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Molecular Glue Discovery: Current and Future Approaches

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Abstract

The intracellular interactions of biomolecules can be maneuvered to redirect signaling, reprogram the cell cycle, or decrease infectivity using only a few dozen atoms. Such “molecular glues,” which can drive both novel and known interactions between protein partners, represent an enticing therapeutic strategy. Here, we review the methods and approaches that have led to the identification of small-molecule molecular glues. We first classify current FDA-approved molecular glues to facilitate the selection of discovery methods. We then survey two broad discovery method strategies, where we highlight the importance of factors such as experimental conditions, software packages, and genetic tools for success. We hope that this curation of methodologies for directed discovery will inspire diverse research efforts targeting a multitude of human diseases.

Graphical Abstract

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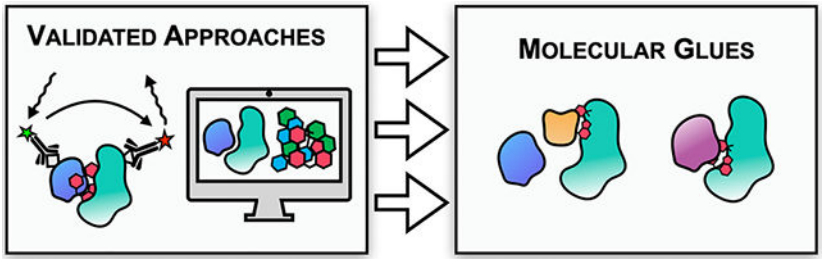
The authors declare no competing financial interest.



1. INTRODUCTION

1.1. Overview.

The workhorses of cellular processes, protein transport and degradation, signal transduction, gene regulation, and metabolism, are proteins acting not alone but in complex concert. These protein–protein interactions (PPIs) come in many forms, including stable complexes of multiple proteins, rapid and transient hetero-oligomerizations, and dynamic polymers. Many human diseases can be traced to aberrant PPIs. This is seen in canonical examples like cancer, where the constitutive activation of K-Ras/Raf or Myc/Max interactions drive transformation, and in neurodegenerative diseases like Alzheimer's, which is marked by the oligomerization of previously minimally interacting β-amyloid proteins.[1-3] The entry of pathogens into host cells also requires the initiation of a PPI, as with the spike protein of the virus SARS-CoV-2 and host ACE2 receptors.[4] Thus, targeting PPIs represents a powerful therapeutic strategy. For example, antibodies, which can effectively function as PPI inhibitors, have already revolutionized the treatment of cancers, autoimmune diseases, and, most recently, SARS-CoV-2 infection.[5] However, antibodies cannot currently target intracellular PPIs and are significantly more expensive to synthesize than small molecules.[6-8] An additional cost is time to discovery. Venetoclax, the sole FDA-approved intracellular small-molecule PPI disruptor (despite innumerable discovery campaigns), took over 30 years to develop.[9] Thus, an alternative small-molecule-based approach could reduce time to discovery as well as costs while broadening the number of druggable targets.

Molecular glues, small molecules that can either stabilize endogenous PPIs or induce non-native ones, are revolutionizing the manipulation of clinically relevant protein interactions. Over the past 25 years, FDA-approved molecular glues have treated millions of patients and generated billions of dollars in revenue.[25] Many of these drugs, such as tacrolimus and rapamycin, are natural products that, unlike antibodies and traditional PPI inhibitors, do not just replace a protein partner (Table 1). Rather, they bind to the PPI interface of a target protein and preclude the native activity. For example, paclitaxel (Taxol), a diterpenoid derived from the Pacific Yew, shifts the conformation of tubulin dimers to impact microtubule dynamics; this in turn disrupts the cell cycle and results in significant antitumor activity.[26,27] The effectiveness of such natural products, chosen by millions of years of natural selection, underscores the promise and superiority of the molecular glue approach to manipulate PPIs.

The research community has taken note of the untapped potential of molecular glues. In the past decade, there have been dozens of reviews expounding upon potential protein

targets, small-molecule characteristics, and PPI biophysics.[25,28-34] Critical to increasing the number of molecular glues on the market is hit discovery. Yet, there are currently no reviews that elaborate methods that produced nondegrader molecular glues and detail the specific experimental conditions that increase the chances of molecular glue discovery.

In this Perspective, we aim to fill this gap, reviewing all papers that successfully identify a validated molecular glue, including its targets and mechanism of action. We also highlight tactics, from methodology (e.g., computational) to experimental conditions (e.g., reagent concentration), that repeatedly result in molecular glue discovery. This discovery begins with a screening method to reveal molecular species that induce or stabilize two or more proteins. For this reason, we will not cover methods to discover proteolysis targeting chimeras (PROTACs) or other bivalent small molecules, as the identification of this subset of molecular glues instead begins with optimizing the covalent linkage between two single-protein binders.[35-37] Given the contemporary focus on PROTACs, however, we include a short commentary that describes their exciting patentability and long-term (>10 years) outlook. This perspective will also not cover the discovery of natural product molecular glues. These molecules were produced by evolution, which faces unique challenges when applied in a laboratory setting, although, directed evolution has recently been harnessed to optimize molecular glues that induce non-native interactions.[38] We hope that this compendium, complete with systematically categorized methods and curated recommendations, will allow researchers to rapidly select methods that match both their capabilities and protein target.

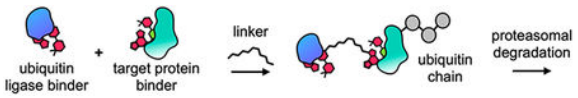
1.2. PROTACS: A Pharmaceutical Gold Rush.

Proteolysis targeting chimeras (PROTACs) are composed of a ubiquitin ligase-binding molecule covalently bound to a target proteing binding molecule via a chemical linker. At first glance, the discovery process for bivalent PROTACs seems daunting. Each binding molecule needs to be identified through screens, followed by linker optimization, which can dramatically modulate PROTAC activity. Fortunately, there is a wealth of shelved target protein binders as well as several validated ubiquitin ligase binding molecules, leaving only linker and pharmacological properties to optimize.

One appeal of PROTACs that is little discussed and nonscientific is their patentability. Each new combination of binder–linker–binder possesses distinct properties and so is a unique molecule to patent. For example, single-atom differences between linkers have been shown to dramatically alter PROTAC activity. Furthermore, most “drug-like” molecules (<500 Da) have already been synthesized and patented, but PROTACs are much larger and so most active PROTACs are initially unpatented.

We contend that eventually the PROTAC “gold rush” will end for several reasons. There are only so many effective target protein binders already discovered. The current protein binders may also lack an effective site for covalent linker attachment. To identify and optimize new protein binders requires similar resources as discovering molecular glues. Finally, there are many proteins that contribute to disease, but cannot be degraded without severe side effects. Certain PROTACs will improve patient care, but their lack of versatility limits their potential. Molecular glues on the other hand are clinically validated to modulate

protein activity via numerous mechanisms including PPI inhibition. Thus, the versatility of molecular glues gives them longevity in drug discovery.



1.3. Classifying Molecular Glues Based on Approved Drugs.

As alluded to above, the term molecular glue encompasses molecules that manipulate PPIs with divergent mechanisms of action and with contrasting functional consequences. Currently, the drug discovery community classifies molecular glues based on their binding, i.e., orthosteric versus allosteric, or structure, i.e., uni- versus bivalent. In this Perspective, we analyze FDA-approved molecular glues to devise a classification system based on their mechanism of action. We find that clinically approved molecular glues can be classified as shielding (type I), redirecting (type II), and novel activity (type III) and that these types inform choices regarding the optimal molecular glue discovery method for a specific protein target (Figure 1 and Table 1).

1.3.1. Type I: Shielding.—Perhaps the most well-known FDA-approved molecular glues are the natural products tacrolimus (FK506), cyclosporine A, and rapamycin, all of which can be classified as type I. Type I molecular glues achieve endogenous PPI inhibition (a “dream” in drug discovery) by inducing a non-native PPI to physically block (“shield”) the target protein’s endogenous activity.[39] Tacrolimus and cyclosporine, both selective inhibitors of calcineurin and thus T-lymphocyte cytokine production, are an integral part of the immunosuppressive regimen for solid organ transplantation and have also been used to treat atopic dermatitis and as disease-modifying antirheumatic drugs (DMARDs).[17-19] Their inhibitory action arises from the recruitment of a protein (FKBP-12 and CyP, respectively) to bind calcineurin and block its interaction and activation of the nuclear factor of the activated T-cell (NFAT) protein. Rapamycin, a less nephrotoxic inhibitor of T and B cell proliferation, functions by blocking the active site of the regulatory kinase mammalian target of rapamycin (mTOR) via non-native FKBP recruitment.[16] Given its antiproliferative and immunosuppressant effects, it is used not only for the prophylaxis of renal transplant rejection but also the treatment of diseases like renal cell carcinoma, acute myeloid leukemia (AML), and tuberous sclerosis complex.[16] Thus, rather than replacing a protein partner, type I molecular glues amplify weak interactions (>1 mM K_d) that already exist between the target protein and a bystander. By recruitment of a bystander protein, they more effectively shield target surface areas to achieve PPI inhibition.

1.3.2. Type II: Redirecting.—Another subset of FDA-approved drugs, tafamidis, paclitaxel, lenalidomide, and dexrazoxane, can be classified as type II molecular glues. These compounds bind an endogenous PPI and “redirect” its conformation or dynamics to abrogate, or occasionally enhance, activity (Figure 1). For example, tafamidis is used to treat both senile and hereditary transthyretin (TTR) amyloidosis, which is caused by the deposition of transthyretin polymers in neuronal and cardiac tissues. It works by stabilizing TTR tetramers via PPI interface binding, thereby precluding monomerization, polymerization, amyloidosis, and subsequent disease.[23] Tafamidis is an excellent example

of how stabilizing one endogenous PPI state over another can elucidate and treat pathology, a model with utility in other aggregation and fibril-related diseases, such as Huntington's, Alzheimer's, and Parkinson's.[40]

Paclitaxel, as discussed in the Introduction, works as an anticancer agent by stabilizing a toxic state of an endogenous PPI (tubulin heterodimers), thereby inhibiting microtubule formation.[27] Notably, compounds with a type II molecular mechanism of action are the most frequently discovered molecular glues to date (Tables 1, 2, and 3). Many of these agents were discovered serendipitously as stabilizers of inactive, but endogenously formed, homodimers.[41,42] Other compounds inhibit proteins that require dynamic cycling between states to carry out their function.[27,43] This subset of type II molecular glues is particularly exciting, as they could target dynamic PPIs that transfer signals (e.g., phosphorylation, ubiquitination, acylation, and lipidation). Proteins involved in physical transport also rely on PPI cycling, as discussed in the example below of *Plasmodium falciparum* invasion, the parasite responsible for malaria.[43] Thus, type II molecular glues can inhibit or activate the formation of particular protein states to harm or aid cellular homeostasis.

1.3.3. Type III: Novel Activity.—Finally, FDA-approved molecular glues like thalidomide and pomalidomide can be termed type III. These compounds, and all other type III molecular glues, induce a non-native PPI to produce a novel activity between those proteins (Figure 1). For example, pomalidomide induces the interaction between the E3 ubiquitin ligase cereblon (CRBN) and a range of non-native substrates, including zinc finger proteins and lymphoid transcription factors.[44-46] The subsequent immunomodulation is useful in the treatment of refractory multiple myeloma and potentially other myelodysplastic syndromes.[47] These drugs demonstrate that novel ubiquitination activity can target disease. However, while non-native degradation is the current research focus, type III molecular glues have also been shown to alleviate disease phenotypes by inducing non-native PPIs that influence homeostatic mechanisms, like calcium channel activity and transcription.[48,49] The biggest challenge in identifying type III molecular glues is understanding which non-native PPIs impact cellular and organismal phenotypes. Tools such as auxin induced degradation and siRNA provide a high-throughput, low cost, readout on whether degradation of a protein target results in a desired phenotypic outcome, providing confidence that *in vitro* small-molecule development will have a clinically relevant outcome.[50,51] Analogous tools to connect nondegradation consequences (trafficking, lipidation, etc.) of non-native PPI induction to phenotypic outcomes currently do not exist. Thus, new approaches to link this vast theoretical landscape to clinical outcomes are necessary for type III molecular glues to reach their full potential. We hope that this Perspective will allow researchers to optimize and apply current methods of molecular glue discovery to both accelerate the identification of molecular glues and eventually generate a high-throughput screening platform to connect individual non-native PPIs to phenotypes.

1.4. Methods to Discover Molecular Glues.

We frame our discussion below of molecular glue discovery methods around two broad categories: activity-based methods (ABMs) and interaction detection methods (IDMs). We then subset each category to detail the experimental aspects that emphasize which methods

are best suited for a particular target. From the 38 methods surveyed, we selected 13 (4 ABMs and 9 IDMs) that each emphasize specific experimental approaches for molecular glue discovery.

2. ACTIVITY-BASED METHODS (ABMS)

Rather than detecting protein interactions directly, activity-based methods (ABMs) measure downstream *in cellulo* or *in vitro* activity, such as cell death, enzymatic catalysis, or protein expression (Table 2). It should be noted that phenotypic screens are a subset of ABMs, where the system being measured (cells, animal models, etc.) contains an active genome that defines the phenotypic assay. Thus, *in vitro* ABMs, such as enzymatic pathway reconstitution assays, are not phenotypic assays but still rely on detection of activity rather than detection of direct protein interactions to evaluate small-molecule efficacy. ABMs are agnostic to the molecular mechanism by which a given activity change is achieved. Therefore, they not only discover specific molecules capable of impacting an activity, ABMs followed by proper validation can also reveal new biological pathways and biomolecules (proteins, RNA, metabolites, etc.) that contribute to that particular activity.

ABMs are currently the only screening method that has led to clinically approved molecular glues. Thalidomide was identified via phenotypic screening in the 1950s and has since been validated as a molecular glue and a treatment for hematological malignancies, despite an initially devastating global impact due to pharmaceutical company and regulatory agency negligence.[52]

The greatest challenge when deploying ABMs is validating the precise molecular mechanism of action by which hit compounds alter an activity, a critical part of the drug approval process since 1998. Thus, although phenotypic screens, which are a subset of ABMs, were popular in the mid to late 20th century, target-based screens have been favored in the last few decades.[53] Nevertheless, the rise of resistance mutations among certain drug classes and an increased appreciation for the multifactorial nature of disease have revived interest in ABMs as an initial step in identifying targets. However, the fundamental challenge of detailing precise mechanisms of action remains. The story of the cardioprotective agent dexrazoxane illustrates this challenge.

Dexrazoxane was discovered over 50 years ago as a cardioprotective agent for anthracycline (doxorubicin) chemotherapy toxicity.[54,55] Initially, its mechanism of action was presumed to be chelation of intracellular iron in cardiomyo-cytes, reducing harmful reactive oxygen species production that results in DNA damage and cell death. Despite some promising data *in vitro* and *in cellulo,* this mechanism could not be conclusively validated *in vivo* with the technologies available. In 1991, Tanabe et al. found that dexrazoxane and other derivatives were capable of inhibiting topoisomerase IIβ activity.[56] Follow-up studies including a crystal structure confirmed that dexrazoxane is also a type II molecular glue that freezes topoisomerase IIβ dimers, inhibiting both enzyme activity and anthracycline binding and causing catalytic protein degradation.[55,57] A recent review thoroughly concludes topoisomerase IIβ binding is the clinically relevant mechanism of action.[58] Had the topoisomerase IIβ mechanism been proposed first, researchers would have

searched for more effective topoisomerase IIβ inhibitors instead of iron chelation drugs for chemotherapeutic cardioprotection. Thus, this example highlights the peril of ABMs: decades of resources can be wasted in pursuing ill-defined, or even false, mechanisms of action.

Although the contemporary pharmaceutical industry steers clear of ABMs, emerging genetic and molecular methods give ABMs newfound potential in initial drug discovery.[59,60] These new technologies exploit the lower cost of DNA sequencing and genetic manipulation to either narrow potential molecular mechanisms or rapidly uncover the biological agents responsible for activity changes. In this section, we dive into four specific examples of ABMs that offer insights into ABM design principles that help overcome hurdles in molecular glue discovery.

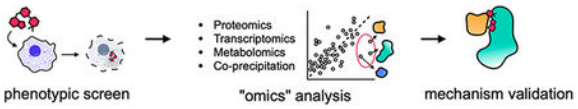
2.1. Snapshot of Activity-Based Methods (ABMs).

Methods: tetrazolium-based cell viability assays (MTS/MTT), viral replication, enzymatic activity, actvity-based protein profiling, enzyme complex reconstitution, biosensor, GFP transcription.

Highlights: (1) Only methods that have successfully led to FDA-approved molecular glues. (2) Can reveal novel protein layers and pathways in pathophysiology.

Challenges: Challenging and costly to determine underpinning mechanism of action. (2) No guarantee of molecular glue discovery.

Outlook: Coupling “omics” technologies with machine learning can rapidly and accurately identify targets.



2.2. *In Cellulo* Activity Methods.

2.2.1. Viral Infection Assay.—In 2011, a team at Bristol-Myers Squibb seeking inhibitors of H1N1 used evolution to overcome the problem of mechanism identification presented by ABMs.[61] Their initial hit discovery method consisted of screening a 1.2 million compound small-molecule library with an *in cellulo* viral infection ABM.[61] They monitored viral neuraminidase activity via fluorescent output in their initial screen, resulting in two hits, compounds **1** and **2**, each with a submicromolar IC_{50} that were optimized to produce compound **3**. Compound **3** possessed a remarkably steep dose–response curve, hinting at oligomerization or aggregation as the mechanism of action. Faced with determining the mechanism driving the change in neuraminidase activity, the team used evolution. The researchers subjected influenza particles to inhibitory concentrations of compound **1** and passaged the particles until they became resistant to compound **1** inhibition. Upon sequencing the resistant particles, the team found multiple mutations in influenza nucleoprotein 1 (NP). Co-crystallization of compound **3** with NP revealed that compound

3 inhibits NP activity by inducing dimerization of endogenously formed NP trimers, thus confirming that it is a type I molecular glue.

Two aspects of this ABM may help future researchers in search of molecular glues. First, noticing the remarkably sharp dose–response slope of compound **3** helped steer researchers toward techniques such as dynamic light scattering and crystallography that elucidate higher-order protein complexes. Therefore, analysis of dose–response data can identify a compound's mechanism of action. Second, the authors' strategy of using resistance mutations to uncover their inhibitor's target is an excellent approach when inhibiting growth phenotypes. For example, any method involving cancerous cells, which typically have high mutagenesis and growth rates, can develop hit-resistant cells and be sequenced to identify genes involved in the mechanism of action of the hit compound.

2.2.2. Signaling Pathway Directed Killing Assay.—The next case demonstrates how ABMs can reveal nodes in cell signaling pathways amenable to modulation by molecular glues. In 2015, Kastrinsky et al. screened 30 tricyclic molecules for their ability to simply kill H1650 lung adenocarcinoma cells, which possess constitutively active ERK and AKT signaling.[62,63] Half of their compounds demonstrated midmicromolar IC_{50}, but the molecular mechanism of action remained undescribed. Two years later, they discovered their hit compounds increased activity of the trimeric phosphatase PP2A via direct binding, which in turn decreased ERK and AKT phosphorylation and subsequent cell growth.[64] It was in 2020 that Leonard et al. used various techniques, including electron microscopy, to confirm these molecules are type II molecular glues that stabilize active PP2A heterotrimers.[65] This work affirms that signaling complex stabilization via direct binding is a viable strategy to impact cell states. This example also gives confidence that an intentional campaign to discover and characterize molecular glues in therapeutically validated signaling pathways can identify compounds with clinically relevant potential.

2.2.3. Single-Gene Selective Cell Killing.—The most contemporary ABM example is also perhaps the current gold standard for utilizing state of the art technologies to both design an ABM screen and rapidly identify a hit compound's molecular mechanism of action. In 2020, Mayor-Ruiz et al. developed a clever *in cellulo* ABM to discover molecules that killed cells via induced protein degradation.[80] The team used a CRISPR Cas9 mutagenesis panel to identify ubiquitin conjugating enzyme E2M (UBE2M) as a candidate that prevents the molecular glue auxin's activity.[82] The researchers replaced wild-type UBE2M with an inactive mutant ($UBE2M^{mut}$) in the KBML cell genome. They then screened a library of 2000 known cytostatic/cytotoxic small molecules for differential killing of wild-type versus $UBE2M^{mut}$ KBM7 cells. The authors used various methods, including proteomics, CRISPR screening, resistance mutation screening, and numerous *in vitro* binding and interaction assays, to determine the molecular mechanism of action of their hit compounds. Their data indicated that the selectively cytotoxic molecules degrade cyclin k by binding at the interface of CDK12, a known cyclin k binder, and the E3 ubiquitin ligase DDB1. However, the exact binding site of the small molecule within this protein complex will remain unknown until a compound-bound protein structure is solved. Key to the success of this study was development of an excellent phenotypic control using CRISPR-

Cas9 mutagenesis and genome engineering to uncover molecules that impact a specific mechanism. This example serves as a blueprint for future ABM-based drug discovery efforts.

2.3. *In Vitro* Activity Methods.

An *in vitro* activity method is one in which multiple proteins interact *in vitro* to perform an activity that can be measured. Only four molecular glues have been discovered by such a method, including Tafamidis.[72,73] New molecular mechanisms of action found by these methods can be rapidly characterized due to the limited number of biomolecular agents involved in the initial screen. These methods are particularly effective when a pathway is known to contribute to a disease, but the most tractable protein target within that pathway is unclear. The modularity of the assays also allows investigators to screen every possible PPI when a hit molecule emerges, unlike with *in cellulo* methods in which innumerable biomolecules could be involved. The *in vitro* activity method we will discuss exemplifies the potential for these approaches to tackle two important areas of pharmaceutical research: protein degradation and dynamic signal transfer (e.g., phosphorylation, ubiquitination, palmitoylation, glycosylation, etc.).

2.3.1. Enzymatic Complex Reconstitution.—In 2011, Ceccarelli et al. sought to inhibit the ubiquitination, and thus degradation, of $p27^{Kip1}$ to induce cell cycle arrest.[77] To achieve targeting, the researchers reconstituted the specific ubiquitination complex responsible for ubiquitination of $p27^{Kip1}$.[83] They then quantified the fraction of ubiquitin-labeled $p27^{Kip1}$ to identify a small molecule, CC0651, that inhibited the accumulation of ubiquitin on $p27^{Kip1}$. The researchers next systematically exchanged all possible protein targets either *in vitro* or in yeast to determine the compound selectivity and potential binders. This approach followed by thermal denaturation studies suggested that hCdc34 is necessary for CC0651 inhibition and is the direct binding target. Cocrystallization of ubiquitin, hCdc34, and CC0651 revealed that the small molecule makes direct contacts with ubiquitin. Paired with experimental evidence, this structure led the researchers to conclude CC0651 stabilizes the hCdc34–ubiquitin PPI to such a degree that the ubiquitin–CC0651–hCdc34 complex is energetically favored over ubiquitin transfer to the E3 ligase complex. In follow up work, the same group applied multiple interaction detection methods, including TR-FRET and molecular docking, to discover active analogues of CC0651.[84] These experiments show that CC0651 is a type II molecular glue because it alters a known interaction to disrupt its function.

Excitingly, the approach modeled in this research can be applied to other PPI-dependent signaling pathways, such as phosphorylation, methylation, glycosylation, or acylation, where proteins interact to transfer a post translational modification and then dissociate to restart the cycle. Molecular glues that prevent protein dissociation will consequently preclude pathway cycling and signal propagation. One challenge in finding these types of molecular glues is eliminating single protein binders during the screening process. However, analysis of this work by Ceccarelli et al. reveals a strategy to bias hits toward molecular glues: higher target protein concentrations. Both hCdc34 and ubiquitin were added to the *in vitro* assay at >200-fold higher concentrations than the other proteins. The dramatically higher protein

concentrations greatly increase the likelihood of a ternary small-molecule–protein–protein interaction and thus the opportunities for molecular glue activity.

2.4. ABM Conclusion.

When reviewing the literature, we were surprised by the lack of type I molecular glues discovered via ABMs (Table 2). All clinically successful type I molecular glues are natural products (rapamycin, fk506, and cyclosporine A), demonstrating that the type I mechanism has survived evolutionary pressure over millions of years to alter mammalian phenotypes. One could also argue that ABMs are most analogous to natural product formation: organisms evolve over time to impact neighboring phenotype via natural products without elucidation of a particular mechanism of action. One reason we believe ABMs have not discovered type I molecular glues, despite their potential, is because the sea of proteins that could shield a given target is too vast. For example, assume a compound induces mislocalization of a transcription factor via a PPI with a plasma membrane protein, but the compound does not bind tightly to either protein individually. How can researchers determine the mechanism of action? Data sets from cutting edge omics techniques can provide some clues. Transcriptomics and proteomics analysis could identify the affected transcription factor, and confocal experiments could visualize changes in localization, but the mechanism would still be incomplete. In this way, type I molecular glues may have been discovered via ABMs, pursued briefly, and abandoned due to high costs in target validation and identification of more mechanistically tractable hits.

Although the consensus has been that ABMs involve more time, effort, and risk than their rewards merit, we posit that ABMs can uncover novel and unpredictable avenues to perturb disease. Moreover, the falling costs of cutting edge analytical and genetic tools enable rapid and affordable deconvolution of a hit compound's molecular mechanism of action. Finally, scientific advances enable ABMs to be designed *in vitro,* avoiding this concern by limiting the scope of potential targets to a desired class and enabling manipulations, such as increased protein concentrations, that increase the likelihood of particular proteins being modulated. Given that ABMs are the only methods that have produced clinically viable molecular glues, they deserve consideration in the design of a drug discovery campaign.

3. INTERACTION DETECTION METHODS (IDMS)

Interaction detection methods (IDMs) provide a readout that explicitly measures the interaction between two proteins upon small-molecule addition (Table 3). As such, no IDMs are *in cellulo* because off-target mechanisms are currently impossible to rule out. IDMs are the only methods that have identified molecular glues intentionally as opposed to serendipitously. This is not surprising given that intentional molecular glue discovery must involve measurement of protein interactions. Moreover, every computational method intentionally identified molecular glues because the interface between two proteins was selected for virtual screening. With that said, the majority of discoveries via experimental IDMs were serendipitous and agnostic to the exact mechanism of action.

Another aspect of IDMs worth noting is their propensity to produce type I ("shielding") molecular glues when compared to ABMs. In our Discussion and Outlook section below,

we explore the untapped potential of type I molecular glues for inhibiting therapeutically relevant PPIs and discuss promising IDM-based approaches for rapid and high yield molecular glue identification.

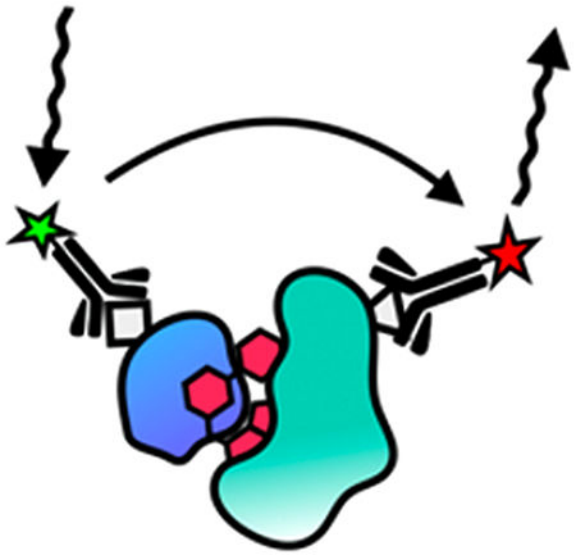
We see IDMs as the most effective method for novel and intentional molecular glue discovery campaigns. Here we review examples of 9 IDMs (5 experimental and 4 computational) to showcase the diverse methods available for molecular glue discovery. Each of the 5 experimental methods were selected to cover a wide range of approaches and mechanisms of action achieved. We decided to highlight multiple computational examples because we see this as an underutilized IDM given its high hit rate and exciting future potential. Finally, we included one outlier example of a molecular glue derived from a crystallography additive. This example, and other more recent fragment-based screens, will hopefully highlight the potential for crystallography experts to apply their skills toward library screening. In this perspective, we classify IDMs into three categories: *in vitro* labeled, *in vitro* label-free, and *in silico,* based on the required laboratory tools, expertise, and protein-target processing.

3.1. Snapshot of Experimental Interaction Detection Methods.

Methods: Time-resolved FRET (TR-FRET), oblique incidence reflectivity difference (OI-RD) microscopy, surface plasmon resonance (SPR), fluorescence polarization (FP), protein crystallization, nuclear magnetic resonance (NMR), mass spectrometry (MS), enzyme-linked immunosorbent assay (ELISA).

Highlights: (1) Capable of intentionally discovering molecular glues. (2) No protein structural information required. (3) Protein modification is not required for unlabeled IDMs. (4) High propensity to discover inhibitory molecular glues (type I or type II).

Challenges: (1) Precise molecular mechanism cannot always be controlled. (2) Protein modification required to use most common (IDMs). (3) Has yet to discover an FDA-approved molecular glue.



3.2. *In Vitro* Labeled IDMs.

The most common and validated methods to discover small-molecule modulators of protein–protein interactions are *in vitro* labeled IDMs. As the name suggests, these methods use covalent labeling of each protein target with a fluorophore or affinity tag to measure the target protein proximity. There are numerous reviews and books covering the basic principles and applications of *in vitro* labeled IDMs, and so our perspective will focus specifically on methodological aspects that influence molecular glue identification. We selected three examples of successful *in vitro* labeled IDMs that each cover a different molecular glue type. All three methods utilize fluorophores, which is not surprising given that fluorescence is the most validated signal to date for PPI detection, but there is an example in Table 2 of labeled molecular glue discovery without fluorophores using an enzyme-linked immunosorbent assay (ELISA).

3.2.1. Time-Resolved Förester Resonance Energy Transfer (TR-FRET).—An early example of an *in vitro* labeled IDM that discovered a molecular glue was carried out in 2012 by Graves et al. and exemplifies a standard workflow for high-throughput type I/II molecular glue screening.[42] The research team's goal was to increase the expression of tumor suppressor p53 by inhibiting its interaction with its negative regulators MDMX and MDM2. The group deployed common and commercially available components to carry out a time-resolved FRET (TR-FRET) assay that detects the interaction between MDMX and p53.[85] This high-throughput method enabled Graves et al. to screen nearly 1 million compounds for inhibitors of the MDMX–p53 interaction. The screen resulted in roughly 1500 hit compounds, with the most potent molecule (RO-2443) inhibiting the MDMX–p53 interaction with a 33 nM IC_{50}. The team assessed the compound's inhibitory mechanism using H^1–N^{15} HSQC NMR and determined MDMX/MDM2 as the protein target. Moreover, they observed broader and diminished HSQC peaks with the small molecule present, which implies that higher-order protein–compound species are formed. Graves et al. determined the compound likely induced a 2:2 protein:compound dimer by deploying static light scattering (SLS), modeling of binding kinetics, and isothermal calorimetry (ITC). To further

validate this hypothesis, they solved a crystal structure of RO-2443 bound to MDMX and observed that the molecule forms tight interactions not only with the MDMX, but also with itself to increase native MDMX dimerization, resulting in inhibition of p53 binding and classification of this molecule as a type II molecular glue. This early example provides a standard workflow for *in vitro* discovery and mechanistic validation of inhibitory molecular glues.

One of our key perspectives is that discovery of molecular glues can be relatively straightforward with proper screening methodology and a validated protein of interest. Tang et al. provided a perfect example of intentional molecular glue discovery in 2021 using high-throughput TR-FRET to identify molecular glues for mutant SMAD4 PPIs.[86] SMAD4 requires interaction with SMAD3 to function as a tumor suppression activity, and mutations in SMAD4 that eliminate this PPI have been observed in multiple cancers (Figure 2A). Tang et al. deployed a TR-FRET assay with >3000 bioactive compounds using lysates from cells transiently transfected with SMAD3/4 to discover molecular glues capable of restoring mutant SMAD4 PPIs (Figure 2B). Two hits restored mutant SMAD4 PPIs both in TR-FRET and coimmunoprecipitation assays with <20 μM EC_{50}s. The hits, Ro-31-8220 and Go-6983, were both PKCIIα inhibitors and shared a bisindolylmaleimide core. The authors tested several other known PKCIIα inhibitors with similar and different chemical cores and observed μM activity for only PKCα inhibitors with the bisindolylmaleimide core, thus ruling out the SMAD4/SMAD3 PPI as a PKCα cross-pathway target. Finally, the team demonstrated that Ro-31-8220 completely restores TGF-IIβ-based tumor suppression in mutant SMAD4 colon cancer cells, validating molecular glues as effective chemical tools to restore pathologically mutated pathways.

3.2.2. Fluorescence Polarization (FP).—In 2017, Kerres et al. carried out a high-throughput inhibition screen that not only discovered molecular glues but also inducers of protein oligomerization.[87] The team sought to inhibit interactions between oncogene BCL6 and its transcriptional corepressor. To this end, they measured small-molecule inhibition of the BCL6–BCL6-interacting corepressor (BCOR) PPI via fluorescence polarization (Figure 3A). They screened 1.7 million compounds against a BCL6/BCOR ratio of 120:1 (Figure 3B). The screen resulted in 202 hits that were down selected to one compound that was cocrystallized with BCL6 and had an 18 μM IC_{50}. When characterizing analogues of their hit compound, Kerres et al. noticed some analogues induced rapid and potent BCL6 degradation while others did not. Moreover, these degrading analogues induced protein precipitation when used in high concentration (>100 μM) methods such as protein crystallization or NMR. Hydrogen–deuterium exchange mass spectrometry (HDX-MS) shed light on the mechanism by identifying a BCL6 region that was protected when degrading analogues, but not nondegrading analogues, were assessed. The team hypothesized that degrading molecules induced BCL6 oligomerization, blocking interactions as a type I molecular glue and leading to ubiquitination and subsequent degradation in cells (Figure 3C). When treated with MG-132, a protease inhibitor, polyubiquitin chains were observed, lending credence to this hypothesis. This hypothesis remained probable, but unconfirmed until Stabicki et al. used electron microscopy to directly observe inhibitor dependent BCL6 protein filament formation in 2020.[88]

Overall, there are three points to emphasize from the BCL6 inhibition campaign. First, the initial FP assay used a relatively high concentration (1.2 μM) of the protein target BCL6 compared to the peptide partner's concentration (10 nM),increasing the probability of generating higher-ordered protein species. Second, assessing each hit compound's efficacy at high concentrations (>100 μM) and carefully recording observations resulted in low-cost mechanistic insights related to protein oligomerization and aggregation. Finally, HDX-MS is emerging as an effective tool to determine complex protein surface contacts in solution when protein solution NMR spectroscopy or X-ray crystallography is not practical.

3.3. *In Vitro* Label-Free IDMs.

The suite of chemical-, antibody-, and genetic-based methods available for labeling proteins, peptides, and small molecules is expanding exponentially. However, there are proteins or peptides that cannot be labeled *in vitro* due to factors such as inaccessible C- and N-termini or challenges with protein folding and stability. Label-free methods to detect protein interactions must be used for these proteins. We highlight three *in vitro* label-free IDMs that successfully discovered molecular glues in this section. However, it should be noted that surface plasmon resonance is another label-free method for detecting protein complex formation, speciation, and kinetics. The technique continues to be invaluable for understanding protein interactions and can be adapted to process high-throughput small-molecule libraries. Moreover, other methods, such as ITC, may emerge as effective label-free *in vitro* molecular glue detectors if throughput is increased greatly.[89]

3.3.1. Nuclear Magnetic Resonance (NMR) and Mass-Spectrometry (MS).—

The first example of a label-free IDM comes from Wendt et al., who used high-throughput NMR and MS in 2007 to detect compound binding to survivin.[92] This method is unique from those mentioned above in that mixtures of compounds were simultaneously incubated with the target protein. The NMR method screened 96 000 compounds by incubating 100 compound mixtures with ^{13}C-methyl labeled survivin, performing ^{1}H /^{13}C-HSQC, and looking for peak shifts that correlate with either the peptide binding site or known survivin homodimer. The mass spectrometry-based method screened 240 000 compounds by incubation, filtration, and washing so that only compounds bound to survivin are identified.

Excitingly, any survivin-binding small molecules discovered via NMR could immediately be classified as binding at either the peptide binding site or survivin dimer interface, providing the research team with mechanistic information early in the discovery process. This example provides a workflow for high-throughput NMR as a label-free molecular glue discovery method that generates large-scale and complex data sets amenable to novel machine learning techniques. Moreover, the initial screen can differentiate between compounds that bind at a PPI interface versus a single protein, allowing researchers to rapidly sort hits based on a desired mechanism of action such as PPI induction. NMR is not without its challenges. It requires high concentrations of stable and soluble protein and struggles to structurally deconvolute medium to large proteins (>35 kDa).[106] Mass spectroscopy on the other hand can screen thousands of molecules for protein binders, but not necessarily molecular glues.[107] However, initial MS hits could be followed up with HDX-MS to determine if hit compounds induce large surface area protection, which indicates PPI formation. Overall,

the work carried out by Wendt et al. lays out two effective label-free methods to discover molecular glues.

3.3.2. Oblique-Incidence Reflective-Difference (OI-RD) Microscopy.—

In 2019, Li et al. intentionally discovered mutant-selective molecular glues using high-throughput oblique-incidence reflective-difference (OI-RD) microscopy.[97] For methodological details on OI-RD microscopy, see reference 108. This technique is similar to SPR in that it is label-free and directly detects the presence of biomolecules on a surface. In this specific case, the research team sought to discover compounds that induce an interaction between pathological huntingtin protein aggregates and the autophagosomal protein LC3B to induce mutant huntingtin degradation. The team first incubated mutant huntingtin, wild-type huntingtin, or LC3B with microarrays spotted with over 3000 compounds. The protein-incubated microarrays were then screened via an OI-RD to identify binders.

Remarkably, the relatively small screen discovered numerous small-molecule binders for each protein target and even found two molecules, 10O5 and 8F20, that bound both mutant huntingtin and LC3B, but not wild-type huntingtin, with sub-μM affinity. The team then treated primary cortical neurons expressing both wild-type and mutant huntingtin with 10O5 and 8F20 and observed degradation of mutant, but not wild-type, huntingtin protein. Addition of autophagy inhibitors eliminated the molecular glue's degradation of huntingtin, confirming autophagy as the mechanism of degradation. Moreover, molecular glue addition increased LC3B mutant huntingtin coimmunoprecipitation and thus the physical interaction between the target proteins. The research from Li et al. provides a workflow for label-free molecular glue discovery that showcases LC3B as a molecular glue target to induce nonproteasomal degradation of protein oligomers or aggregates.

3.4. Computational and Crystallography IDMs.

A number of molecular glues were discovered via *in silico* screening. An *in silico* IDM is any method that computationally predicts small-molecule–protein binding to identify a molecular glue. Most of the experimental IDMs and methods discussed earlier found molecular glues serendipitously in detection of PPI inhibition. However, four *in silico* IDM-based campaigns set out specifically to discover molecular glues and were successful. It is important to note that availability of the PPI structure is currently required for all *in silico* IDMs.[109] Recent developments in the area of *in silico* protein design may provide new possibilities, which we elaborate on in the Discussion and Outlook section below. Additionally, recent advances in artificial intelligence, in particular machine learning, could be promising for future molecular glue discovery programs.[110,111]

3.4.1. Snapshot of In Silico Interaction Detection Methods (IDMs).

Methods: Docking, ligand-based virtual screening (LBVS), molecular dynamics.

Highlights: (1) Guarantees molecular glue mechanism discovered intentionally. (2) High screening capacities (10^9) molecules and secondary hit rates (1-10%). (3) Easily compatible with various machine learning approaches. (4) Efficacy scales with improving computational power and technology.

Challenges: (1) Small-molecule resolution PPI structural informaton is required. (2) $>10^2$ molecules need to be screened via experimental IDM for validation. (3) No FDA-approved molecular glues discovered to date.

3.4.2. Docking and Ligand-Based Virtual Screening (LBVS).—Tan and co-workers in 2012 employed *in silico* technologies with the combination of both structure-based docking and ligand-based virtual screening (LBVS) to identify compounds inhibiting proliferating cell nuclear antigen (PCNA) activity through stabilization of the known trimmers of PCNA.[94] A trimer structure model enabled docking of a two hundred compound library using the Glide and Molegro software, leading to two hits with sub-μM IC_{50}s. Tan et al. performed a ligand-similarity search of the commercial database ZINC, which is often used for LBVS, to find eight more hit compounds with similar or lower potency.[112] Further experiments confirmed that these molecules carried out their effects by directly stabilizing endogenous PCNA trimers and thus inhibiting the monomer units from carrying out necessary cell cycle functions.

This example by Tan et al. provides two key insights into computational screening of molecular glues. First, even though docking managed to select the most potent compounds, LBVS revealed structure–activity relationships that identified which moieties are responsible for protein binding and stabilization. These insights are of paramount importance when improving a hit molecule's pharmacokinetics and dynamics without negatively impacting activity. Second, virtual screening drastically lowers the number of compounds tested experimentally and therefore allows researchers to rapidly advance to low-throughput disease related assays, such as cell growth inhibition.

Nemetski et al. performed docking in 2015 to discover a PPI stabilizer and confirmed their *in silico* finding by crystallizing the compound in complex with both protein partners.[43] In their work, the authors sought to stabilize *Plasmodium falciparum* aldolase with thrombospondin-related anonymous protein (TRAP) to lock these necessarily dynamic proteins in a single conformation and thus prevent malaria infection. Unlike in the previous example, the authors had to locate a possible binding pocket from their crystal structure using the PocketFinder tool from the docking software ICM. Next, the team virtually screened a library of 315 102 molecules from Chembridge Corporation against not only *P. falciparum* aldolase–TRAP interactions but also against the *P. berghei*–TRAP6 interaction to increase the number of hits. Sixty compounds were selected for further biological evaluation, and, depending on the method, at least 13 compounds displayed some activity, suggesting they stabilized the PfAldolase–TRAP interaction. A crystal structure of the compound **24**, PfAldolase, and TRAP complex confirmed this molecule bound directly at the PPI interface.

This second example highlights the application of binding 856 pocket identification software as an effective tool to assist in docking screens. Also, the work by Nemetski et al. is an example of molecular glues freezing dynamic protein cycling to inhibit a biological process.

Recently, Lin and co-workers used docking to stabilize a non-native PPI to target the Middle East respiratory syndrome coronavirus (MERS-CoV), implying a similar methodology could discover compounds against SARS-CoV-2, the agent responsible for COVID-19.[104] First, the team crystallized the N-terminal domain of the MERS-CoV nucleocapsid protein (N-NTD) and determined its structure. Intriguingly, previous crystal structures of N-NTD produced monomeric proteins, but their protein crystals were composed of N-NTD dimers. Two nonendogenous N-terminal amino acids from protein expression facilitated this dimerization, pointing to a cryptic and previously unknown dimerization hot spot (Figure 4). Lin et al. docked a set of three small-molecule databases (Sigma-Aldrich, Acros Organics, and ZINC) to this hot spot using LIBDOCK software. After multiple rounds of docking and filtering, they found three compounds to select for further study. One of the compounds showed μM activity against Vero E6 cells infected with MERS-CoV, and inhibited MERS-CoV by promoting oligomerization of nucleocapsid proteins within cells (Figure 4). The best hit, P3, was crystallized with full length nucleocapsid protein, revealing that the compound induces eight-membered nucleocapsid ring formation that completely inhibits the protein's active interface. This work proves that inhibiting protein activity by inducing an unnatural PPI is effective. Moreover, this demonstrates that searching for simple PPI dimerizers can inadvertently result in molecules that produce higher-ordered protein oligomers.

3.4.3. X-Ray Crystallography and Molecular Dynamics (MD).—Finally, Zhang and co-workers in 2012 combined X-ray crystallography, docking molecular dynamics (MD), and mutagenesis to establish previously identified potassium channel potentiators as molecular glues.[113] In their research, the authors cocrystallized calmodulin (CaM) and the CaM-binding domain (CaMBD) of a calcium-activated potassium channel (SK) with compounds from the 1-EBIO family, which are known to nonselectively potentiate SK. Successful crystallization and structure determination of the PPI revealed one of the additives used for crystallization, phenylurea, bound at the interface between the two proteins, and was later confirmed as a weak channel modulator like 1-EBIO. Zhang et al. applied docking and MD to approximate insoluble compound binding where no crystal structure could be obtained. Overall, all compounds were found to bind in the same pocket as phenylurea at the PPI interface, and mutagenesis studies confirmed that this binding pocket is functionally relevant to molecule binding and protein activity. The experiments used in this paper (crystallography, MD, and electrophysiology) are essentially low-throughput small-molecule fragment screens and so have limited application as an initial screen. However, this method suggests that analyzing data from previously solved crystal structures could reveal small-molecule additives at the interface of proteins that could be starting fragments for a novel molecular glue.

3.5. IDMs Conclusions.

A wide range of IDMs with various advantages and disadvantages have been deployed to discover molecular glues for diverse proteins of interest (Table 4). Despite this plethora of approaches, IDMs have yet to produce a clinically approved molecular glue. We contend that this is not due to a fundamental flaw with IDMs, but rather results from a general lack of intentional discovery campaigns for molecular glues prior to the 21st century. Recent efforts have proved that identifying effective molecular glue hits for diverse purposes and protein targets is straightforward and rapid with contemporary technologies and validation assays.[86,97,104] Our intention is to expand the focus of the ongoing molecular glue renaissance from simple protein degradation to include inhibition and activation of protein activity. In concluding our review of IDMs, we discuss methodological changes that increase the likelihood of molecular glue discovery.

Molecular glues definitionally require multiprotein complexes to alter cellular activity. This key aspect results in molecular glue activity being highly dependent on the protein concentration. Nearly all methods discussed herein used protein concentrations near or above 1 μM. For example, when Kerres et al. performed a fluorescence polarization assay between BCL6 and BCOR, they added 120-fold more BCL6 protein (1.2 μM vs 10 nM), resulting in hits that almost exclusively interacted with BCL6.[87] Moreover, their assay discovered molecules that not only dimerize, but also oligomerize BCL6, which induced BCL6 degradation in cells. Using high protein concentrations (>1 μM) will exponentially increase the chance of finding molecular glues, including glues to dimerize a target for inhibition, in a small-molecule screen.

When screening a large library (>10 000 compounds) for inhibitors of a given PPI, it can be difficult and expensive to determine which of the hundreds of hit molecules inhibit the PPI via a molecular glue mechanism versus a traditional single-protein binding mechanism. There are abundant technologies available for accurate deconvolution (SPR, ITC, DLS, FRET, FP, etc.), but the fastest and most cost-effective way is to model the molecules' dose–response curves. For example, Graves et al. performed a 12-point dose–response curve and modeling to accurately distinguish between a 1:1 versus 2:2 small-molecule:protein interaction. This subtle difference in protein binding mechanism, which could not be distinguished by ITC, demonstrates the efficacy of modeling and fitting protein–small-molecule binding interaction curves.[42] Gathering mechanistic hints from dose–response curves was also utilized in the ABM case study carried out by Gerritz et al.[61] Finally, this analysis is nearly cost-free, given that dose–response curves are necessary when validating hit compounds.

Another challenge facing the drug discovery industry is the vastness of chemical space, with over 230 million purchasable compounds available in the ZINC database and 166 billion compounds available for *in silico* screening from GDB17.[114-116] Computational methods narrow the chemical space to a subset of likely binders that can be fully surveyed by moderate- to high-throughput IDMs. Among *in silico* techniques, docking remains the method of choice. The docking software Glide is known for its excellent performance and accuracy and has had the most success in discovering molecular glues.[117-120] Docking of molecular glues is ideal when a crystal structure of the PPI complex is available, is very

difficult when only individual structures are known, and is impossible when protein structure information is absent. New tools to predict protein structures, PPI binding interfaces, and small-molecule binding pockets are emerging to overcome this challenge but have yet to be used for molecular glue discovery.[121] The outlook and potential for these technologies are discussed more below.

4. DISCUSSION AND OUTLOOK

A major lesson we learned in gathering and analyzing the various methods covered in this Perspective is that a wide variety of common and high-throughput approaches can succeed in discovering molecular glues. However, we took note of several areas where concerted efforts may be particularly fruitful in rapidly identifying molecular glues at a relatively low cost. These include increasing molecular docking efforts and analyzing published crystal structure data for additives at protein and unit cell interfaces. We additionally found that kinetic modeling of dose–response curves is nearly cost-free and effective at uncovering molecular glue hits that may otherwise have been abandoned. These hits could then be optimized via standard medicinal chemistry pipelines to generate a mechanistically novel class of molecules that may be more effective *in cellulo* or *in vivo.* We also identified long-term data gathering efforts that could serve as a foundation to accelerate future molecular glue screening efforts. For example, generating a subset of validated “shield proteins” would provide researchers with a promising PPI goal. Moreover, pairing large-scale data sets with computational approaches like machine learning has the potential to further accelerate efforts. Below, we describe these latter two future directions in more detail to spark broader discussion surrounding nondegrading molecular glues within the drug discovery community.

4.1. Type I Inhibitors.

The type I mechanism relies on inducing interactions between a target protein and a bystander “shield” protein. The exciting outlook for type I discoveries is identifying a versatile “shield” protein that can be used in any high-throughput screen and against diverse target proteins (cytosolic, nuclear, membrane bound, etc.) (Figure 5). By focusing on a shortlist of “shield” proteins, researchers can compare their insights to build a knowledgebase that leads to accelerated and effective molecular glue discovery. Some specific qualities of this versatile shield protein are uniform and abundant expression within many cellular compartments and tissues, low to no negative impact on homeostasis when sequestered to an unnatural PPI, and multiple previously determined crystal and NMR structures. Two proteins fit this description and have already inhibited proteins of interest via molecular glues: ubiquitin and LC3B.

Ubiquitin and its many homologues are small proteins that are covalently linked to proteins, triggering their proteasomal degradation. Truly as ubiquitous as its name implies, ubiquitin is expressed at an appreciable concentration in every subcellular compartment, cell line, and tissue in all eukaryotes on earth. This single fact would be enough to explore ubiquitin as a versatile shield protein, but ubiquitin shines in other respects. Its structure has been solved hundreds of times by both X-ray crystallography and NMR spectroscopy, even at subangstrom resolution. Moreover, induced ubiquitination of target proteins to trigger

degradation via small molecules, such as lenalidomide and PROTACs, is a clinically validated mechanism to alleviate disease, thus, confirming minimal off-target effects when ubiquitin is sequestered from its natural interactions in homeostasis.

LC3B and its homologues are similar to ubiquitin in several ways. LC3B is conserved among all eukaryotes, is expressed in the cytoplasm and nucleus of all cell lines and tissues, is directly involved in a form of protein degradation (macro-autophagy), has had its structure solved by X-ray crystallography and NMR spectroscopy, and is amenable to *de novo* PPI formation with molecular glues. Moreover, the associations of LC3B with protein aggregates induces their degradation via autophagy.[97] This added ability increases the applications of LC3B shielding to degradation of protein macrostructures, including aggregates and oligomers. We therefore believe IDM campaigns to identify type I molecular glues are more likely to have clinical success if they induce the interaction between ubiquitin or LC3B and the pathological target protein. Although ubiquitin and LC3B are compatible with any IDM given their small size, solubility, and available structures, only certain methods quickly produce large enough data sets to fuel machine learning for accelerated future discovery. For this reason, we recommend docking and LBVS followed by TR-FRET and cocrystallization or simply screening using C-13 protein NMR, which produces structural data sets ideal for training a machine learning algorithm to predict molecules that not only interact with either LC3B or ubiquitin but interact at a structural site more likely to result in a successful molecular glue.

Inducing the proximity of ubiquitin may induce protein degradation, given its endogenous role in the cell. There are cases in which degrading a target protein, even to a small degree, could lead to negative impacts on homeostasis, and so, inducing a ubiquitin PPI may not be fruitful. In these cases, there is always one shield protein that is by default colocalized with the target protein and will not have off-target effects, the target protein itself. Molecular glues that induce non-native homodimerization or oligomerization have a large impact on protein activity. An excellent showcase of this strategy is the campaign carried out by Kerres et al. to discover inhibitors of BCL6.[87] The molecules they identified ranged from stabilizing inactive BCL6 homodimers to inducing oligomers that lead to degradation. Similarly, Lin et al. set out to inhibit protein activity via unnatural homodimerization and were ultimately successful.[104] These two examples also demonstrate that both experimental and computational tools produce type I molecular glues. However, choosing a specific method will depend on the target protein's properties, such as crystal structure availability, amenability to chemical or affinity labels, and *in vitro* expression, purification, and solubility. The two most straightforward, cost-effective, versatile, and promising approaches we would recommend are docking using the Glide software or, if a crystal structure is not available, TR-FRET *in vitro* either with purified proteins or even with cell lysates as demonstrated by Tang et al.[86] It should be noted that FP is not recommended to detect homodimerization of proteins because it relies on changes in relative size of 5- to 10-fold or greater to produce an excellent signal-to-noise. FP may be applicable in cases where oligomerizers of small proteins (~10 kDa) are sought because the oligomer size would be significantly larger relative to the small monomer, resulting in a high FP signal. Overall, type I molecular glues such as rapamycin and tacrolimus have been excellent in the clinic, inspiring us to recommend more discovery efforts to produce type I molecular glues.

4.2. Machine Learning to Discover Molecular Glues.

A key takeaway from our analysis is the success of molecular docking, which has been proven to rapidly discover molecular glues. Another computational technology that can be employed is machine learning, which to date has accurately predicted protein folding, protein–protein interaction interfaces, and druggable binding pockets. For example, the Alphafold program by DeepMind, which depended on data from the publicly funded and managed Protein Data Bank database, accurately predicts an individual protein structure from its amino acid sequence.[122] To assist in molecular glue discovery, Alphafold could be combined with protein–protein binding interface prediction tools to produce a virtual model of a PPI. To discover type I and type III molecular glues, the PPI interface prediction tool can be modified to identify the highest affinity interface between two proteins that do not currently interact.[121,123] Next, a small-molecule pocket-finding tool can predict the most tractable site for molecular glue binding at the virtually constructed PPI interface.[124] Finally, docking small-molecule libraries to these pockets will provide hundreds of molecular glue hits that can be screened with IDMs for activity. In sum, this approach would expand computational molecular glue discovery toward not only proteins lacking structural information but also non-native PPIs that are necessary for type I and type III molecular glues.

Machine learning requires not only thousands of positive data but also diverse examples and negative data to produce accurate predictions. The number of publicly available protein structures with molecular glues is in the low hundreds. However, most of these structures are molecular glues with human E3 ligases, reducing the prediction accuracy for non-E3 ligase PPIs. Furthermore, only molecular glues with activity in biological assays are reported. Inactive compound structures represent a negative data set gold mine for machine learning but are almost always unpublished. Although machine learning has yet to discover molecular glues, pooling both published and unpublished data from academia and industry would serve as a springboard for future machine learning efforts.

We propose that building, curating, and maintaining a molecular glue database for the 21st century are the most effective ways to facilitate machine learning-based molecular glue discovery (Figure 6). This would entail gathering and curating known PPI structures with and without bound molecular glues as well as small-molecule structures that are validated as molecular glues by IDMs. Another challenge when developing an accurate machine learning model is the lack of high-quality data sets with negative results. The database, therefore, should also include a negative data set of small-molecule structures that had no impact on PPI affinity. Finally, and perhaps most importantly, ensuring the database is free and publicly available would allow machine learning models to be accurately compared to identify the most effective approaches.[125,126]

LBVS is a computational method capable of using only a small-molecule structure to identify chemical motifs and physical shapes with a probable activity, e.g., PPI induction. LBVS works by first digitizing small-molecule structural information into simplified molecular input line entry systems (SMILES) or 3D shapes. In a typical screen, SMILES and 3D shapes from purchasable libraries are screened virtually using docking and then physically in IDMs to identify hits.[127] Next, compounds with the highest similarity to hits

are identified, purchased, or synthesized and then screened to rapidly find active analogues. The pool of analogues generated by LBVS increases the chance of finding not only more potent molecules but also molecules with other beneficial properties, such as increased oral bioavailability or selectivity, that are necessary in drug development.

As mentioned before, thousands of positive and negative data points are needed to apply machine learning. Millions of compounds have been screened in IDMs to find molecular glues, resulting in thousands of validated hit compounds with diverse structures. If this wealth of data is made publicly available, then machine learning and LBVS could be combined to accurately predict a subset of chemical space with an increased likelihood of molecular glue activity. A large (>100 000) small-molecule library can then be compiled through a trained machine learning model to both cover molecular glue chemical space and increase the diversity and drug-like properties of the library. This specialized library could then be screened against innumerable validated molecular glue targets. We predict this molecular glue-focused library will not only discover novel molecular glues but also reveal the chemical principles dictating which scaffold and motifs lead to potent molecular glues.[114]

4.3. Summary.

Small-molecule molecular glues represent an enticing therapeutic strategy with over 15 drugs FDA-approved for hundreds of clinical applications to treat millions of patients. Their success and potential have led to concerted molecular glue discovery campaigns in the past decade to impact clinically relevant proteins. In this Perspective, we reviewed the most effective methods and approaches to date and propose two promising scientific focus areas with high potential to rapidly develop clinically relevant molecular glues. We also emphasized specific experimental factors that can be incorporated into molecular glue discovery methods to improve hit rates, target specific proteins, and lead to desired mechanisms of action. We hope that this compendium will inspire and embolden research teams to discover molecular glues that modulate clinically relevant protein activities and uncover new mechanisms to alter cellular processes more broadly.

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Biographies

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He is now a postdoctoral researcher at Argonne National Laboratory in the group of Dr. Antonopoulos, where he studies metabolic interactions in microbial cocultures and environmental factors influencing CRISPR Cas9 kill switch efficacy.

Clemence Delalande received both a B.S. and M.S. in Pharmacy and Medicinal Chemistry from Paris Descartes University, France. She went on to receive her Ph.D. in Chemistry from the University of Bern, Switzerland, before becoming a postdoctoral fellow in the Chemistry Department at the University of Chicago. Dr. Delalande's expertise lies in medicinal chemistry, computational chemistry, biochemistry, and pharmaceutical sciences. She identified a new potent and selective tool compound against the cation channel TRPM4 via ligand-based virtual screening, chemical synthesis, and computational technologies developed in the group of Prof. Jean-Louis Reymond. She also developed covalent inhibitors of protein S-acylation through rational design.

Saara-Anne Azizi received her B.A. in Biological Chemistry and Classical Languages & Literature from Dartmouth College and her M.Phil. in Chemistry from the University of Cambridge, before landing at the University of Chicago Pritzker School of Medicine via the Medical Scientist Training Program (MSTP). After earning her Ph.D. in Chemistry, Dr. Azizi is now finishing her third year in medical school.

Vivian Lu received her B.S. in Chemistry and Biochemistry from the University of Chicago before joining the Broad Institute of MIT and Harvard as a research associate within the Center for Development for Therapeutics. As a member of the structural biology team, she supports multiple early drug discovery projects through the generation of protein crystal and cocrystal structures.

Dionysios Antonopoulos is Division Director for Biosciences at Argonne National Laboratory (USA). He received his PhD from the University of Illinois at Urbana–Champaign. Prior to joining Argonne, he was a USDA postdoctoral fellow at Michigan State University and NIH research fellow in the Division of Infectious Diseases at the University of Michigan. As a microbiologist he has focused on a mechanistic understanding of the factors that shape and stabilize microbial community function within a variety of environmental systems, from mammalian gut microbiomes to soil and subsurface ones.

Gyorgy Babnigg earned his Ph.D. in Molecular Physiology and Pharmacology from the University of Chicago. He then completed postdoctoral training at the University of Chicago and Argonne National Laboratory. Currently, he is a bioinformatician/molecular biologist in the Biosciences Division at Argonne National Laboratory. Dr. Babnigg is interested in studies focused on the structure–function relationship of proteins and protein–protein complexes using structural bioinformatics. He has helped to develop methods for the heterologous expression of protein–protein complexes for x-ray crystallography. He has also successfully applied droplet-based microfluidics to fixed-target serial crystallography, drastically lowering the protein requirement for crystallization of proteins and protein–protein complexes. In addition, Dr. Babnigg has built several database systems and web and desktop applications to support large multi-institution efforts.

ABBREVIATIONS USED

ABM	activity-based method
AML	acute myeloid leukemia
APC	allophycocyanin
BCOR	BCL6-interacting corepressor
CaM	calmodulin
CaMBD	CaM-binding domain
CRBN	cereblon
DMARD	disease-modifying antirheumatic drug
ELISA	enzyme-linked immunosorbent assay
FP	fluorescence polarization
FRET	Förster resonance energy transfer
HDX-MS	hydrogen–deuterium exchange mass spectrometry
hCdc34	human Cdc34
IDM	interaction detection methods
IMID	immunomodulatory drugs
ITC	isothermal calorimetry
LBVS	ligand-based virtual screening
MD	molecular dynamics
MERS-CoV	Middle East respiratory syndrome coronavirus
MS	mass-spectrometry
mTOR	mammalian target of rapamycin
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFAT	nuclear factor of activated T-cells
NMR	nuclear magnetic resonance
N-NTD	nucleocapsid N-terminal domain
NP	nucleoprotein 1

OI-RD	oblique-incidence reflective-difference
PCNA	proliferating cell nuclear antigen
PPI	protein–protein interaction
PROTACs	proteolysis targeting chimeras
SLS	static light scattering
SMILES	simplified molecular input line entry systems
SPR	surface plasmon resonance
TRAP	thrombospondin-related anonymous protein
TR-FRET	time-resolved Förster resonance energy transfer
TTR	transthyretin
UBE2M	ubiquitin conjugating enzyme E2M

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SIGNIFICANCE

Many molecular machines or signaling cascades rely on protein-protein interactions with differing half-life and affinity domains. The interactions of these biomolecules can be maneuvered to redirect signaling, reprogram the cell cycle, or decrease infectivity using only dozens of atoms. Such “molecular glues,” which can drive both novel and known interactions between protein partners, represent an enticing therapeutic strategy. Here, we review the techniques that have led to the identification of small molecule molecular glues.

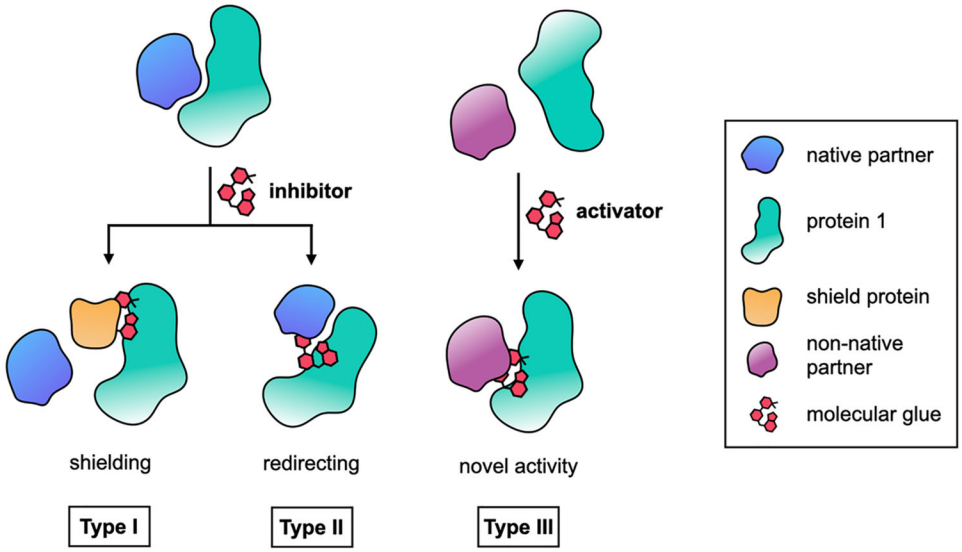

Figure 1.

Cartoon depicting a molecular glue classification system based on its mechanism of action. Type I molecules (left) induce a non-native protein protein interaction (PPI) to “shield” a protein of interest from carrying out its function. Type II molecular glues (middle) inhibit a protein’s function by redirecting an endogenously formed PPI. Type III molecular glues (right) induce a non-native PPI to produce a novel activity (ubiquitination, transcription, etc.).

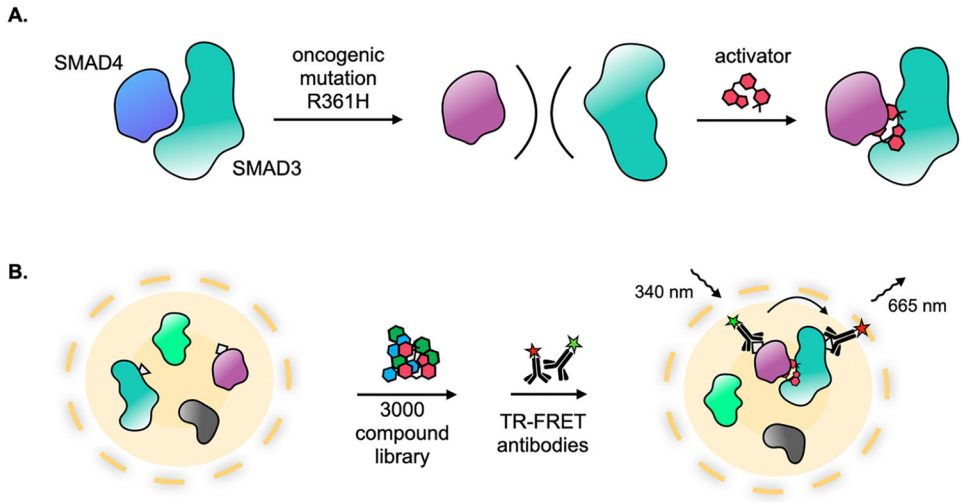

Figure 2.

Type III molecular glue discovery enabled by time-resolved-FRET (TR-FRET). (A) The oncogenic SMAD4 mutation R361H eliminates its interaction with SMAD3, so Tang et al. sought identify molecular glues that reactivate mutant SMAD4's interaction with SMAD3. (B) Cells overexpressing epitope-tagged SMAD3 and SMAD4 were lysed and then subjected to screening for molecular glues using an antibody-based TR-FRET approach.

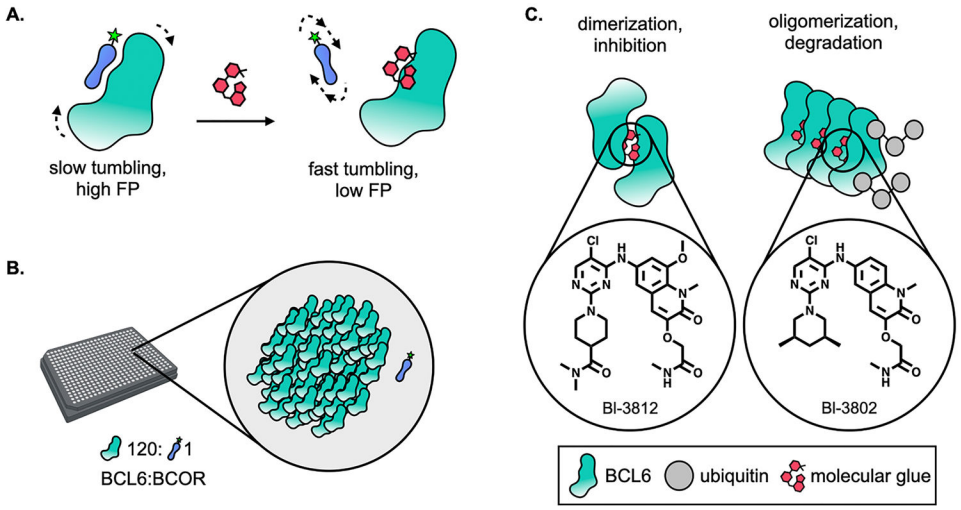

Figure 3.

Molecular glue discovery via fluorescence polarization (FP). (A) Protein binding decreases tumbling velocity of a fluorophore tagged biomolecule, increasing the FP signal. (B) The high BCL6 protein concentration relative to the BCOR peptide increased the chance of discovering a BCL6 molecular glue because molecular glue homodimerizers scale exponentially with protein concentration. (C) Relatively small changes in molecular glue chemical structure resulted in protein oligomerization versus dimerization.

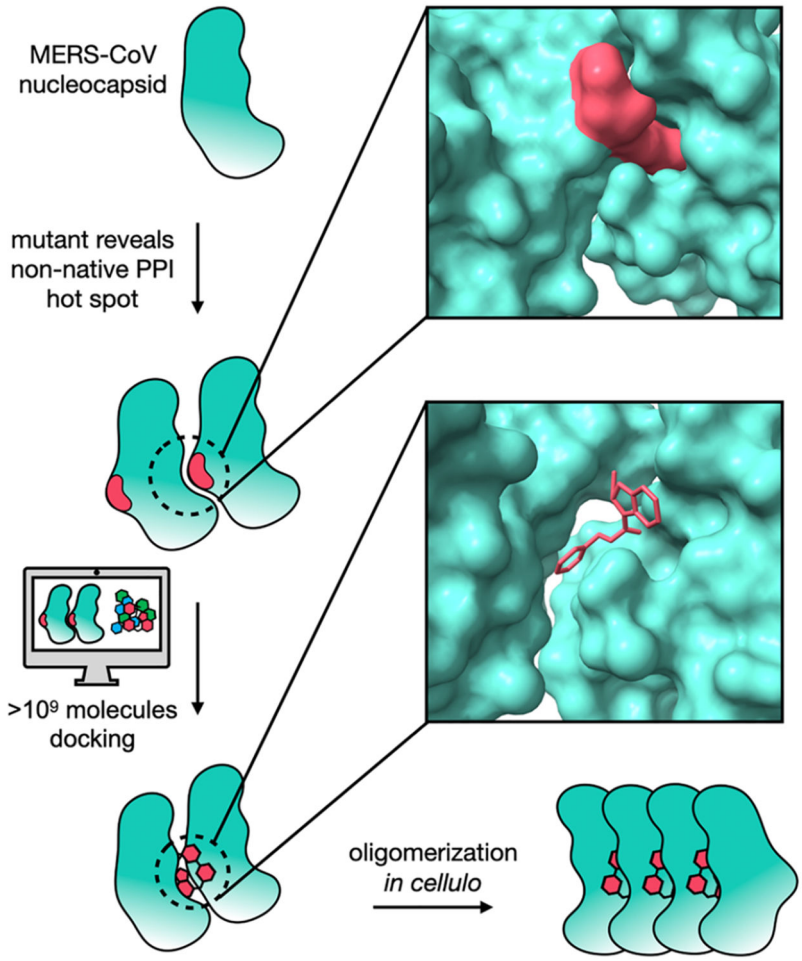

Figure 4.

Computationally identified molecular glue inhibits MERS-CoV replication. A mutant of the MERS-CoV nucleocapsid resulted in low-affinity dimerization and enabled protein crystallization. Docking at the dimerization site identified a molecular glue that oligomerized nucleocapsid proteins *in cellulo* resulting in MERS-CoV inhibition (PDB 6KL2 and 6KL5).

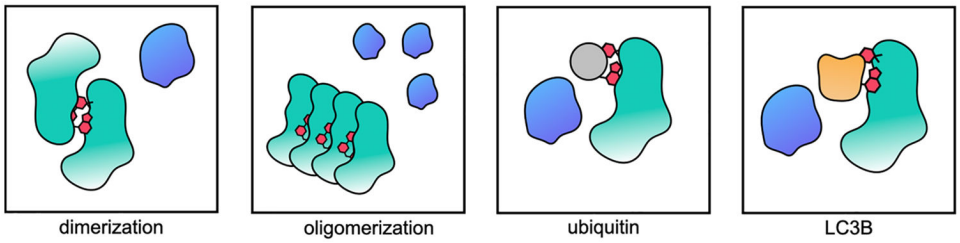

Figure 5.

Recommended future approaches to discovering type I molecular glues. Triggering homodimerization or oligomerization with molecular glues can effectively inhibit or even degrade a target protein. Ubiquitin and LC3B are abundant and evolutionarily conserved proteins that are already validated as effective molecular glue targets to inhibit protein activity. Moreover, these proteins can be used in nearly any method described herein including NMR and X-ray crystallography.

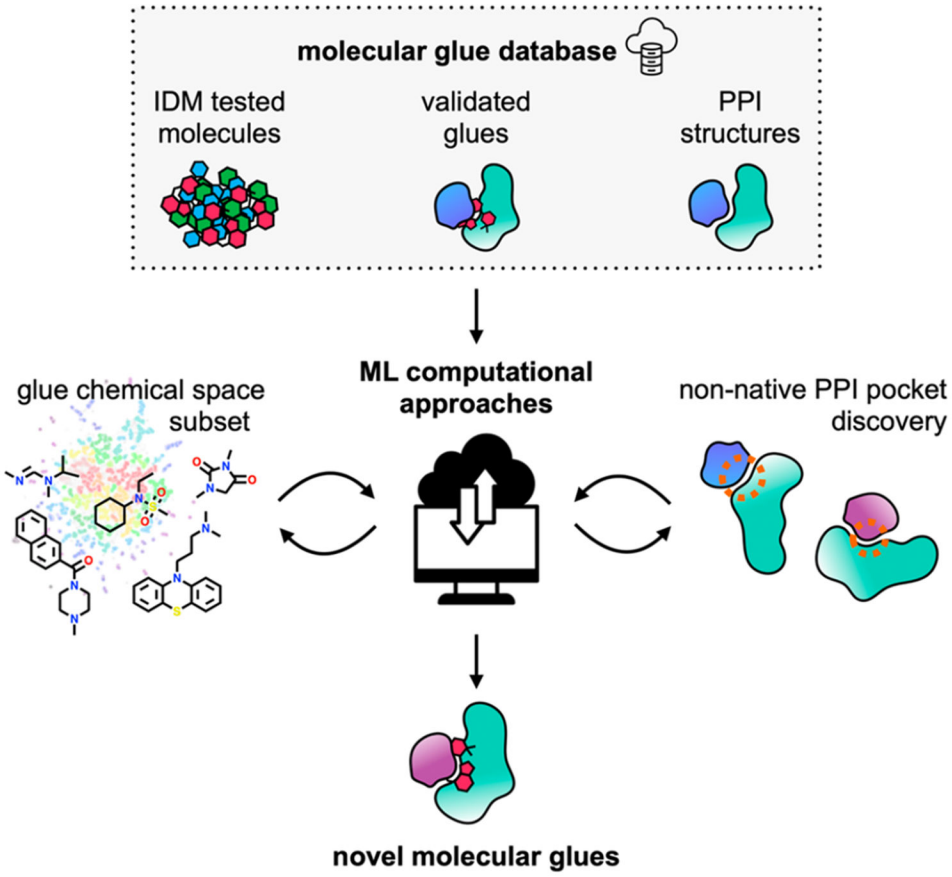

Figure 6.

A public database drives machine learning (ML) based molecular glue discovery. First, a publicly available molecular glue database (top) should be assembled that consists of interaction detection method (IDM) screening results, validated molecular glues and their target PPIs, and all PPI structures. Next, the database can fuel ML approaches to predict non-native PPI small-molecule binding pockets and a subset of chemical structures that are more likely to bind PPI interfaces (middle). Together, these combined advances will generate novel molecular glues (bottom) and discover chemical and biophysical principles that underlie molecular glue activity.

Table 1.

Current List of FDA-Approved Molecular Glues, Their Mechanism, and Impact

International name	FDA brand name	PPI	MOA	Analogs	Annual revenue	Year FDA approved	Refs
Paclitaxel	Taxol	ß-Tubulin	Type II	Docetaxel	$2.5 billion (2001)	1992	10, 11
Thalidomide	Contergan, Thalomid	CRBN/IKAROS-family	Type II/III	Immunomodulatory drugs (IMIDs)	$25 million (2018)	1998	12, 13
Rapamycin	Rapamune, Sirolimus	FRB (mTOR1)-FKBP	Type I	Numerous "Rapalogs"	$276 million	1999	14-16
Cyclosporine A	Neoral, Sandimmune, and Gengraf	CyP-CaN	Type I	Voclosporin	$2.0 billion	2000	17
Tacrolimus (FK506)	Prograf, Advagraf, Protopic, others	FKBP-CaN	Type I	Pimecrolimus	$6.4 billion	2005	18, 19
Lenalidomide	Revlimid	CRBN/CK1α	Type II	IMIDs	$12.8 billion	2005	12, 20
Dexrazoxane	Totect, Zinecard, Cardioxane	TopIIα/β	Type II	N/A	$11-12 million	2007	21, 22
Pomalidomide	Pomalyst	CRBN/IKAROS-family	Type II/III	IMIDs	$3.3 billion	2013	12
Tafamidis	Vyndaqel and Vyndamax	Transthyretin tetramer	Type II	N/A	$2.0 billion	EU 2011, US 2019	23, 24

Table 2.

Activity-Based Methods (ABMs) That Discovered Novel Molecular Glues

Method	PPI	MOA	Throughput	Hit rate	Molecule	Initial potency	Year	Refs
Mouse studies and SAR	CRBN-MEIS2 and CRBN-IKAROS	Type II/III	NA	NA	IMiDs	0.157 μM	1953-2014	12, 13, 66
Cell killing	Topoisomerase II dimer	Type II	NA	NA	Dexrazoxane	12 μM	1972	55
Patch clamp	CaM/CaMBD	Type III	NA	NA	1-EBIO	490 μM	1996	48, 67
Cell killing	UBR7-TP53	Type III	NA	NA	Asukamycin & manumycin A	10 μM	1998-2020	68, 69
Structure activity relationship (SAR)	RBM39-DCAF15	Type III	28	3.57%	Indisulam (E7070)	28.5 μM	1999-2019	70, 71
SAR	SMRT-PPARα	Type III	NA	NA	GW6471	0.24 μM	2002	49
***In vitro* aggregation assay**	transthyretin	Type II	28	3.57%	Tafamidis	0.055 μM	2003-2012	72, 73
Fluorescent biosensor assay	S100A4	Type II	400	3%	Perphenazine	41 μM	2008-2010	74, 75
Actin polymerization assay	Arp2-Arp3	Type II	400,000	<0.01%	CK-0944636 (CK636)	32 μM	2009	76
Viral replication assay	influenza nucleoproteins	Type **I**	1,200,000	<0.01%	Compounds 1-5	0.06 μM	2011	61
***In vitro* enzyme complex reconstitution**	hCdc34-U2	Type II	NR	NR	CC0651	1.72 μM	2011	77
Cell killing	B56a-PP2A	Type II	124	0.80%	DT-061/RTC-5 (SMAP)	12.6 μM	2015-2020	63, 65
SAR	BRD4	Type II	NA	NA	BiBET	100 pM	2016	78
Chemogenomics	PDE3A-SLFN12	Type II	NA	NA	DNMDP	0.3 μM	2016	79
Mutant selective cell killing	CDK12/cyclinK-DCAF15	Type II/III	2000	0.20%	dCeMM 1-4	0.4 μM	2020	80
Transcription factor luciferase assay	CDK12-DDB1	Type II/III	65,790	1.52%	HQ461	1.3 μM	2020	81

Table 3.

Interaction Detection Methods (iDMs) That Discovered Novel Molecular Glues

Method	PPI	MOA	Throughput (molecules)	Hit rate	Molecule	Initial potency (IC50)	Year	Ref
Mass spectrometry (MS)	CD40L trimer	Type II	NR	NR	BIO8898	25 μM	2003	[90]
Docking (Glide)	SOD1 dimer	Type II	1,500,000 docked, 100 tested	15%	Compound 2	-	2005	[41]
Enzyme-linked immunosorbant assay (ELISA)	TNF-Alpha dimer	Type II	NR	NR	SPD00000304	22 μM	2005	[91]
NMR, MS	Survivin dimer	Type II	96,000 NMR, 250,000 MS	0.1-0.35	Compound 8	5 μM	2007	[92]
Surface binding fluorescence	14-3-3/PMA2	Type III	37000	<0.01%	Epibestain	1.8 μM	2010	[93]
Crystallization	CaM-SK2-b	Type III	NR	NR	Phenyl Urea	1.6 mM	2012	[48]
Docking (Glide)	PCNA trimer	Type II	300000 docked, 200 tested	2%	PCNA-11	0.1 μM	2012	[94]
TR-FRET	MDMX dimer	Type II	996,864	0.15%	RO-2443	0.033 μM	2012	[42]
Docking (Molsoft)	TRAP-PfAldolase	Type II	315,102 docked, 30 tested	3.3%	Compound 24	NR	2015	[43]
Homogenous time-resolved fluorescence (HTRF) assay	PD-1 dimer	Type **I**	>290	NR	BMS-202	NR	2015	[95]
Docking (Glide)	MT-Hec1	Type II	350,000	6.7%	SM15	20 μM	2017	[96]
Fluorescence polarization (FP)	Bcl6 dimer/oligomer	Type **I**	1,700,000	0.013%	Pyrimidine-R4	20 μM	2017	[87, 88]
Oblique-incidence reflective difference (OI-RD) microscopy	Hungingin(m)-LC3B	Type III	3375	0.059%	10O5 and 8F20	0.2 μM	2019	[97]
Surface plasmon resonance (SPR)	TNF-alpha trimer	Type II	2000	0.1%	UCB-9260	22 μM	2019	[98]
FP	B-Catenin-B-TrCP	Type III	350,000	<0.01%	NRX-1532	206 μM	2019	[99]
Covalent Fragment MS	14-3-3-ERα	Type II	1,600	0.5%	Compound 1	100 μM	2019	[100]
Docking (Glide)	14-3-3-ChREBP	Type II	633 docked, 200 tested	6.5%	Compound 1	0.3 μM	2020	[101]
Covalent Fragment X-ray Crystallography	14-3-3-NF-κB	Type II	800	0.375%	TCF521	ND	2020	[102]
Fragment X-ray Crystallography	14-3-3-TAZ/p53	Type II	500	0.4%	AZ-003	ND	2020	[103]
Docking (Libdock)	N-NTD oligomer	Type **I**	230,000,000 docked, 3 tested	NR	5-benzyloxygr amine	32 μM	2020	[104]
Time resolved-FRET (TR-FRET)	SMAD4-SMAD3	Type III	2036	0.39%	Ro-31-8220	3.9 μM	2021	[86]
FP	14-3-3-CFTR	Type II	5760	0.122%	CY007424	31 μM	2022	[105]

Table 4.

Advantages and Disadvantages of Interaction Detection Methods (iDMs)

Method	Advantages	Disadvantages
Mass spectrometry (MS)	No protein labeling required, commonly available reagents/equipment	protein binding only, pooled sample testing for throughput
Docking (Glide)	Massively high-throughput, high postdocking hit rate, very low cost, improves with availability of public data	Crystal structures required, followup IDM required postdocking
Enzyme-linked immunosorbant assay (ELISA)	Protein labeling may not be required, very high-throughput	expensive reagents at high concentrations, less validated
Nuclear magnetic resonance (NMR) and MS	Massive amount of structural data from initial screen, high-throughput	Very expensive, requires high protein concentration and solubility, pooled sample testing for throughput
Surface binding fluorescence	High-throughput, low cost	false positives due to small molecule fluorescence, requires EGFP labeling of protein
Crystallization	Publically available dataset (PDB) has yet to be screened	Requires high protein solubility, expression, and propensity to crystallize
Time resolved-FRET	Very high-throughput, high signal to noise, low cost, low false positives	Requires labeling of both proteins
Fluorescence polarization (FP)	high signal to noise, very high-throughput, low cost	requires large size difference between proteins (5+ fold), requires protein labeling
Oblique-incidence reflective difference (OI-RD) microscopy	no protein lable required, high-throughput	protein binding only, unique microscopy setup required
Surface plasmon resonance	Simultaneous on/off kinetics, binding affinity, and protein speciation measurements, no protein label required	moderate-throughput, less common equipment required