ADVANCED SCREENING STRATEGIES FOR MOLECULAR GLUE DISCOVERY

Despite strides in small-molecule drug development over the last century, approximately 85% of the proteome remains undruggable.[1] This means that the majority of proteins evade targeting by conventional small-molecule drugs that have a straightforward inhibitor or activator mechanism of action. Such proteins may lack available binding pockets for small-molecule interactions. In other cases, a small molecule has been identified that targets the protein, but off-target effects prevent it from being further developed. As a result, many diseases cannot be treated with conventional small-molecule medicines. In addition, our increasing knowledge of the molecular mechanisms of disease provides opportunities for therapeutic intervention that cannot be approached with traditional small molecules.

One class of therapeutics addressing this challenge is molecular glues (figure 1).[2] "Molecular glues are molecules that can encourage two proteins to interact when they otherwise may not," says Peichuan Zhang, director at WuXi AppTec. "As a consequence, they can induce proximity to help stabilize existing interactions or create new, nonnative interactions." This mechanism

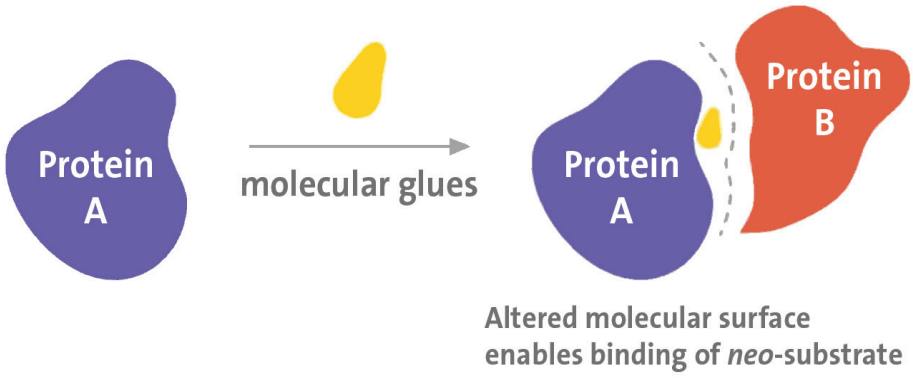

Figure 1: Molecular glues enable new interactions between proteins

is catalytic—meaning it may induce the colocalization (and the functional consequence of that colocalization) between many pairs of target proteins. This allows the medicine to achieve efficacy at low concentrations.

This interaction could lead to degradation, stabilization, inhibition, or activation of the target protein and ultimately result in the desired therapeutic outcome.[3]

For example, to promote degradation, a molecular glue can promote the binding between an E3 ligase (a housekeeping protein responsible for tagging other proteins for degradation) and a second protein (the target protein). By creating a favorable binding surface between these two proteins, the molecular glue induces their proximity; that allows the ligase to ubiquitinate the target protein, marking it for degradation. An example of a nondegradative molecular glue is one that targets the Ras-MAPK signaling pathway, which is associated with many cancers. This molecular glue binds both RAF kinase and MEK (two proteins in the Ras-MAPK signaling pathway), thus preventing MEK phosphorylation and inhibiting downstream signaling.[4]

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—**DAVID MADGE,** WUXI APPTEC'S VICE PRESIDENT OF DISCOVERY SERVICES

Surprisingly, there are a number of drugs used to treat cancer and neurodegenerative disorders that were later discovered to function as molecular glues. For example, cyclosporin helps prevent transplant rejection, but the drug's mechanism of action as a molecular glue was identified 8 years after FDA approval.[5] Another molecular glue, thalidomide, was used for decades before its molecular glue activity was identified in the early 2000s.[6,7] As of January 2025, about a dozen molecular glues had entered clinical studies.[8]

"The vast majority of the molecules that we now consider to be molecular glues were discovered serendipitously," said David Madge, WuXi AppTec's vice president of discovery services. "That's starting to change, and there are a number of new platform technologies that are now facilitating molecular glue discovery."

WuXi AppTec has developed numerous screening methods and libraries, as well as in vitro and in vivo assays, to identify and evaluate potential molecular glues. These screening methods have created a toolbox for more strategic and systematic discovery of molecular glues. This white paper describes the tools used to study molecular glue activity, including screening methods to identify potential molecular glues, in vitro assays to evaluate binding, protein degradation, selectivity, and functional outputs, as well as in vivo testing to validate in vitro findings and pharmacokinetic properties.

MOLECULAR GLUE SCREENING METHODS

Molecular glue drugs rely on a three-part interaction between two proteins and the molecular glue to induce a therapeutic effect. Because they create a new surface and depend on interactions with two proteins rather than one they are more complex than traditional active site inhibitors. Minor changes to the molecular glue's structure can have outsize, poorly understood effects that influence the interaction, which complicates structure-activity relationship modeling. This makes it even more important to identify promising hits from large-scale screening, as there are limited options for downstream optimization. Some of the most effective methods for molecular glue discovery include the use of DNA-encoded libraries, affinity selection mass spectrometry, and high-throughput screening such as time-resolved Förster resonance energy transfer (TR-FRET) and spectral shift assays.

DNA-ENCODED LIBRARIES

DNA-encoded libraries (DELs) are small-molecule or peptide libraries built using combinatorial chemistry.[9] These libraries are created using a split-and-pool approach, an iterative process used to prepare many molecules by first separating a mixture of compounds into different groups. Each group is coupled to a different building block, and the groups are then recombined before another round of splitting and coupling. The split-and-pool approach enables chemists to create millions to billions of compounds rapidly. For DELs, a unique DNA barcode attached to each compound (extended with each synthetic step) allows scientists to identify the hit compound after an affinity-based selection process.

Researchers can screen DELs either in suspension as a pooled library or with multiple copies of each molecule attached to an individual bead. In either case, the goal is to identify compounds that induce complex formation between two target proteins.

- **To screen with in-solution DELs,** the first target protein is immobilized on a matrix. The other protein is preincubated with the DEL and then added to the first target for interaction. If the second target protein does not interact with the first one, it will be washed away. Any interacting molecules that bind the first target can then be released using heat and identified by their unique DNA barcode (figure 2).

- **On-bead screening** uses fluorescently labeled target proteins, each with a different label. These proteins are incubated with the on-bead DEL consisting of DNA-tagged beads attached to the molecule library. If the molecule forms a ternary complex, the complex can be identified using a fluorescence-based readout, and molecular glue hits can be identified by their corresponding DNA barcodes.

Screening molecular glues with DELs provides a large screening capacity and can be completed quickly.

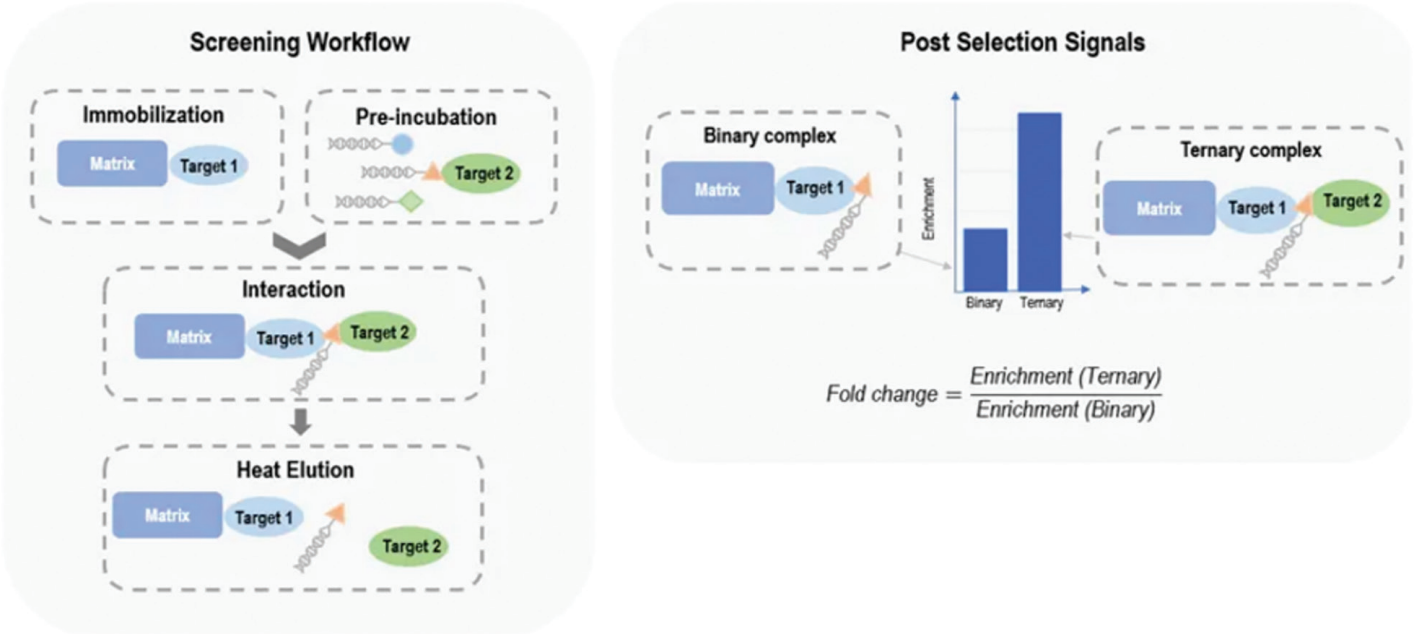

Figure 2: Screening workflow for in-solution DEL screening. A higher fold change suggests higher cooperativity of the compound.

AFFINITY SELECTION MASS SPECTROMETRY

Like screening DELs, affinity selection mass spectrometry (AS-MS) identifies molecular glue hits based on affinity. The first step in AS-MS is to incubate a pooled molecule library (for example, 400,000 small molecules arrayed as 2,000 pools of 200 compounds) with target proteins. Ternary complexes formed will be larger than individual proteins or compounds, which allows them to be separated by chromatography. Because the library used in AS-MS does not contain DNA barcodes, hits must be identified using mass spectrometry (figure 3).

Using mass spectrometry to identify compounds has the advantage that they appear in their native format during screening and are not subject to potential interference from a DNA barcode. However, screening with AS-MS is not as high throughput as DELs and requires specialized equipment.

HIGH-THROUGHPUT SCREENING

Molecular glues can also be screened using a one-compound-per-well high-throughput screening (HTS) approach. This method uses proximity-based assays such as TR-FRET or spectral shift technology to detect ternary complex formation and molecular glue activity.

HTS has several advantages. Having only one compound per well eliminates any interference that could occur in a pooled well of compounds (e.g., aggregation). And if incorporated, TR-FRET, spectral shifts, and degradation assays provide much more information than a yes or no binding result, which is the outcome of other screening methods. For example, the Crelux spectral shift technology from WuXi AppTec uses fluorophores attached to a target protein to measure the strength of the interaction. The basis of this technology is that the polarity of

ASMS: Affinity Selection Mass Spectrometry

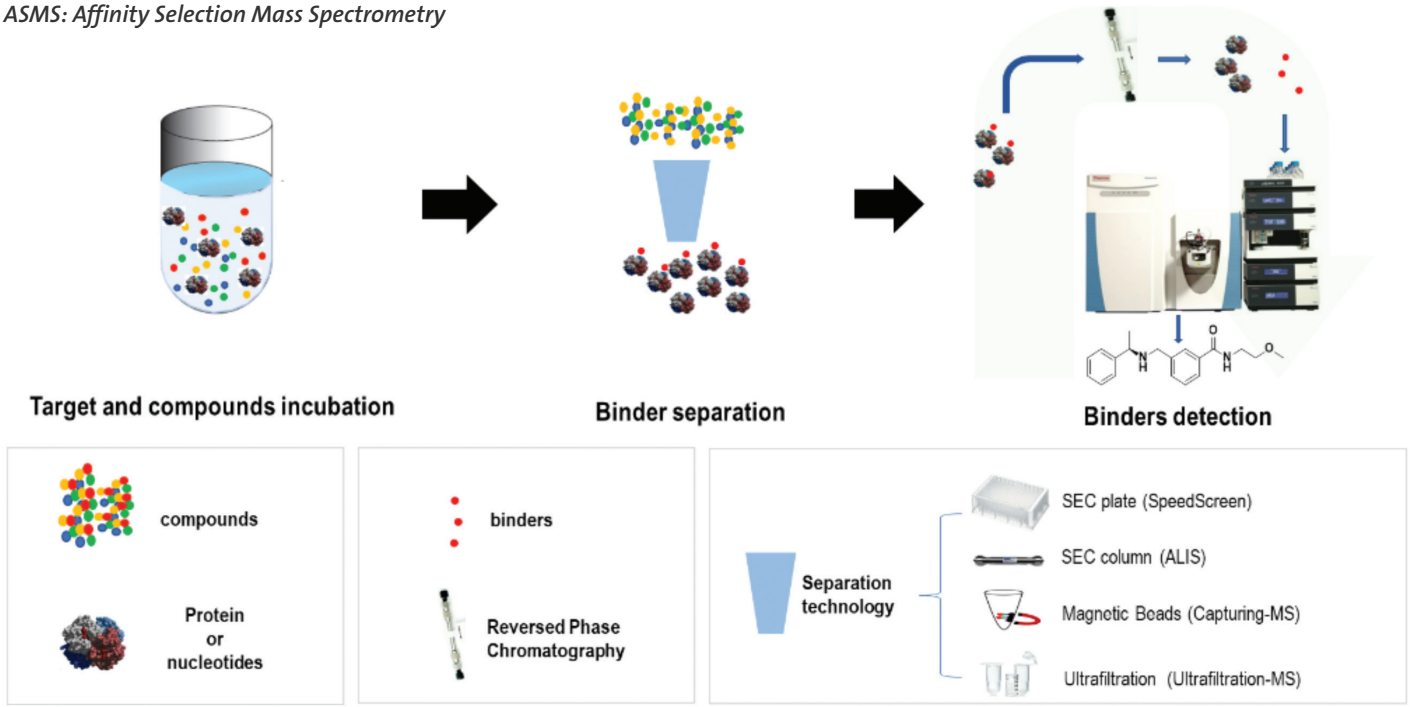

Figure 3: The AS-MS workflow involves first incubating target proteins with compounds to identify binding molecules, then separating and detecting those molecules.

the environment around the fluorophore changes in response to binding events. When binding occurs, the fluorophore's emission at 650 nm and 670 nm changes, and this shift can be dependent on molecular glue concentration (figure 4).

VALIDATION AND OPTIMIZATION OF SCREENING HITS

Once the top molecular glue hits have been identified, in vitro and in vivo assays are used to understand how they bind, how they affect their target protein, and what functional consequence they produce. Zhang adds that these assays are very helpful in the early stage for hit triage.

Binding

Molecular glue binding can be assessed using each target protein individually or as a complex with both proteins. Assessing each target individually confirms binding to each target, while comparing the results from binary and ternary binding can show cooperativity (figure 3). Several assays are used to understand binding:

- **Biophysical assays** measure physical interactions to understand complex formation. These assays provide quantitative readouts of binding affinity, as well as association and dissociation kinetics. Biophysical assays include surface plasmon resonance, microscale thermophoresis, temperature-related intensity change, and biolayer interferometry. These assays can help scientists study how binding kinetics change with the concentration of the hit compound and how binding alters the surface properties of the proteins.

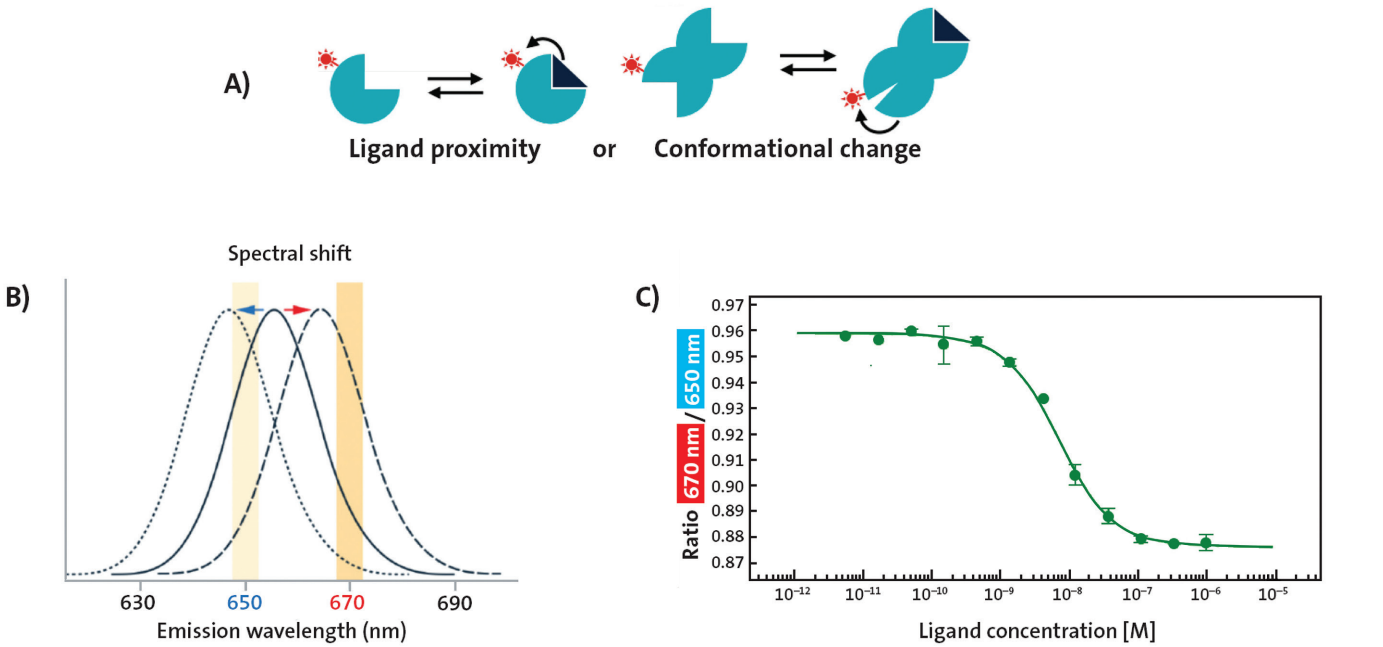

Figure 4: Spectral shift technology measures a change in the fluorophore emission spectrum upon binding or conformational change of a protein. A) Illustration of relationship between ligand binding and spectral activity. B) Shift in wavelength caused by conformational change. C) Relationship between ligand concentration and wavelength ratio.

- **Biochemical assays,** such as TR-FRET, evaluate properties including proximity, enzyme kinetics, and protein-protein interactions. These assays typically use labeled substrates, enzymes, or energy transfer to provide quantitative readouts like fluorescence or luminescence.
- **Cellular assays,** such as NanoBiT and NanoBRET, provide data on molecular glue– target binding, proximity, and complex formation within the context of living cells (unlike assays done using purified proteins).

Protein ubiquitination and degradation

For molecular glues targeting the degradative machinery, such as E3 ligases, a first step in understanding their action is to examine whether binding triggers protein ubiquitination and degradation. This can be achieved with biochemical assays like TR-FRET, which use fluorescently labeled ubiquitin molecules; or with cellular assays, including a pull-down assay, to capture ubiquitinated proteins from cells.

Western blots, including near-infrared western blots, can be used to quantify protein levels and determine the extent and kinetics of degradation. Testing different concentrations of the molecular glue makes it possible to observe how its concentration affects protein degradation (figure 5). High-throughput western blot technologies enable scientists to quickly evaluate multiple proteins and compounds at various concentrations in an automated manner.
In-cell Western blots can also quantify endogenous protein levels directly in cultured cells using immunofluorescence. For CRBN-related molecular glues, a CRBN knockout cell line can be used to compare inherent protein instability to molecular glue–induced degradation in a CRBN-expressing cell line.

SCREENING LIBRARIES FROM WUXI APPTEC:

(1) **A diversity DEL,** which covers the entire known drug space, based on over 6,000 bioactive scaffolds. This library contains over 200 sublibraries, each constructed from a different synthetic route, totaling approximately 50 billion structures. This library is ideal for targets that lack reported molecular ligands or binding sites.

(2) **Glue-focused DEL,** based on previously known molecules with molecular glue activity. These libraries begin with reported small-molecule ligands or core scaffolds of molecular glues and expand the chemical space using extensive chemical modifications. This type of library is useful for targets with existing molecular glues to help increase screening performance and hit rates. Examples of this type of library include one focused on an immunomodulatory imide drug targeting the E3 ligase cereblon (CRBN).

(3) **Small-molecule library** of over 370,000 compounds for affinity selection mass spectrometry and high-throughput screening.

	DNA-encoded library screen (DEL)	Affinity screen mass spectrometry	High-throughput screening
Scope of screening capability	1 million–100 billion structures	10 k–1 million structures	10 k–1 million structures
Time for screening	4 weeks	1–3 months (scale dependent)	2–4 months (scale/method dependent)
Typical readout	In-solution DEL On-bead DEL	Affinity	Function
Initial medicinal chemistry support for screening data	• Primary structure-activity relationship analysis • Hit resynthesis and validation • Hit optimization	• Cherry-picking and hit validation • Hit optimization	• Cherry-picking and hit validation • Hit clustering and optimization
Suited for	• Novel target with no suitable screening library • Seek for novel pocket or mechanism of action	• Suited for any soluble target • Protein/RNA/DNA • Seek for novel pocket or mechanism of action	• Target assays that can be miniaturized • Cell assay available

Table 1: Screening capabilities available from WuXi AppTec

Selectivity, functional assays, and in vivo *pharmacology*

For degradative molecular glues, demonstrating binding and degradation is a first step to validating and prioritizing molecular glue hits. Selectivity testing, functional assays, and in vivo pharmacology are then used to further characterize hits and identify those that should proceed to development.

It is critical that molecular glues target only the intended proteins, as off-target interactions can cause unwanted biological effects. For example, researchers often use western blots to determine whether related proteins are degraded, but this method cannot provide a picture of the entire proteome. Mass spectrometry can help scientists understand the effect of molecular glues on the whole proteome. For some molecular glues, such as CRBN-related ones, degradation-profiling panels are available to help in assessing unwanted degradation of common unintended targets.

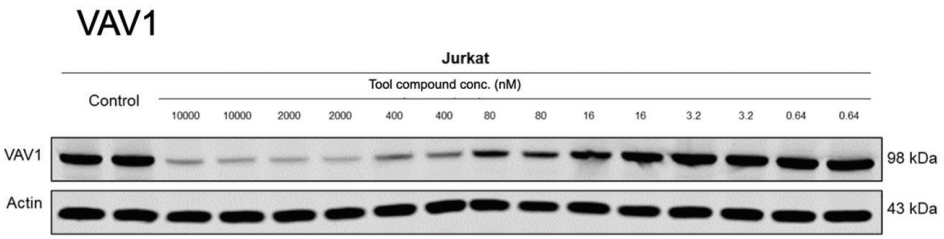

Figure 5: Western blot quantifying VAV1 degradation in Jurkat cells at different compound concentrations

Once it is established whether hits induce any off-target activity, the next step is to determine whether the outcome of molecular glue binding has the desired downstream effect. These assays differ according to the molecular glue's function (induced degradation or some other proximity-induced functional effect) and depend on the target protein. For example, degradative molecular glues focus on observing the consequences of degradation on the target proteins. If the hypothesis is that degrading the target protein changes the proliferation rate of a specific cell type, for example, assays should be designed to measure proliferation rates. For nondegradative molecular glues, if the hypothesis is that bringing two proteins together changes the transcription levels of downstream genes, quantifying those transcripts would be an appropriate assay.

After candidates pass through in vitro studies, the next step is to evaluate their behavior in a living organism, including ADME and DMPK testing. While these studies are not unique to molecular glues, they help scientists understand the molecular glue activity in animal models. For example, in vivo pharmacokinetic studies conducted in animal models can help drug developers understand how the body processes the drug, while metabolite identification assays aid in assessing its efficacy and safety profile. As mentioned earlier, because of their mechanism of action, molecular glues that induce degradation can have effects at lower concentrations compared with other small-molecule drugs and exhibit long-lasting inhibition of the target protein.[10]
These types of in vivo studies help evaluate molecular glue hits for further development toward the clinical stage (Phase 1, 2, and 3 trials) and ultimately, approval.

CONCLUSIONS

Molecular glue discovery is a complex process involving screening thousands or millions of compounds and then confirming their binding properties and function both in vitro and in vivo (figure 6). Having the right screening strategies to identify the most promising hits is essential.

WuXi AppTec has developed a comprehensive toolbox to facilitate the entire molecular glue discovery process, including

- Screening capabilities using DEL, AS-MS, and HTS
- Protein production and crystallography capabilities

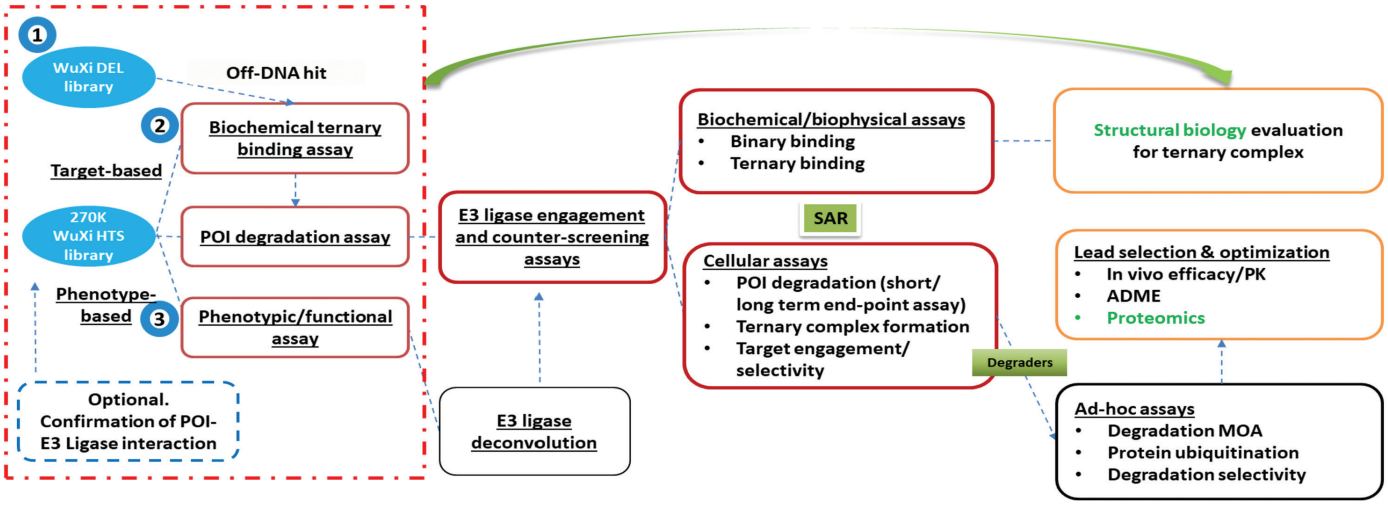

Figure 6: Workflow representation for advancing molecular glue drug discovery

- Full testing support, including biochemical, biophysical, and cellular assays
- Cell lines, tumor models, and animal models to support *in vitro* and *in vivo* assays
- *In vivo* pharmacology studies to test safety, efficacy, and toxicity

Molecular glues open new opportunities for targeting undruggable proteins, which can have a tremendous impact for patients with limited treatment options. By providing avenues to engage with disease pharmacology in ways that traditional small-molecule drugs cannot, molecular glues can help turn undruggable protein into ones that can be effectively targeted, paving the way for treatments for diseases once considered untreatable.

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