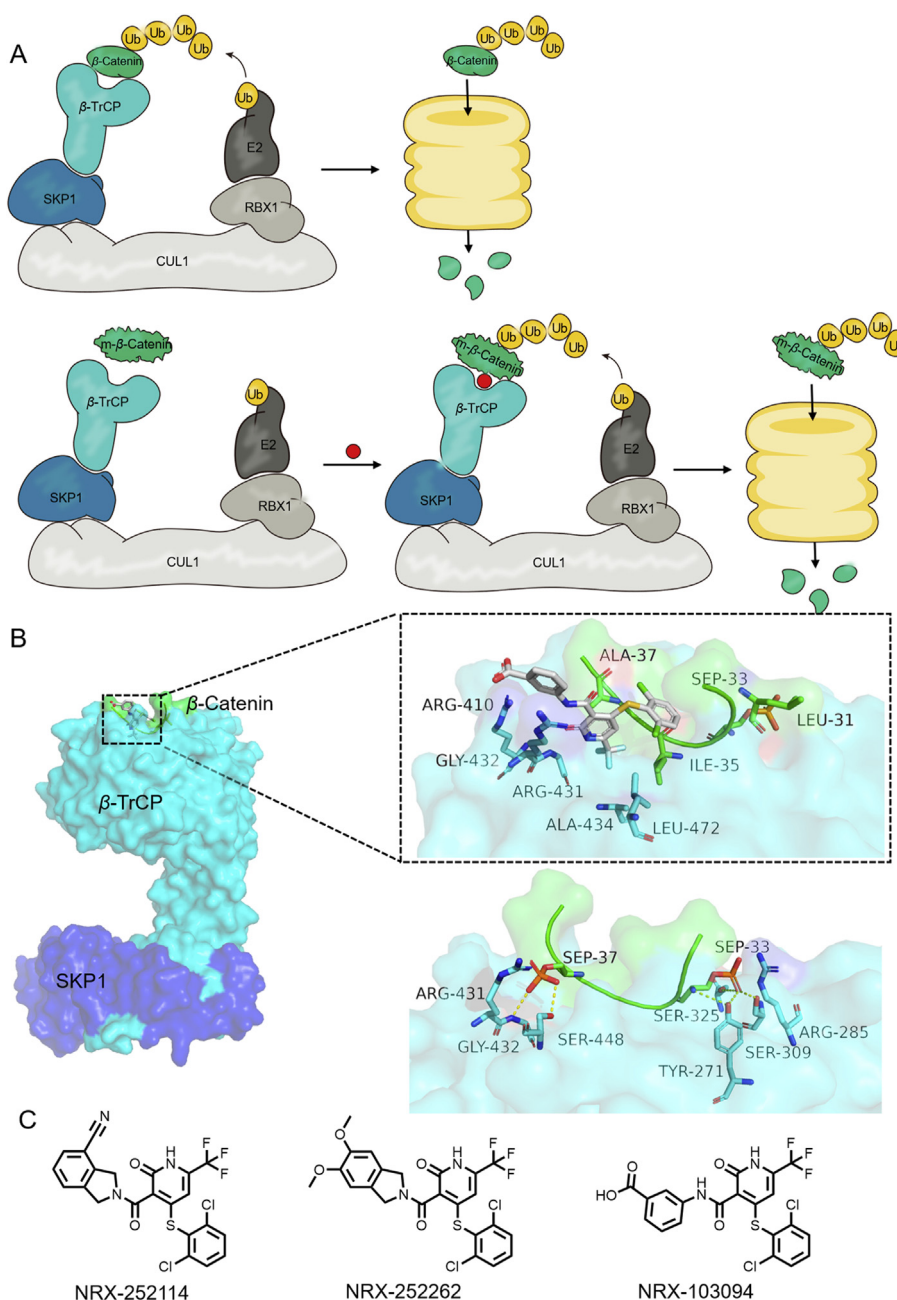


Figure 10 (A) Schematic diagram of the action mode of molecular glue NRX-103094. (B) NRX-103094 mediate the hetero-dimerization of β -catenin– β -TrCP (up, PDB:6M91), and comparison with wild-type (down, PDB:1P22). (C) Structures of NRX series as molecular glues.

have been no reports suggesting neo-substrate will interact with CRBN in the absence of IMiD⁷⁹. Moreover, for the IMiDs/CRBN system, β hairpin requires less sequence specificity, and most IMiDs are structurally similar and have many neo-substrates. However, the interaction between α -helix of RBM39 and DCAF15 is more specific, making it difficult to find neo-substrate. Nevertheless, they both support the concept that molecular glues depend on the secondary structural characteristics of target proteins, rather than the classically defined ligand binding pocket, representing a potential method for expanding the druggable space in the proteome.

4.3. DDB1-CDK12/cyclin K

The molecular glue degraders mentioned above target the substrate receptors (such as CRBN and DCAF15) of E3 ubiquitin ligases to recruit substrates^{80–82}. Three types of small molecules that induce the hetero-dimeric target complex CDK12–cyclin K and DDB1 binding will be briefly described in the following paragraphs. It is noteworthy that, unlike the cases described above, the interaction of CDK12–cyclin K induced by these molecular glues is functionally independent of the substrate receptors (Fig. 9A). Molecular glues may not necessarily bind directly to the target (cyclin K in this case) but rather may use an intermediate protein (CDK12 in this case), and the transfer of ubiquitin is largely dependent on the spatial location of the E2 and substrate protein. The molecular glue toolkit is expanding to bypass the requirement in consideration of whether the target tissues/cells express specific DCAF and thus has a wider range of applications.

Mayor-Ruiz et al.⁸³ revealed that dCeMM2/3/4 (Fig. 9B) prompt the interaction between CDK12–cyclin K and a CRL4 ligase complex. Similarly, the new type compound HQ461 was discovered and designed by phenotype-based high-throughput screening and biochemical reconstitution, which can directly occupy and modify the ATP binding pocket of CDK12 for DDB1 binding⁸⁴.

Likewise, CR8, discovered through systematically mining databases, is a new type molecular glue that induces proximity of CDK12–cyclin K and DDB1–CUL4–RBX1 E3 ligase core (Fig. 9C)⁸⁵. To achieve this, CR8 locates at the ATP-binding pocket of CDK12 and bridges the CDK12–DDB1 interface. More importantly, the localization of 2-pyridyl in the surface-exposed moieties of CR8 confers its gain-of-function glue activity by forming discrete contacts with Ile909, Arg928 and Arg947 residues in DDB1 β -propeller domain C (BPC, also involved in DCAF binding). Stabicki et al.⁸⁵ demonstrated that modification of surface-exposed regions of the inhibitor can develop the target-binding molecules into molecular glues, the same as the modification of BI-3802. The parent compound (*R*)-roscovitine⁸⁶ and natural product flavopiridol⁸⁷ both stimulate CDK12–cyclin K interacting with DDB1, albeit with lower binding affinity than CR8 (Fig. 9C). In contrast, the exposure volume of the covalent CDK12 inhibitor THZ531 is bulky, which is predicted to have steric hindrance with DDB1, losing the ability to recruit CDK12–DDB1 instead⁸⁸.

4.4. SCF ^{β -TrCP}– β -catenin

Simonetta et al.⁸⁹ reported the rational design and identification of potent molecular glues for the oncogenic transcription factor, β -catenin, and its homologous RING-E3 ligase, SCF ^{β -TrCP}. Under normal conditions, the phosphorylated β -catenin binds to β -TrCP at Ser33 and Ser37, leading to ubiquitylation and degradation (Fig. 10A)⁹⁰. However, mutations in these β -catenin phosphodegron attenuate β -catenin– β -TrCP interactions, leading to an enhanced oncogenic transcriptional program. Phosphorylated residue Ser37 mediates key electrostatic and hydrogen bonding in PPI interactions, and the removal of the phosphate of Ser37 leads to a reduction in binding affinity, while simultaneously forming a small hydrophobic pocket between the PPI interface (Fig. 10B). Using the concept of molecular glues, this research group discovered that NRX-103094 can occupy the mutated interface

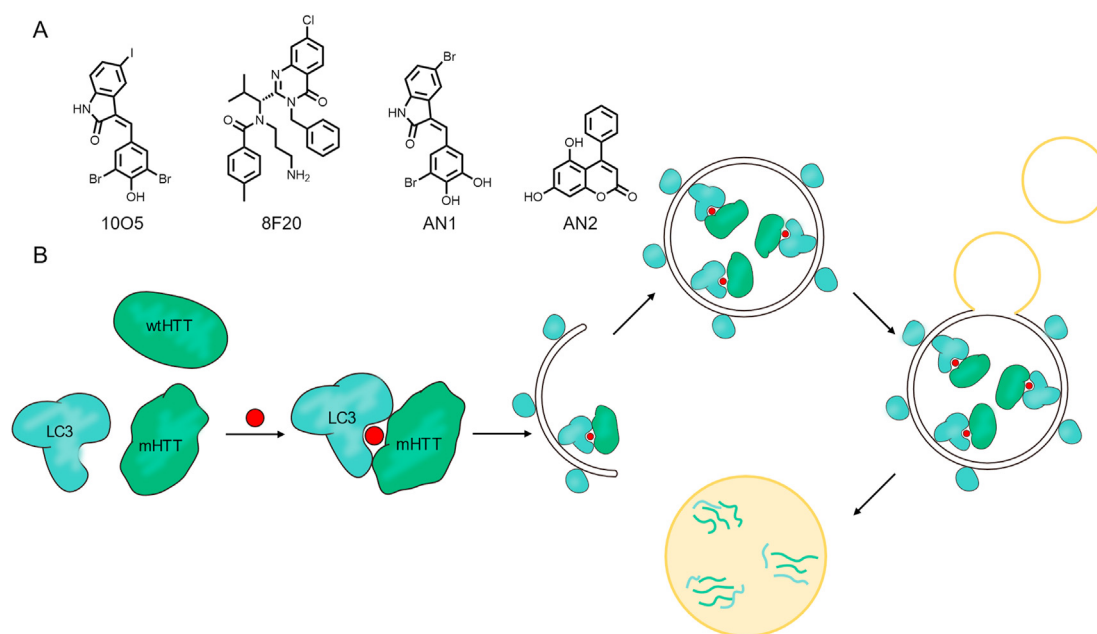


Figure 11 (A) Structures of ATTEC. (B) Schematic diagram of the action mode of ATTEC.

(Fig. 10C). As shown in the complex of the S37A β -catenin mutation and β -TrCP, the Ala434 and Leu472 residues of β -TrCP and Leu31 and Ile35 residues of the β -catenin form hydrophobic pockets, which are filled by the trifluoromethyl substituent of NRX-103094.

4.5. LC3-mHTT

Intracellular protein degradation mainly occurs through the ubiquitin–proteasome system (UPS) or autophagy–lysosome pathway^{91,92}. Although most current studies focus on E3 ligases of UPS, Li et al.⁹³ first applied the concept of autophagosome-tethering compounds (ATTEC), which eliminates pathogenic proteins by mediating target proteins to the autophagosome protein microtubule-associated protein 1A/1B light chain 3 (LC3) for autophagic degradation. Mutant huntingtin (mHTT) protein causes a neurodegenerative disorder disease, Huntington's disease (HD)^{94,95}. mHTT contains an expansion of the polyglutamine (polyQ) tract (more than 35 glutamine residues) and forms aggregates in neurons⁹⁶. Such aggregates or large proteins can resist being degraded by proteasomes, so PROTACs is an inefficient approach in this condition.

Fei's group⁹³ developed a high-throughput drug screening platform based on small molecule microarray (SMM) and oblique-incidence reflectivity difference (OI-RD) technology, that can quickly, sensitively and without labeling find small molecules binding to target proteins from thousands of small molecule compounds. They selected four compounds that tether mHTT and LC3 to incorporate mHTT into double-membrane autophagosomes for further degradation through autolysosomes (Fig. 11A). Such compounds specifically interact with mHTT but not with wide-type HTT (wtHTT) by distinguishing the emergent conformation of the expanded polyQ tract, which is distinct from the

short polyQ stretch in wtHTT, achieving allele-selective. LC3 is polymerized and amplified to form a membrane structure, which wraps the mHTT to form a complete autophagosome during the process of autophagy. After fusion with the lysosome, the encapsulated substances can be degraded (Fig. 11B). However, to parse the interactive interface of the compound and LC3, comprehensive pharmacological and structural studies still need to be performed.

4.6. GLP-1–GLP-1R

Although molecular glue degraders are the most common molecular glues mediating hetero-dimerization of target proteins identified in recent studies, it should be noted that in addition to acting as a degrader, molecular glues can also be used to increase ligase-substrate binding affinity by stabilizing hetero-dimerization, acting as PPI enhancers or stabilizers to augment substrate–ligase interactions. Such molecular glues can be therapeutically useful against targets previously considered undruggable, such as the PPI between the target protein and its natural substrate, which may be weakened by mutations.

The glucagon-like peptide 1 receptor (GLP-1R) acts as an important target of type 2 diabetes^{97–99}, but the half-life of its endogenous ligand GLP-1(7–36) is only 2 min, followed by hydrolysis to remove the N-terminal dipeptide His7-Ala8 to produce metabolite GLP-1(9–36)¹⁰⁰. His7 and Ala8 are key residues of GLP-1(7–36) in the interaction between GLP-1(7–36) and GLP-1R, as His7 forms a hydrogen bond with Gln234, and Ala8 generates van der Waals interactions with Glu387 and Leu388. And therefore, compared with GLP-1(7–36), GLP-1(9–36) has a weaker affinity for GLP-1R. Bueno et al.¹⁰¹ reported the discovery of a molecular glue called LSN3160440 (Fig. 12A), which participates in and enhances the interactions between GLP-1R and

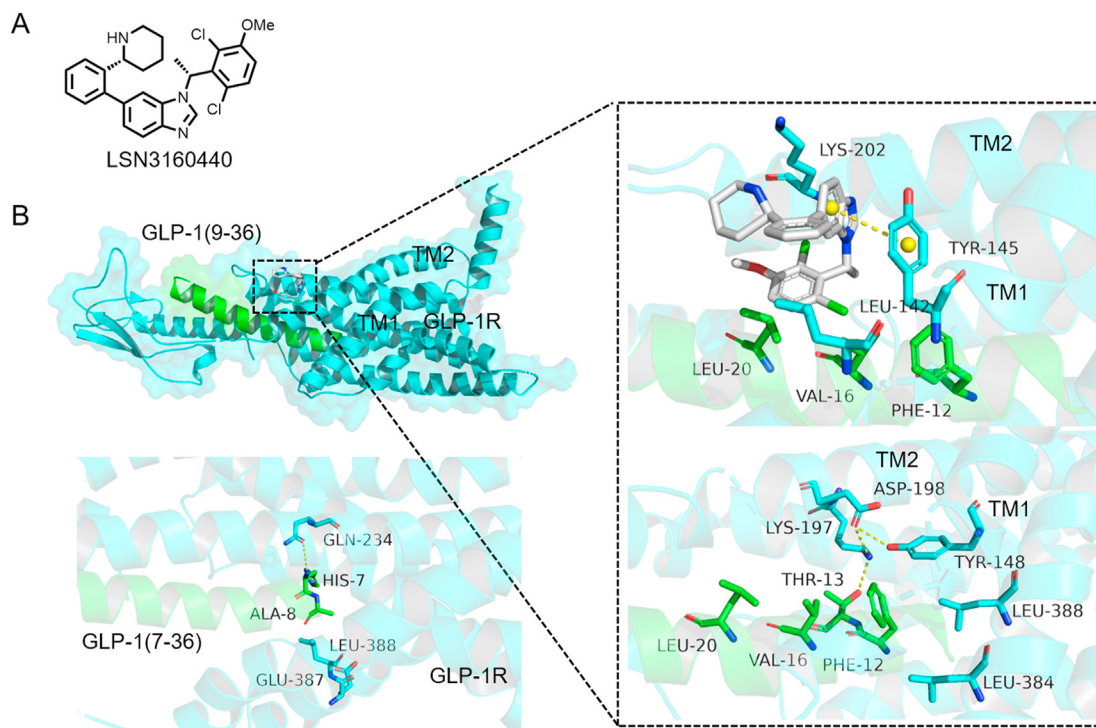


Figure 12 (A) Structure of LSN3160440. (B) GLP-1(9–36)–LSN3160440–GLP-1R complex (right, GLP-1R: cyan, GLP-1: green; PDB:6VCB), and comparison with GLP-1(7–36)–GLP-1R complex (left, PDB:5VAI).

GLP-1(9–36). LSN3160440 binds between the TM1 and TM2 interfaces of GLP-1R, and the benzimidazole group of LSN3160440 interacts with GLP-1R in a variety of ways, such as van der Waals contacts with Leu142, π - π stacking with Tyr145, and frequent water bridging with Lys202 (Fig. 12B). In the absence of His7 and Ala8 residues, LSN3160440 can act as a molecular glue to establish new bridging contacts between Val16 and Leu20 residues of the GLP-1(9–36) and the GLP-1R, enhancing the affinity of PPI.

5. Biochemical techniques for the development of molecular glues

The development of biochemical techniques has made a significant contribution to the discovery of molecular glues, and this review lists most of the discovery strategies discussed above (Table 1)^{102,103}. The following paragraphs primarily introduce the relatively latest technologies or methods and divide them into three stages of molecular glue development: starting point, optimization and confirmation.

5.1. Starting point: Rational design

Although most molecular glues have been discovered by chance, or as secondary development of existing compounds, we can still obtain some insights into the direction of screening and rational design from some of the reports.

Some key residues on the PPI interfaces contribute a lot to the total binding energy of the interaction, termed “hot spots”^{104–106}. For potential PPI or PPI with weak binding affinity, molecular glues can function as “hot spots” to mediate protein–protein binding by occupying the protein sites that can be anchored. Therefore, screening small molecules that can interact well with these sites can be regarded as the starting point for the identification of molecular glues. The screening strategy can be divided into structural screening based on shape complementarity and hydrophobic interactions, and pharmacophore screening based on identifying the key characteristics of interacting proteins. For example, a large number of novel thalidomide analogs were discovered and developed through phenotypic screening^{67–69}, and antiviral molecule P3 was obtained by structure-based virtual screening⁶¹.

For existing protein–protein interactions, the tethering strategy¹⁰⁷ may capture the fragments with an inherent binding affinity for pockets, which can be used as the starting point for the development of molecular glues^{107,108}. The host protein 14-3-3 binds with the phosphorylated motifs of client proteins to play a critical role in disease-related signaling networks, for example, the stabilization of 14-3-3 and estrogen receptor α derived phosphopeptide (ER α -pp) is an effective strategy for the treatment of ER α -positive breast cancer¹⁰⁹. Based on the tethering strategy, Ottmann’s group¹⁰⁹ introduced a cysteine at position 42 of 14-3-3 to act as a handle and identified several fragments that binding to the complex of 14-3-3 and ER α -pp with increased binding affinity (Fig. 13A). While inverting the strategy with the reactive handle on the estrogen related receptor gamma (ERR γ), a client-derived

Table 1 The discovery strategies of molecular glues and the specific techniques exploited within these studies.

Target protein	Molecular glues	Discovery strategy	Techniques	Refs.
PD-L1	BMS-202	Serendipity	NMR, DSF, crystal	32
REV1	JH-RE-06	Serendipity	Crystal, SDS-PAGE	40
MDMX	RO-2443, RO-5986	Serendipity	NMR, SEC-SLS, crystal, TR-FRET	46
BCL6	BI-3802	Serendipity	IF, Cryo-EM, WB, TR-FRET	53
	CCT369260	Serendipity	Nano-BRET, IF, MSD	54
MERS-CoV	P3	Virtual screening	SAXS, IF, crystal, thermostability	61
DDB1 ^{CRBN} -substrate	Thalidomide, lenalidomide, pomalidomide	Serendipity	Proteomic, LC-MS/MS, crystal, TR-FRET	62,63
	CC-122, CC-220	Rational design	Crystal, WB, TR-FRET	64,65
	CC-885	Rational design	IP, crystal, pull-down, IF, LC-MS/MS, SPPIER	67
	CC-90009	Rational design	WB, proteomic, crystal	69
	SJ6986	Rational design	WB, MD simulation	68
DDB1 ^{DCAF15} -RBM39	Indisulam, E7820	Serendipity	IP, LC-MS/MS, CRISPR/Cas9, pull-down, Cryo-EM, TR-FRET	77,78
DDB1-CDK12/cyclinK	dCeMM2, dCeMM3, dCeMM4	Chemical screening	WB, CRISPR/Cas9, pull-down	83
	(R)-CR8	Data mining	Co-IP, ITC, crystal, TR-FRET, nano-BRET	85
	HQ461	HTS	CRISPR/Cas9, Co-IP, WB, pull-down, CXMS, LC-MS/MS, DSF, thermostability	84
SCF ^{β-TrCP} - β -catenin	NRX-252114, NRX-252262, NRX-103094	HTS, rational design	FP, crystal, TR-FRET, pull-down	89
LC3-mHTT	1005, 8F20, AN1, AN2	HTS	SMM, OI-RD, HTRF, MST, pull-down, IF	93
GLP-1-GLP-1R	LSN3160440	HTS, rational design	Crystal, MD simulation, IF	101

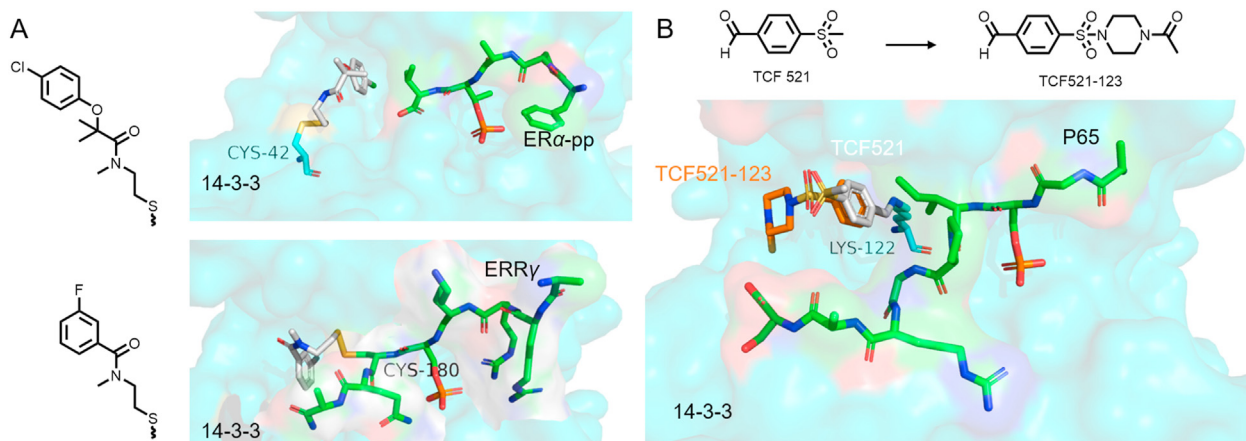


Figure 13 (A) Crystallographic overlay of 14-3-3(C42)-ER α (PDB:6HMT); 14-3-3-ERR γ (C180) (PDB:6Y3W) and the structures of fragments they screened out. (B) Crystallographic overlay of 14-3-3/P65 with TCF521 (PDB:6YOW) and 14-3-3/P65 with TCF521-123 (PDB:6YPY).

peptide of 14-3-3, they identified several fragments that stabilize the complex of 14-3-3 and ERR γ by targeting the Cys180 residue of ERR γ (Fig. 13A), which provide the starting point for the discovery of molecule glues¹¹⁰.

Inspired by cysteine, lysine has been used in covalent drug targeting methods as well^{111,112}. Wolter et al.¹¹³ reported an unprecedented concept for identifying initial fragments of molecular glues based on lysine covalent imine formation. Lys122 is located at the PPI interface between 14-3-3 and the NF- κ B subunit derived peptide P65, which is an ideal target residue for molecular glues screening (Fig. 13B). Based on this, they screened several fragments containing aldehyde groups, and grew these fragments to obtain molecular glue TCF521-123. The methods of disulfide capture or covalent anchoring reported are applicable to the early discovery of molecular glues, and further structural optimization and sufficient confirmation are required to fully validate the utility¹⁰⁸.

5.2. Optimization: Structural modification

When the starting point for a structure-enabled design campaign is successfully finished, such as an active fragment or small

molecule lead compound was achieved, to improve both the potency and cooperativity of the molecular glues, structural modification is highly desirable. According to different systems, corresponding optimization strategies need to be adapted to local conditions, but there are also some unique methods for reference.

BI-3802 targeting BCL6 protein was designed by exposing small groups in the solvent region, suggesting that modification of molecular structures in the exposed regions of the PPI interface or the target binding pocket surface could lead to the discovery of new molecular glues⁵³. Likewise, molecules such as CR8 were optimized using this strategy. However, it's noteworthy that large groups should be avoided in the solvent-exposed region, which may result in steric hindrance and adversely affect the action of molecular glues, such as the CDK12 inhibitor THZ531⁸⁵.

Analysis of the existing binding pockets of small molecules and targets to find space for further action also provides a strategy for molecular glues optimization. NRX-1933 was identified from a high-throughput screen (HTS), and the crystal structure of NRX-1933- β -catenin showed that the N-terminus of β -catenin has the potential for movement. Therefore, Simonetta et al.⁸⁹ proposed such a modification strategy: designing compounds to rearrange of β -catenin Leu31 and, as a result, inducing a larger, more

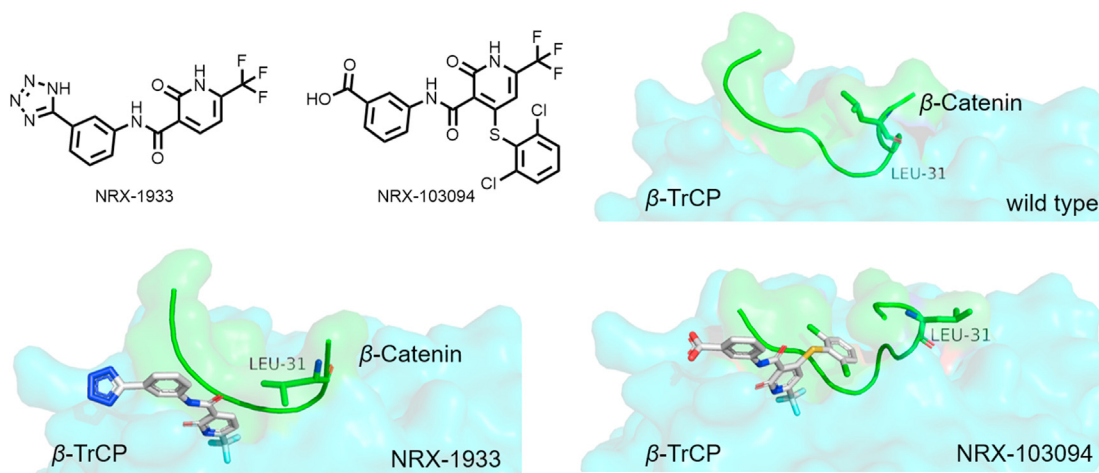


Figure 14 Structural optimization strategy of NRX-103094. β -TrCP-NRX-1933- β -catenin complex (PDB:6M93), other proteins PDB have been mentioned above.

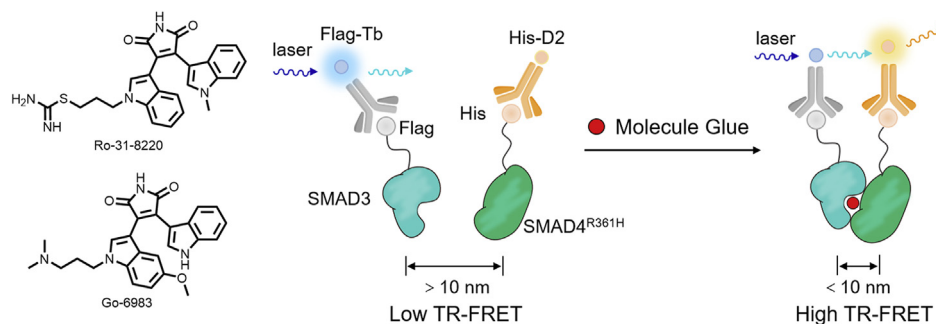


Figure 15 Schematic illustration of TR-FRET assay and the structures of the resulting molecular glues Ro-31-8220 and Go-6983.

druggable binding pocket (Fig. 14). Further optimization of NRX-1933 gave NRX-103094, a molecular glue that mediates SCF ^{β -TrCP}– β -catenin dimerization.

5.3. Confirmation: Identification method

Biophysical techniques can be used to verify the mechanism of molecular glues, and some specific experimental tools allow for monitoring protein polymerization, such as X-ray crystallography, Förster resonance energy transfer, polyacrylamide gel electrophoresis, and differential scanning fluorimetry (DSF). Here, we focus on some burgeoning and distinctive biophysical and biochemical techniques that have been adapted for the identification and confirmation of molecular glues.

Time-resolved fluorescence resonance energy transfer (TR-FRET) utilizes the energy transfer of two fluorescence groups for detection purposes. With the help of the TR-FRET assay, Tang et al.¹¹⁴ reported that the bisindolylmaleimide derivatives, Ro-31-8220 and Go-6983 acted as molecular glues to restore SMAD4^{R361H} PPI with SMAD3. When the molecular glues induce proximity of SMAD4^{R361H} and SMAD3, Flag-Tb and His-D2 fluorophores are brought closer together, thus achieving efficient energy transfer and generating TR-FRET signals (Fig. 15).

The *in situ* proximity ligation assay (PLA) allows the observation of intracellular PPI at the endogenous level. The antibodies bind to the target proteins, and are recognized by secondary antibodies that are coupled with short single-stranded oligonucleotide pairs. When the target proteins are adjacent (<40 nm), oligonucleotides hybridize and form a continuous circular DNA structure, and amplification of the signal can be detected by a fluorescence microscope¹¹⁵. Separation of phases-based protein interaction reporter (SPPIER) is an assay based on fluorophore phase transition, and can dynamically display protein–protein binding induced by small molecules in living cells. The protein encoded by green fluorescent protein (GFP) forms high-fluorescent GFP droplets when protein polymerization is induced, and thus emitting a detectable signal. Chung et al.¹¹⁶ successfully utilized this technique to detect the interaction between CRBN and GSPT1 induced by IMiDs such as CC-885.

6. Conclusions

Molecular glues play physiological roles by stabilizing existing PPIs, establishing new protein interactions, and occupying the binding sites of original pathogenic proteins and inhibit their subsequent pathways. Therefore, molecular glues are presented as potential strategies of drug design that will be utilized in the treatment of diseases involved in immunology, neurology, cancer

and others. Importantly, molecular glues depend on the secondary structural characteristics of targets and not necessarily on the classically defined ligand binding pockets, hence this feature broadens the range of drug therapeutic targets and makes drugs available for previously undruggable targets, bringing hope and paving a new path for drug development.

However, many challenges remain in subsequent drug development and modification. Molecular glues have mostly been discovered by accident or by secondary sifting through previous databases. Therefore, most current researches pay close attention to the screening strategies and the establishment of mechanism verification methods. It is essential to understand the action mechanism of molecular glues to screen and identify molecular glues according to different modes of action. Although this review classifies the different types of molecular glues mediating protein polymerization into homo-dimerization, hetero-dimerization and homo-polymerization, molecular glues can be essentially classified into two types according to their functional processes. First, a molecular glue binds to a target protein, and forms interaction surface (mostly known as “hot spots”) that can mediate the target protein binding with the “recruited protein”, resulting the aggregation of the target protein with “recruited protein”. Without such molecular glue, the target protein will not polymerize with the “recruited protein”. The cases such as IMiDs, CR8, or BI-3802, function in this mode. On the other hand, a molecular glue can bind to the establishing PPI interface, enhancing or stabilizing their interaction affinity. However, it is worth noting that the existing protein–protein interaction may only have a weak binding force. For example, E7820 and LSN3160440 act in this mode.

For pre-existing PPI complementary surface, there have been some successes with structure-based screening or structural optimization targeting this region. Additionally, more compatible interfaces than expected may emerge between unrelated proteins. For unformulated protein targets, we may be able to use docking software to find complementary interfaces between two proteins and design the novel molecular glues.

For molecular glues, whether accidentally discovered, or obtained through fragment-based drug discovery, subsequent structural modifications are needed. Since molecular glues often regulate protein interactions through the solvent exposure region, new molecular glues may be obtained by structural modification from it. Meanwhile, as the PPI interface is usually narrow, the design of molecular glues that can induce migration or rearrangement of residues on protein–protein binding surfaces and thus inducing larger pockets suitable for molecular glues, is also an excellent strategy for consideration.

Although this review focuses on small molecules, it should be noted that other macromolecule, such as macrolides and peptides

may also have potential application prospects as molecular glues. Macrolides were the first molecular glues to be discovered^{21,117}, which paved the way for the discovery of subsequent molecular glues. Cyclosporine exerts immunosuppressant activity by inducing the binding of cyclophilin and calcineurin simultaneously¹¹⁸. The similar FK506/rapamycin systems have been well studied and verified, and other macrolide molecular glues have also been developed^{119–121}. As early as 1992, Schreiber et al.¹²² discovered that antigenic peptides act as glues between major histocompatibility complex (MHC) presenter proteins and T-cell receptors to establish an immune response. In recent years, cyclic peptides and helical peptides have been widely used in the development and design of PPI inhibitors^{123–129}, demonstrating their ability to target the surfaces of undruggable proteins. These peptides possess a large binding interface and satisfactory binding affinity, making them promising candidates for recruiting other proteins, and thus, it is reasonable to speculate that cyclic peptides and helical peptides may also be developed as molecular glues in the future.

Taken together, with advances in crystallography and biochemical technology, the class of molecular glues is constantly expanding, and researchers are gaining a deeper understanding of their mechanisms of action. Rational design, optimization strategies and detection technologies are continuing to develop, which bring hope for breakthroughs in undruggable targets.

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Author contributions

Hong Yao conceived the formulation of overarching research goals and superintended the whole study. Hongyu Wu collected the data and drafted the manuscript. Chen He, Yilin Jia, Shengtao Xu, Zheyang Zhu, Dahong Li and Jinyi Xu revised the manuscript. All the authors have read and approved the final manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

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