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EMERGING DRUG DISCOVERY STRATEGIES FOR TARGETED PROTEIN DEGRADATION

The era of drug discovery dominated by small molecules faces a steady challenge from new therapeutic modalities. Peptides, oligonucleotides, and cellular and gene therapies treat disease in situations where small molecules may otherwise fail.

Another emerging modality assembles traditional small-molecule subunits into a larger molecule that initiates targeted protein degradation. Proteolysis-targeting chimeras (PROTACs®) are higher molecular weight, bifunctional chemical compounds that bind both a protein of interest (POI) and a second protein that marks proteins for elimination, bringing the two species in close proximity to induce POI degradation.[1–3]

Targeted protein degradation offers the ability to reach disease-causing proteins that are difficult to target with conventional small-molecule therapeutics. Researchers have used PROTACs to target proteins in the cytosol, nucleus, and plasma membrane.[4] Two PROTACs entered clinical testing in 2019, and at least 20 compounds are in clinical trials to treat cancer, immune disease, neurodegenerative disease, cardiovascular disease, and viral infection.[5]

PROTAC drug discovery follows the same general steps as small-molecule drugs: target validation, compound optimization, and in vitro mechanistic studies, along with in vivo efficacy, metabolic, and toxicity tests. PROTACs' complex mechanism of action amplifies the challenges of a discovery and development pipeline. Choosing specific screening and analysis techniques to characterize and optimize PROTAC function can create a workflow streamlined for PROTAC development.

"Over the last 20 years of research, synthetic bifunctional small molecules are starting to prove their worth in the field," Kris Rutten, director of discovery services at WuXi AppTec, says in a webinar.[2] "The development of these molecules can help us better elucidate the biological functions of numerous pathways, and it will inspire the next generation of therapeutic tools."

MECHANISMS OF BIFUNCTIONAL MOLECULES

In a PROTAC therapeutic strategy, a ligand for a POI is chemically linked to a second ligand specific for an enzyme called E3 ubiquitin ligase. The PROTAC binds to the POI and the E3 ligase and pulls them together. The ligase then attaches multiple ubiquitin subunits at various lysine residues on the POI, marking it for degradation by a protein complex called the ubiquitin-proteasome system (figure 1).

Known colloquially as the cellular garbage disposal, the ubiquitin-proteasome system (UPS) is the primary intracellular mechanism for degrading and recycling damaged proteins. PROTACs hijack the UPS to induce degradation of any protein for which a binding ligand can be identified.[6]

Unlike small molecules, which typically work by interfering with a protein's active site, such as in the case of an enzyme or receptor, PROTACs can interact with any part of a protein to recruit it, allowing many more opportunities for success. While scientists have estimated that only 10–15% of the human proteome is druggable, an as-yet unquantified larger set of proteins is "ligandable" and amenable to a PROTAC therapeutic approach.[7,8]

After a POI is marked and degraded, the PROTAC molecule is released. The PROTAC then has the unique ability to perform additional rounds of binding and degradation, acting in a catalytic fashion and achieving greater activity from a lower dose. Even low-affinity ligands can induce the efficient degradation of target proteins.[9]

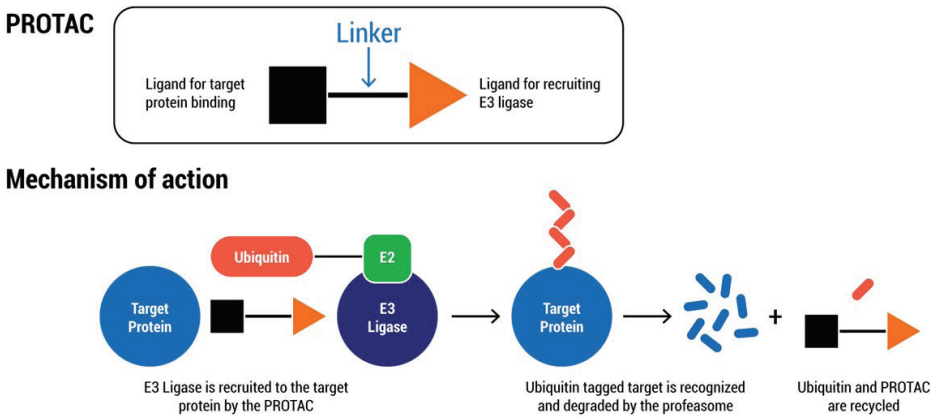

Figure 1: A schematic of the structure of a PROTAC (A), and the mechanism of using it to induce targeted protein degradation (B).

Credit: WuXi AppTec/C&EN BrandLab

CHALLENGES

The discovery and development of effective PROTACs is not trivial. All the typical difficulties associated with a drug discovery program are amplified as suitable ligands for two different proteins must be identified and incorporated into a single molecule.

PROTAC development includes assays that validate and monitor the binding of the bifunctional molecule and its two protein partners. The formation of ternary

complexes can create unusual and surprising situations related to the kinetics and thermodynamics of binding. For instance, tight binary interactions can sometimes exist with minimal ternary complexation, often for reasons that are not completely understood. Similarly, a hook effect can occur where higher drug concentrations cause the preferential formation of binary, inactive complexes, and it may be important for each binary interaction to be of similar magnitude to mitigate this effect.[10] Additionally, the two ligands and linker region may create distinct sets of potential off-target interactions.

With molecular weights typically around 750 kDa, PROTACs sit firmly beyond accepted parameters for drug-like small molecules. Their size introduces limitations for permeability, solubility, and bioavailability. Significant consideration of these factors should be applied throughout a PROTAC development pipeline, and special formulations may be required to achieve bioavailability.

Importantly, the challenges of PROTACs can be surmounted; two Arvinas-developed PROTACs being used in Phase 2 clinical trials are showing efficacy as orally bioavailable preparations that induce the degradation of hormone receptors linked to breast and prostate cancers.[11]

CONVENTIONAL LIGAND SYNTHESIS AND SCREENING WITH DNA-ENCODED LIBRARIES

DNA-encoded library (DEL) technology is well suited to the discovery of ligands for a POI, especially when the ligands are intended for PROTAC development. Molecules in a DEL carry a linker that is a good starting point to conjugate an E3 ligand to generate a bivalent PROTAC molecule. This section will discuss how to use DELs to identify conventional ligands binding to a POI, and the next section will explore how the linker choice affects PROTAC bioavailability and other drug-like properties.

Molecules in a DEL are synthesized in cyclical split-and-pool approach. First, different building blocks (BBs) are conjugated with a bifunctional linker, which consists of starting tags, a spacer, and a headpiece. Then, each BB carrying the bifunctional linker is connected to a DNA sequence that encodes the identity of the BB. Finally, all the different BBs are pooled together and split into groups. Each group then has a new BB and its corresponding DNA sequence added.

These procedures repeat for two–four rounds, depending how many synthesis cycles are designed. Library diversity increases exponentially with this split-and-pool approach. For example, if a pool of 100 common scaffolds is subjected to just three iterations of 100 different modification reactions, the final pool will contain 100 million molecules. Libraries from different synthesis cycles can be further pooled together to generate a superpool, further increasing the final diversity.

A mixture of molecules in a DEL can be screened for binding to a target protein using affinity pull-down methods that need minimal assay development and lab equipment. Barcode amplification and next-generation sequencing quickly identify these “hit” molecules by their unique DNA barcodes. Figure 2 shows classes of proteins for which WuXi AppTec has used proprietary DELs to identify

molecules that bind to the proteins, which can be a starting place to identify ligands for POI in a PROTAC.

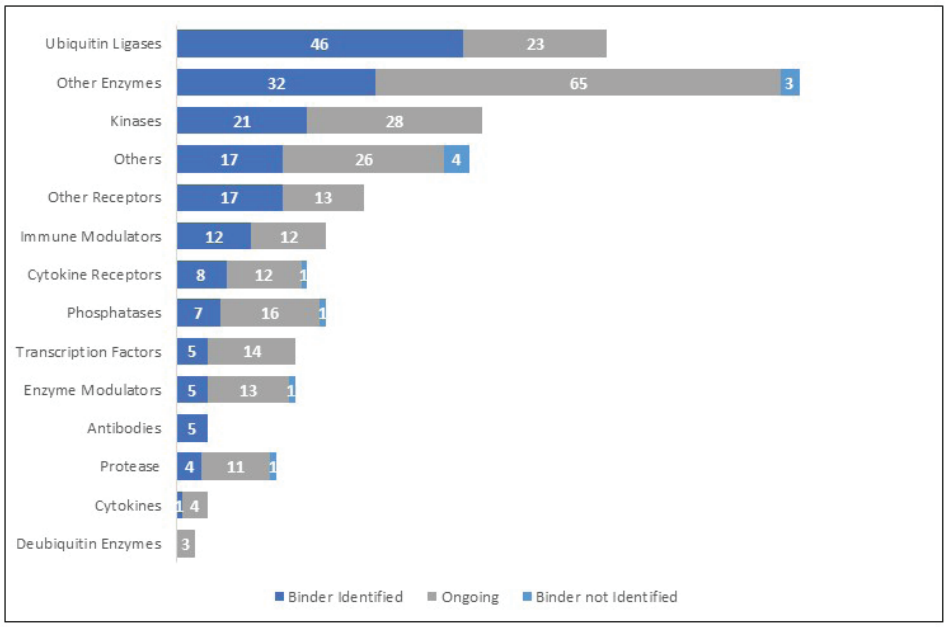

Figure 2: Protein classes screened for ligands using WuXi AppTec's proprietary DELs.
Courtesy of WuXi AppTec

LINKER OPTIMIZATION

While synthesizing bifunctional molecules is theoretically as simple as connecting a POI ligand an E3 ligand, the structure of the linker joining the two ligands requires careful consideration.

The linker provides opportunities to improve physicochemical properties of a PROTAC molecule. The length, lipophilicity, and rigidity of this region can have major effects on POI and E3 ligase binding, cellular permeability, metabolic stability, and other physicochemical drug-like parameters.

Linkers may be linear and flexible, such as common polyethylene glycol–based and alkyl linkers. Alternatively, fused or spiro ring systems create a more rigid structure. Rigid linkers are increasingly being explored for their ability to constrain molecules in a bioactive conformation, and heterocycles can improve solubility. Linkers that are too short may create protein-binding conflicts, but the precise length and rigidity of the linker can affect efficacy in unforeseen ways because of the thermodynamic complexity of ternary complex formation.[10] A diverse library of linkers and the tools to screen them shortens the development timeline.

DIRECT DISCOVERY OF PROTAC MOLECULES VIA SOLUTION DEL

The next two sections will explore how DEL technology can be used to synthesize and screen diversified bifunctional PROTAC libraries. Different cycles of DEL synthesis can be used to construct different combinations of E3 ligands, linkers, and POI ligands. Any of the three components can carry the DNA barcodes. A

library often contains a fixed, known E3 or POI ligand, and the linker and other ligand is diversified and screened against its binding partner.

WuXi AppTec has accumulated a DEL with over 9 billion bifunctional molecules containing varied DNA conjugation sites (figure 3). The ready-to-go libraries with complete PROTACs contain fixed, well-established CRBN and VHL ligands. There are also libraries of molecules in intermediate state of synthesis. Members of these libraries contain various warheads and linkers, and an open site for ligand synthesis. These intermediate libraries enable rapid ligand-focused bifunctional library construction for either E3 or POI targets.

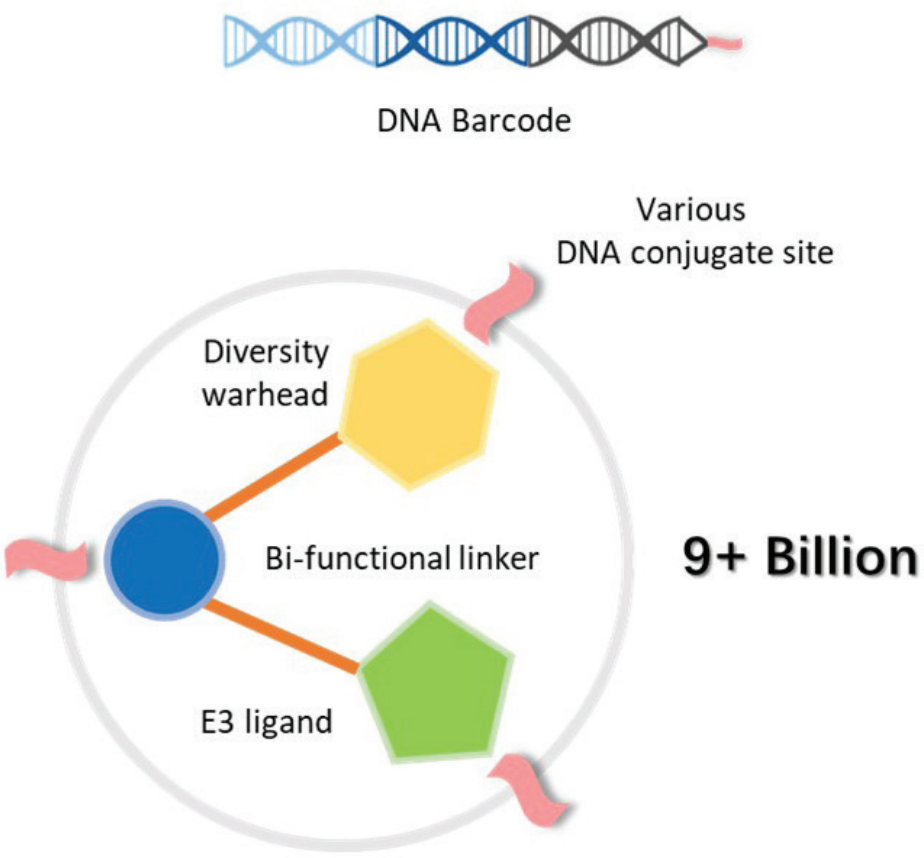

Figure 3: A schematic of a bifunctional PROTAC synthesized using DNA-encoded library technology. The moiety labeled E3 ligand represents a fixed ligand, which could also be a known POI ligand. The DNA barcode can be attached to any of the three components (E3 ligand, bifunctional linker, or POI ligand, labeled "diversity warhead" here). WuXi AppTec maintains a proprietary DEL of more than 9 billion bifunctional molecules.

Courtesy of WuXi AppTec

The screening method for DELs containing bifunctional molecules is different from that used to screen conventional ligands. The aim of bifunctional DEL screening is to identify molecules that will induce ternary complex formation between an E3 ligase, the small-molecule PROTAC, and a POI.

Several screening methods are being tested at WuXi AppTec for screening bifunctional DELs (figure 4). Although they have slightly different details, all are set up to find molecules that favor ternary complex formation.

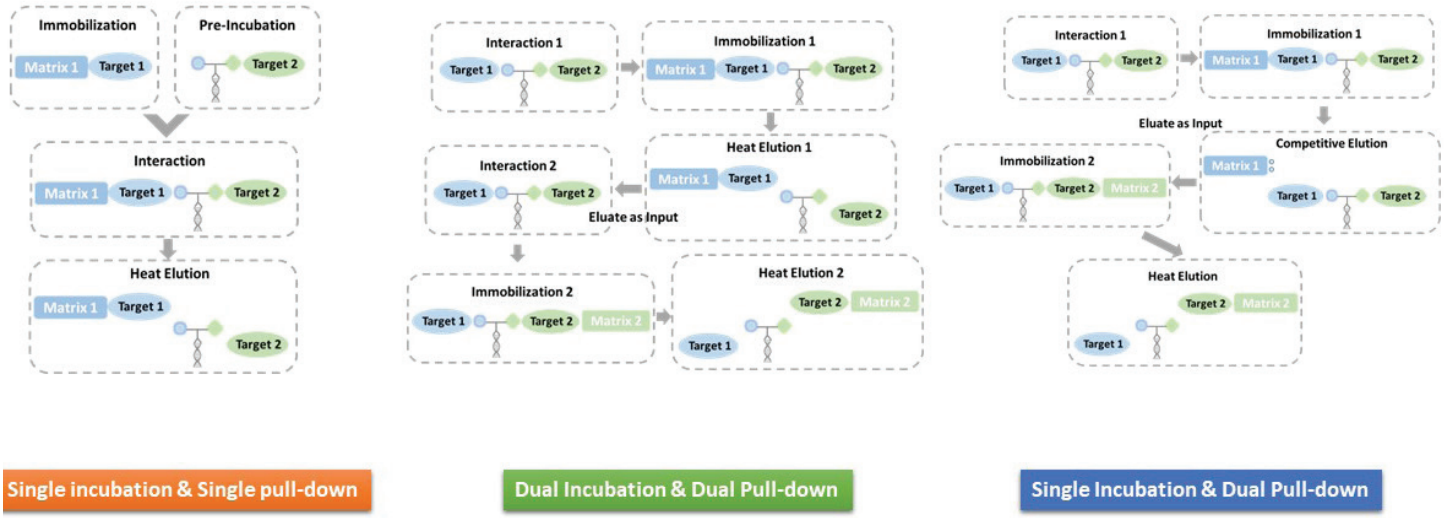

Figure 4: Schematic of methods to screen a DNA-encoded library of bifunctional molecules, such as PROTACs. Target 1 represents the POI, and Target 2 represents the E3 ligase in the case of PROTAC molecules. Usually, two conditions will be screened and compared in parallel: 1. Both POI and E3 proteins present simultaneously; 2. Only POI or E3 present.

Courtesy of WuXi AppTec

DIRECT DISCOVERY OF PROTAC MOLECULES VIA BEAD DEL

Along with solution DEL technology, WuXi AppTec also specializes in one-bead-one-compound (OBOC) DELs. In an OBOC DEL, a small molecule and DNA barcode are attached to a microbead. Both conventional OBOC DELs and specially designed bifunctional OBOC DELs can be used for screening. Bifunctional OBOC DELs enable direct detection of ternary complex formation.

During a bifunctional OBOC DEL screening, the library is incubated with both E3 ligase and the POI. Antibodies specific to the E3 ligase and POI will be used to detect the presence of both proteins. If the small molecules on bead can simultaneously recruit both the POI and E3 ligase, the corresponding specific antibodies can recognize these small molecules and beads. Based on the fluorescent signal, flow cytometry can detect and sort out these positive compounds that can induce ternary complex formation.

Compared with solution DEL, an OBOC DEL provide a more direct readout of bispecificity. Additionally, using a preinstalled photocleavable linker to connect the bifunctional molecule to the microbead means hit compounds can easily be released from the microbead for biochemical or cell-based screening (figure 5).

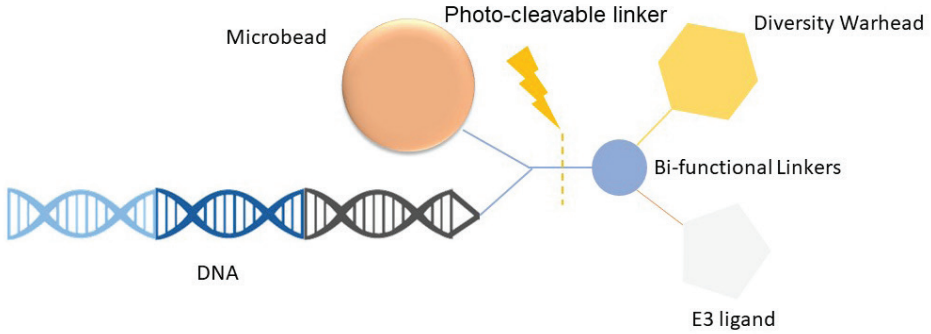

Figure 5: A schematic of a bifunctional molecule in a one bead one compound DNA-encoded library. Using a preinstalled photocleavable linker to connect the bifunctional molecule to the microbead means hit compounds can easily be released from the microbead for biochemical or cell-based screening.

Courtesy of WuXi AppTec

BIOLOGICAL CHARACTERIZATION

After PROTAC molecules have been synthesized, the next step in development includes quantifying binding events. Tests for binding involve advanced biochemical, biophysical, and cellular methods to study the formation, stability, and kinetics of the PROTAC-POI-E3 ligase complex.[12] Additional methods are then used to determine if complex formation leads to effective protein degradation.

Common assays to study binary complex formation (both PROTAC-E3 ligase and PROTAC-POI) include time-resolved Förster resonance energy transfer (TR-FRET), fluorescence polarization, surface plasmon resonance, microscale thermophoresis, and nano-bioluminescence resonance energy transfer (NanoBRET).

Ternary complex formation can then be monitored using some of these same technologies, as well as cellular assays such as NanoLuc® binary technology (NanoBiT). The NanoBiT assay fuses peptide subunits to each protein (POI and E3 ligase) involved with the PROTAC function. When the two tagged proteins interact, the peptide subunits combine and generate a luminescent signal. AlphaScreen is another commonly used assay to determine protein complex formation; here, the signal is based on energy transfer between proximal beads.

After measuring ternary complex formation, the next step of determining PROTAC function involves measuring protein ubiquitination and the ultimate goal, degradation. For this analysis, Western blots, in-cell Western assays, AlphaLISA, TR-FRET, and HiBiT are commonly used. In-cell Western assays and HiBiT provide highly quantitative measurements of protein degradation compared with Western blots. To confirm that degradation occurs through the UPS, small-molecule UPS modulators, molecules that compete for binding with the E3 ligase, or E3 knockdown/knockout approaches can be used to restore protein levels. RT-PCR can confirm that protein decreases are not due to transcriptional inhibition.

Researchers have a large toolbox of analytical techniques to interrogate the dynamics of protein-PROTAC interaction, ternary complex formation, ubiquitination, and degradation. Proper choice of techniques depends on the particulars of a drug discovery effort. It is important to note that because PROTACs act catalytically, assay results can change depending on the time of

measurement. Assays with light-based signals for protein interaction, such as NanoBiT and NanoBRET, provide a steady readout of kinetic information.

DMPK OPTIMIZATION

PROTACs that pass in vitro tests progress to pharmacokinetic testing. This phase of development includes predicting or measuring a drug candidate's absorption, distribution, metabolism, and excretion (ADME). Common ADME experiments encompass a battery of in vitro tests to predict or measure logP, solubility, Caco-2 permeability, plasma protein binding, microsome- or hepatocyte-based metabolic stability, and metabolite profiling. In vivo studies include monitoring exposure levels, biodistribution, clearance, and excretion.

PROTACs present unique challenges and characteristics in many of these assays. For instance, solubility often increases significantly in simulated fed-state intestinal fluid. Recovery is thus improved in Caco-2 permeability assays using this fluid in one chamber, and bioavailability can accordingly be improved when PROTACs are administered after a meal.[13]

Because binding to plasma proteins is typically very high for PROTACs, routine equilibrium dialysis methods are not suitable for plasma protein binding assays, according to Liping Ma, a scientist in the drug metabolism and pharmacokinetics (DMPK) service department at WuXi AppTec, in a webinar.[3] Tailored assays such as ultracentrifugation-based methods are preferred. Metabolic linker cleavage is a common phenomenon, in addition to oxidation of the parent molecule. High performance liquid chromatography methods to analyze the samples from metabolic studies can be complicated by multiple chiral centers that necessitate chiral columns for separation of peaks.

In vivo tests show that PROTAC excretion usually occurs primarily by liver metabolism and biliary clearance, with little urinary or fecal elimination.[13] Because much less data exist for PROTACs compared with small-molecule drugs, understanding of PROTACs' DMPK behavior will continue to develop.

FORMULATION STRATEGIES

The high lipophilicity of PROTACs is beneficial for membrane permeability, but high fat solubility often results in low aqueous solubility, which ultimately limits absorption in a patient's gastrointestinal tract. Formulation techniques can overcome inherent solubility challenges with bifunctional molecules.

Nano milling reduces a drug's particle size, which increases its surface area and thus increases the dissolution rate. Several parameters must be optimized depending on PROTAC chemical and physical stability, including the proper milling vehicle selection and the duration and speed of milling. In other cases, a PROTAC can form an amorphous solid dispersion upon spray drying or hot melt extrusion, and polymers can be added to this dispersion to fine-tune its solubility.[14] Lipid-based emulsions can improve drug solubility, and sometimes, permeability.

Using smaller E3 ligase ligands, such as those for CRBN, can help reduce total molecular weight and improve solubility. Molecular glues that essentially omit the linker can also reduce molecular weight and achieve efficacy in select cases.

Despite these hurdles, with careful design, some PROTACs can be made orally bioavailable without special formulation.

PROTACs are a technically challenging yet highly promising treatment modality capable of targeting a range of disease conditions. Using and understanding state-of-the-art methods for screening libraries and for in vitro and in vivo validation assays is helpful if not necessary for a robust discovery pipeline that yields effective compounds.

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