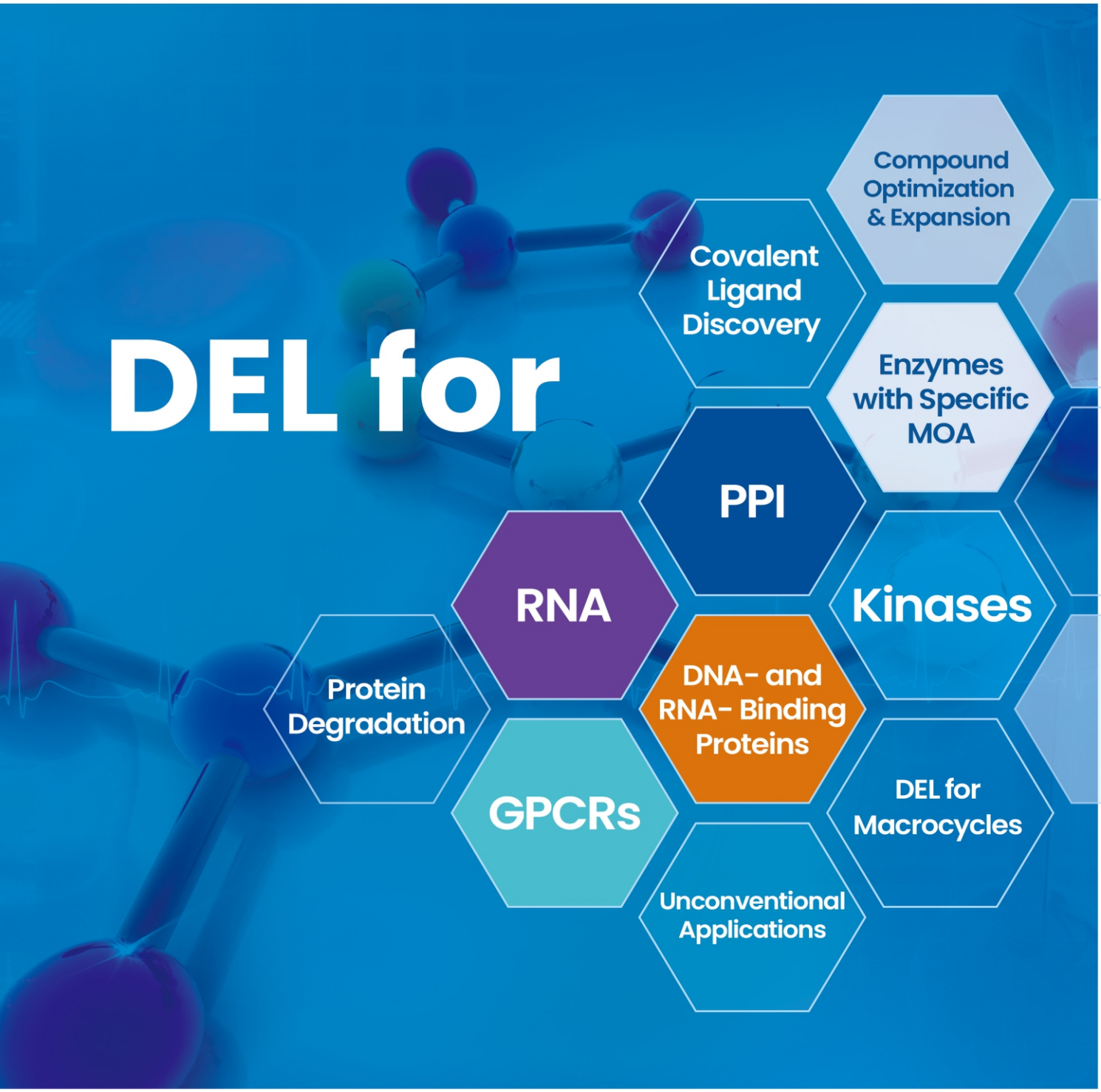
HITGEN



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DEL for Protein-Protein Interaction

Protein-protein interactions (PPI) are physical and chemical contacts between two or more protein molecules. As a fundamental aspect of almost all biological processes, any interference of the sophisticated PPI network could result in potential physiological disorder or disease. Although some PPI networks have been well-established for their roles in tumor development and therefore have been recognized as potential oncological targets (Figure 1), PPI-based drug discovery is challenging due to its possible event-driven conformational alteration and interface accessibility variation. DNA-encoded Library (DEL) selection, an affinity-based small molecule selection process, is considered a powerful PPI-based drug discovery tool. With strong understanding of the target PPI mechanism of actions (MOAs) and rational design of interaction partner group, DEL is capable of providing selective hits with desirable MOA and potentially, designated function.

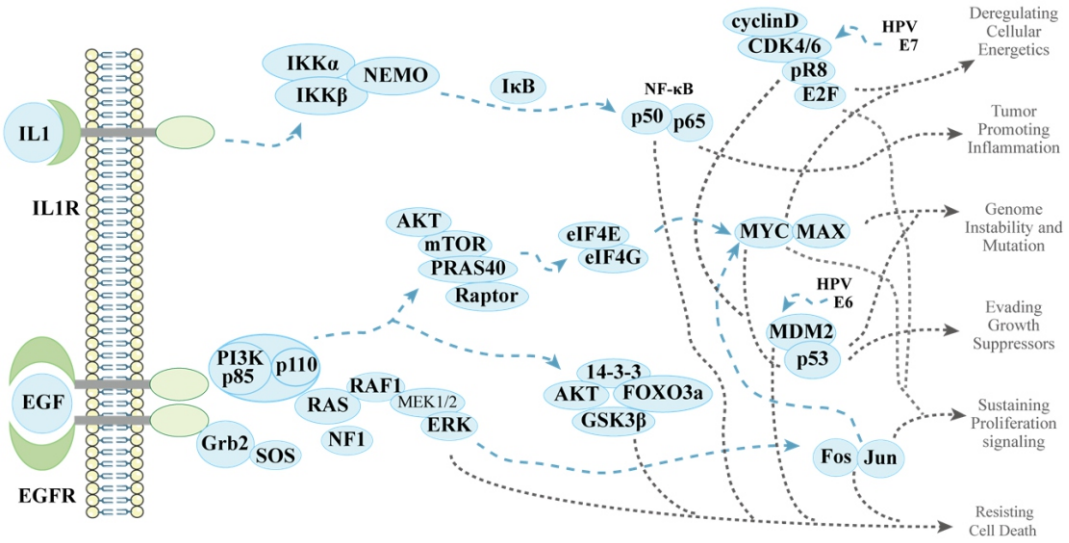


Figure 1. an example of PPI network in tumor development related processes.

Direct PPI blocker identification

One of the biggest challenges for PPI disruptor identification is that the “coverage” of small molecules might differ from that of the interacting motifs in PPI process. This is largely due to the aforementioned intrinsic nature of PPI that the interaction sites are more of a shallow and large interface rather than a deep and well-defined pocket. With its great diversity, DEL has been providing PPI interface binders for over a decade at HitGen, and a large portion of the identified binders has been later confirmed as bona fide PPI blockers in the follow-up validation studies. For example, aiming to target the interactions between the IL17A and IL17RA for treatment of autoimmune and autoinflammatory diseases, HitGen has conducted a DEL selection, and novel IL17A/IL17RA PPI interrupter have been identified and compounds through medicinal chemistry optimization have now been nominated as preclinical candidates.

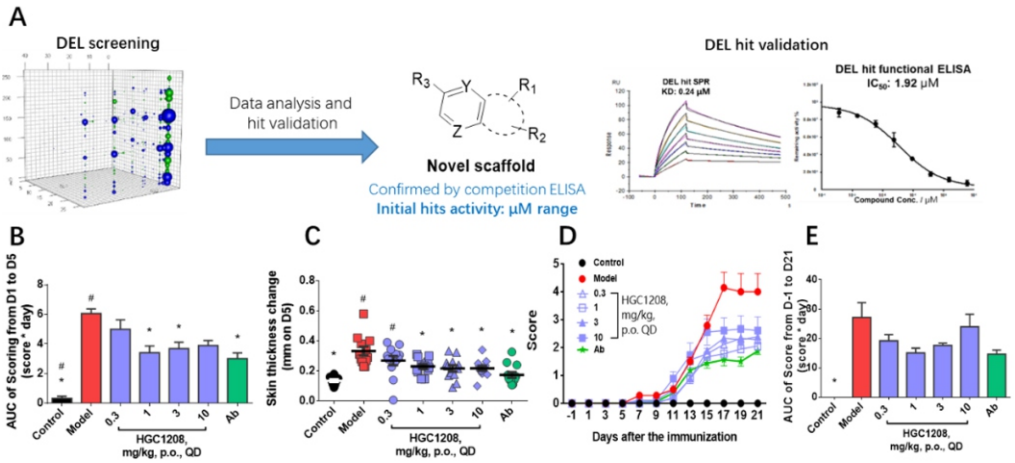


Figure 2. A) A successful hit series identified by DEL for direct IL17A/IL17RA interaction disruption. DEL-derived compounds showed efficacy in IMQ-Induced psoriasis model (B, C) and EAE model (D, E).

"Smart Selection Campaign": function oriented hit identification

PPI could be modulated due to the conformational alterations of the interacting proteins upon particular signaling event, such as ligand binding, co-factor interaction, pH variation, and others. These PPI regulation processes could lead to pathway activations upon complex formation, or switches to be turned on and off with domain orientation change upon presence or absence of co-factors. At HitGen, "Smart Selection Campaign" was developed to explore the binding capability alteration and conformation-based regulation in PPI ligand discovery. By conducting DEL screening in the absence or presence of partner proteins, co-factors, activators and/or inhibitors, this hit identification process has already provided vast amount of information of potential function of the candidate.

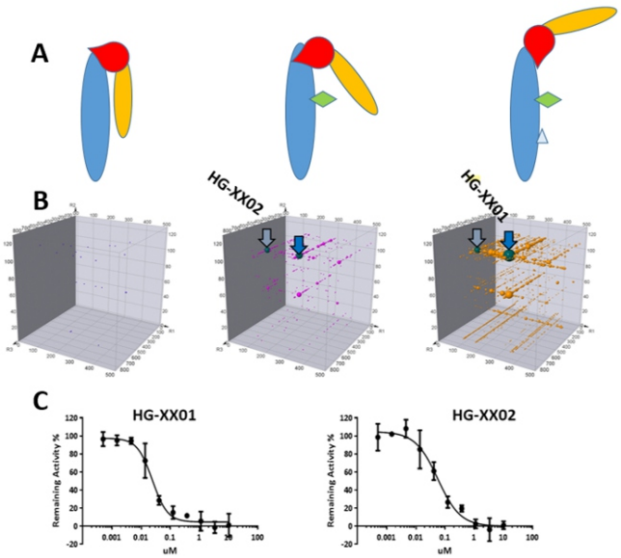


Figure 3. A) Conformation change upon presence of different cofactors for a PPI target introduces different binding capabilities. B) Different series of compounds identified from DEL selection with conformational specificity. C) Confirmed hits from different series.

Identification of specific binder with Special MOA

In a particular DEL selection conducted at HitGen, the PPI network for the target of interest was complicated because different pathways are activated upon binding to different co-factors. On the one hand, target protein could form a heterodimer with partner protein 1 and further form a ternary complex with protein 2 to initiate the downstream signaling pathway. On the other hand, the same target protein could form a different heterodimer with partner protein 3, leading to a bypass pathway. By including all the partner proteins in our DEL selection with different combinations to reflect all possible scenarios, several hit series were identified, and one of the series could specifically bind to the target-protein 1 complex. In the subsequent hit validation studies, we also found that this binder series could stabilize the target-protien 1 complex and more importantly, this stabilization prevents the formation of either the ternary complex, or the target-protein 3 complex. Therefore, we have identified a specific PPI stabilizer series with dual functions at once.

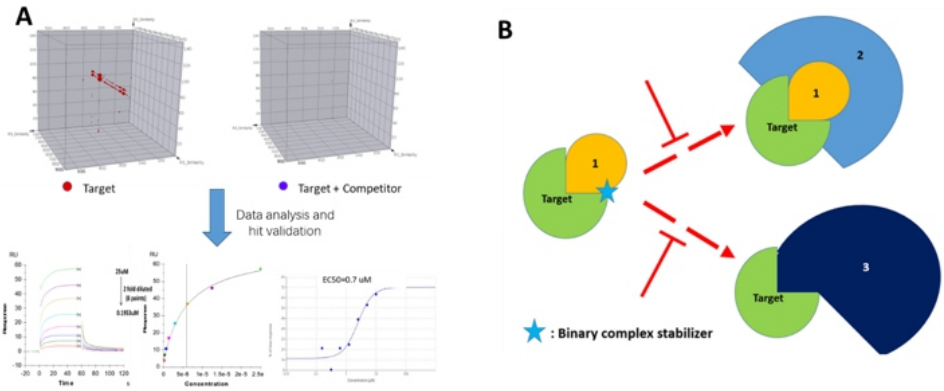


Figure 4. A) a series of hits identified by DEL and confirmed with orthogonal methods. B) Further MOA study demonstrated that in the presence of the confirmed hit compound, two PPI cascades have been interfered based on a special MOA.

DEL for PPI at HitGen

With profound understanding of the PPI target MOA and highly case-dependent DEL selection plan, all possible conformational alterations and different interactions with different partners will be included in our selection to simulate the actual PPI processes and maximize the chance of identifying robust hits with good affinities and properties. HitGen's "Smart Selection Campaign" (Figure 5) along with our trillion size DEL, have enabled PPI-targeted drug discovery with easier design and more straightforward execution.

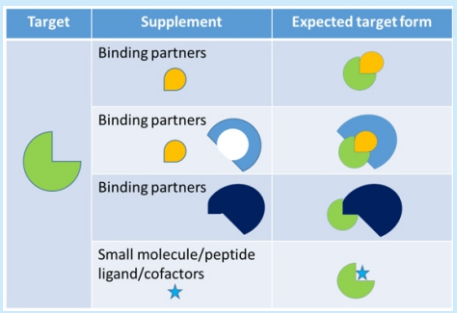


Figure 5. An example of possible selection groups to explore all possible target forms in one selection campaign.

DEL for GPCRs

G protein coupled receptors (GPCRs) are a family of multi-transmembrane proteins with typical 7 transmembrane regions. Many marketed drugs are known to target GPCRs. Given the difficulty in preparing the purified protein with right conformation, traditional GPCR ligand discovery utilizes cell based high throughput screening (HTS), virtual screening and in fewer cases, protein based screening. DNA encoded library (DEL) selection for GPCRs has also been intensively explored at HitGen and a variety of selection approaches are utilized to enable GPCR ligand discovery.

Cell based DEL selection

Cell based DEL selection has been applied for GPCR targets, especially for targets that are more difficult to be purified. DEL selection is a binding process with binding equilibrium driven mainly by target concentration, therefore, target density on the surface is the key for a successful screen. A direct comparison of expression level and enrichment ratio in cell based DEL selection was reported (ACS Comb Sci. 2015 Dec 14;17(12):722-31), when below certain expression level, the nM binding ligand could not be enriched. Another confounding factor is the complexity of other cell surface membrane proteins, for a regular protein based selection, the target purity can be regarded as 100%, however, for cell based selection, target purity may be less than 1%. Though with difficulty, cell based DEL selection for GPCRs has been validated at HitGen. Target was prepared by overexpression on HEK293 cells, and a positive ligand on-DNA conjugate was made to validate selection condition and served as a positive control for DEL selection (Figure 1). DEL selection revealed relatively weak features and the corresponding compound was validated as an antagonist with uM potency.

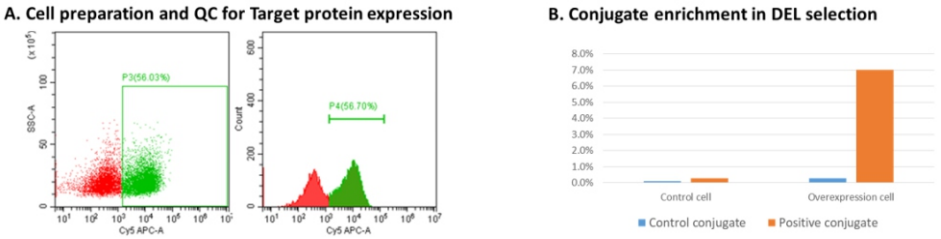


Figure 1. A) FACs to confirm the overexpression of target GPCR. B) The positive conjugate was successfully enriched in DEL selection.

Membrane prep based DEL selection

Preparing cells with high level of GPCR overexpression is labor intensive and difficult to achieve, as high exogenous proteins sometimes impedes cell proliferation. As an alternative, membrane prep based selection provides a new approach to expand DEL applications. It maintains the conformation of GPCR in the natural membrane environment, offers higher target density by various strategies and saves the effort of purifying protein from membranes. HitGen has validated membrane prep based DEL selection for a GPCR, with the purified detergent stabilized protein based selection performed in parallel for comparison, similar features were enriched for both selections, and the corresponding compounds were validated as nM binders (Figure 2).

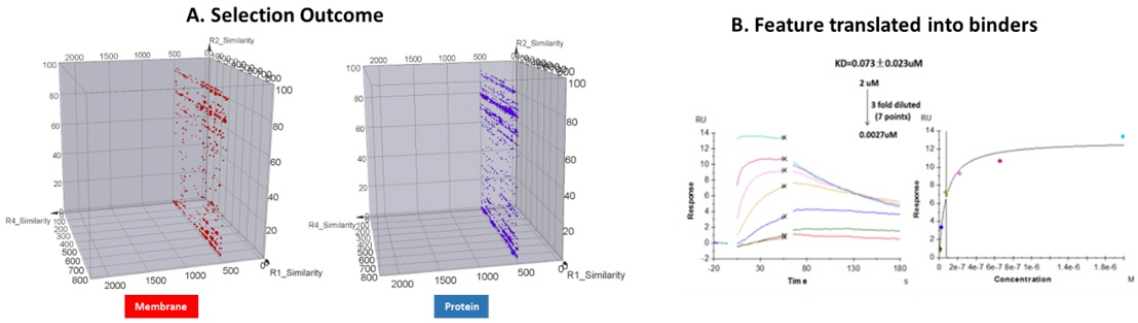
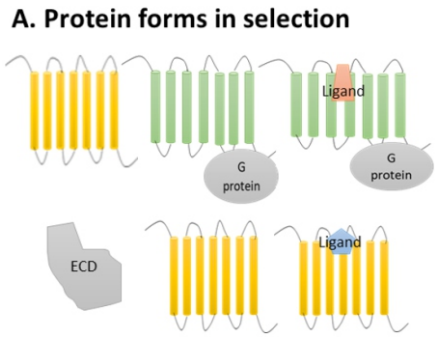


Figure 2. A) Both membrane and protein based selection revealed similar features. B) SPR validated nM binding compound from DEL selection.

Purified protein based DEL selection

Due to the hydrophobicity of the transmembrane region, GPCRs tend to precipitate in aqueous buffer. Therefore, different methods including detergent, nanodisc and other particles are utilized to stabilize the protein. Detergents provide hydrophobic protection for GPCRs and maintain the protein in lipid-free system, however, detergents must be kept in the downstream process. Nanodisc offers a more membrane like structure for GPCRs and sometimes is capable of stabilizing the more difficult proteins, but it is more laborious to make and the scaffold binders need to be excluded. HitGen has performed several detergent/nanodisc stabilized GPCR selections, and different agonists or antagonists were identified with targets complexed with or without G proteins (Figure 3).



B. Representative DEL hits

Cmpd	MW	cLogP	HBA	HBD	IC50/EC50 (μM)
1	580	6.5	7	1	0.012
2	448	1.5	9	2	1.1
3	463	4.7	5	1	0.002
4	344	4.8	3	2	<1
5	434	5.4	5	1	<1

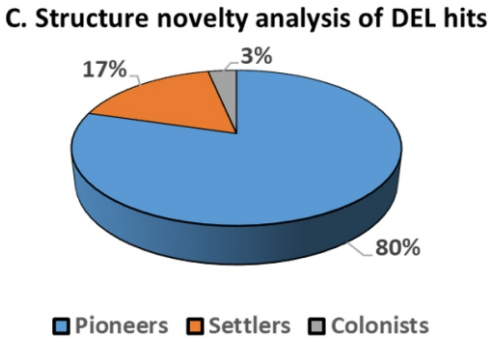


Figure 3. A) Different forms of GPCRs used in DEL selection. B) Chemical properties and potency of representative hits from DEL selection. C) Structure novelty analysis of DEL hits according to reported method (ACS Med Chem Lett. 2020 Nov 12; 11(11): 21142119).

DEL for GPCR at HitGen

The aforementioned approaches provide versatile selection strategies using DEL for ligand discovery against GPCR targets. Protein based selection gives a higher target concentration and the binding process is maintained in a simpler context. If the stabilized protein can be obtained, protein based selection affords a quick option for GPCR ligand discovery. If purified protein is difficult to be prepared, membrane prep and cell based DEL selection offer alternative approaches. In cell based selection, target is maintained in the natural context with the presence of interaction proteins and cofactors, but high target density is the key and sometimes very difficult to achieve. As an alternative, membrane prep based selection also preserves the target in the cell membrane, saves the efforts to solubilize the protein, and enables target enrichment by immobilization strategies. HitGen has built up good experience in GPCR biology, offers various strategies for GPCR DEL selection and provides customized selection solutions for your GPCR:

- Cell/membrane prep/protein based selection offers different selection strategies.
- Different protein forms/constructs allow for ligand identification of different binding sites and gonist/antagonist mechanisms.
- Large diversity of DEL molecules maximizes hit rate, especially for orphan receptors.
- Quick turnaround time and demonstrated successful cases.

DEL for DNA- and RNA-Binding Proteins

While DNA-encoded library (DEL) selection has been applied to a variety of targets with multiple candidates progressed into different stages of drug development, the utilization of DEL in DNA- and RNA-binding proteins remains questionable. This is largely due to the fact that each DEL compound contains a long stretch of DNA barcode, which when used to screen against DNA- or RNA- binding proteins (transcription factors, DNA polymerases, helicases, DNases, RNases, chromatin modifying complexes, etc.) , would lead to significant false positives. To address this potential interference caused by DEL DNA tags, HitGen has made significant efforts in fine-tuning selection conditions as well as developing deconvolution algorithms. These practices, along with our trillion size DEL and 10+ year of DEL experience, have made it possible to conduct meaningful ligand discovery for DNA- or RNA-binding proteins.

DEL selection for transcription factors

Transcription factors (TFs) are involved in multiple biological processes and reflect a delicate system in gene expression regulation. TFs are generally considered as challenging targets because on one hand, most of them lack endogenous ligands (except for nuclear receptors); on the other hand, although most TFs bind to essential cofactors or DNA fragments, the interface of these interactions are generally large, flat and transient. In addition, the potential binding of DNA binding domains (DBD) to the DEL DNA tags further complicates DEL selection strategies. To alleviate these confounding factors, HitGen has employed an array of technologies such as using target proteins with different domains or mutations, counter targets, consensus DNA blocking in DEL selection (Figure 1).

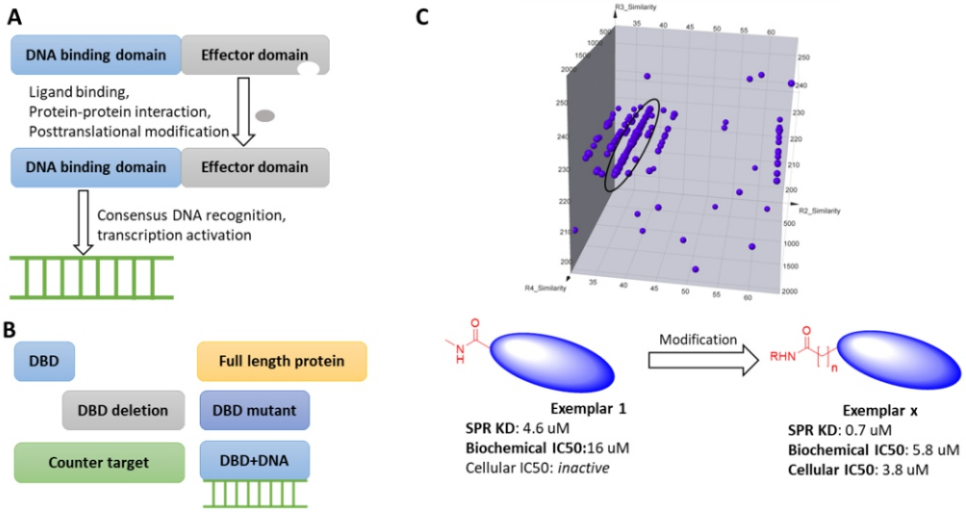
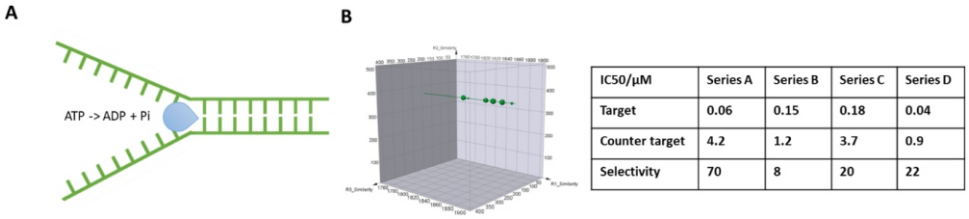


Figure 1. A) Schematic of transcription factor activation. B) Different strategies used in DEL selection. C) Representative signals and hit parameters.

DEL selection for DNA helicases

DNA helicases unwind DNA structures in an ATP-dependent manner and are essential for DNA replication and repair. Defects in DNA helicases can result in different genetic disorders in which genomic instability and predisposition to cancer are common features, warranting them as potential cancer treatment targets. However, the DNA binding nature of this family also presents potential hurdles of high background and false positives when employing DEL selection for their ligands. At HitGen, by implementing our customized selection strategies, we have successfully performed DEL selection for multiple helicase targets and identified different series of hits with good selectivity (Figure 2).



IC50/μM	Series A	Series B	Series C	Series D
Target	0.06	0.15	0.18	0.04
Counter target	4.2	1.2	3.7	0.9
Selectivity	70	8	20	22

Figure 2. A) Schematic of DNA helicase function. B) Representative selection signals and hit parameters.

DEL selection for DNA polymerases

DNA polymerases create DNA molecules by assembling nucleotides in chain reactions. Due to their crucial functions in many DNA metabolic processes, multiple human diseases have been linked to mutations or defects of DNA polymerases, making them potential drug targets. In addition, inhibition on DNA polymerase also represents a good strategy on anti-microbial drug development. At HitGen, exquisitely designed screenings involving DNA substrates and different ligands have afforded multiple hits and yielded well-behaved drug candidates with good enzymatic inhibition activity and selectivity (Figure 3).

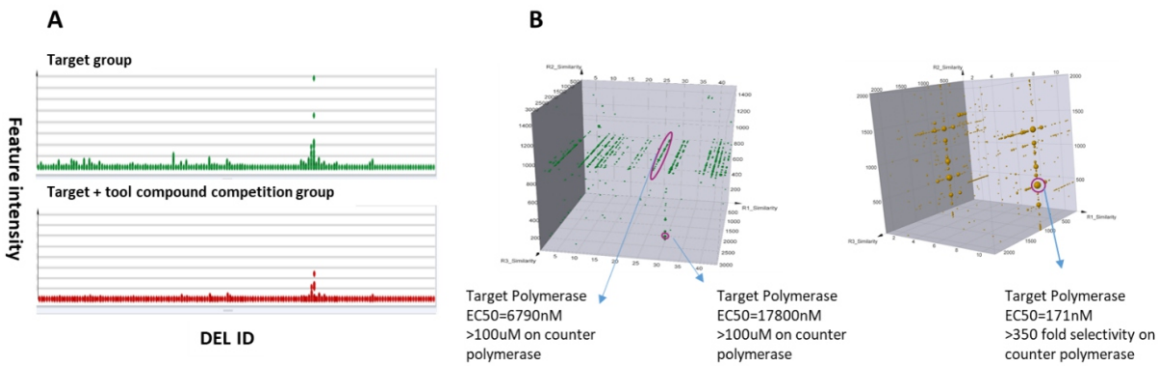


Figure 3. A) An example of screening output for a DNA polymerase target. B) Representative signals and hit parameters.

Computational approaches to find the needle in the haystack

In our pursuit of identifying small molecule ligands for DNA-binding proteins, it's crucial to discern the true positive signals from the noise introduced by the inherent affinity of these proteins for the DNA tags.

DEL for DNA- and RNA-Binding Proteins

At HitGen, we meticulously scrutinize the enrichment of DNA motifs from the Unique Molecule Identifier region (UMI region, which is a region with random bases) within the target samples, providing us with a comprehensive understanding of the overall DNA binding levels. Furthermore, we delve into the structure-signal relationship of the DEL molecules, enabling us to distinguish features that are enriched due to common DNA motifs, rather than pharmacophores on the DEL compounds. This rigorous process ensures us to minimize false positives and enhance the precision of our ligand discovery.

How about nucleases and RNA binding proteins?

Due to their DNA cleavage nature, DNA nucleases have been regarded non-compatible with DEL selection for a long time. With relentless efforts, HitGen is now able to conduct DEL selection for numerous nuclease targets by introducing proper mutations in those targets to abolish their DNA cleavage activities, and by depleting specific metal ions required for the reactions.

RNA helicases, RNases, RNA-dependent RNA polymerases, as well as other RNA processing/exporting proteins represent a large category of RNA-binding proteins. Although some RNA-binding proteins interact with DNA significantly, the binding affinity could be accurately assessed to evaluate the potential interference during DEL selection therefore presents less of a hurdle. At HitGen, we have successfully conducted multiple DEL screenings for various RNA-binding protein targets with validated hits identified.

DEL selection for DNA- and RNA-binding proteins at HitGen

HitGen is at the forefront of DEL design and screening, with numerous elegant strategies developed to tackle the most challenging targets. Contact us for DNA- and RNA-binding protein projects. Your chance of successful ligand development will be maximized with our demonstrated abilities:

- DNA binding compounds excluded from selection given the nature of DEL.
- Comprehensive screening solutions to cover various target types.
- Proprietary algorithms to double-check on DNA-binding false positives.
- Proven track record of our screening experience and successful cases.

DEL for RNA

While proteins have traditionally been the primary target class for drug discovery, the landscape is rapidly evolving. RNA targets have recently garnered significant attention, driven by advances in RNA interference technologies and the development of small molecule drugs like Risdiplam. Despite the progress in RNAi drugs, small molecules remain a crucial component in drug discovery due to their unique advantages such as cell permeability, stability, and the ability to modulate a broad range of biological targets. As a powerful tool for small molecule ligand discovery, DNA-encoded libraries (DELs) for RNA target hit identification presents unique challenges. HitGen's DEL technology is at the forefront of these efforts, uniquely equipped to navigate these challenges and pioneer the discovery of small molecule ligands for RNA targets.

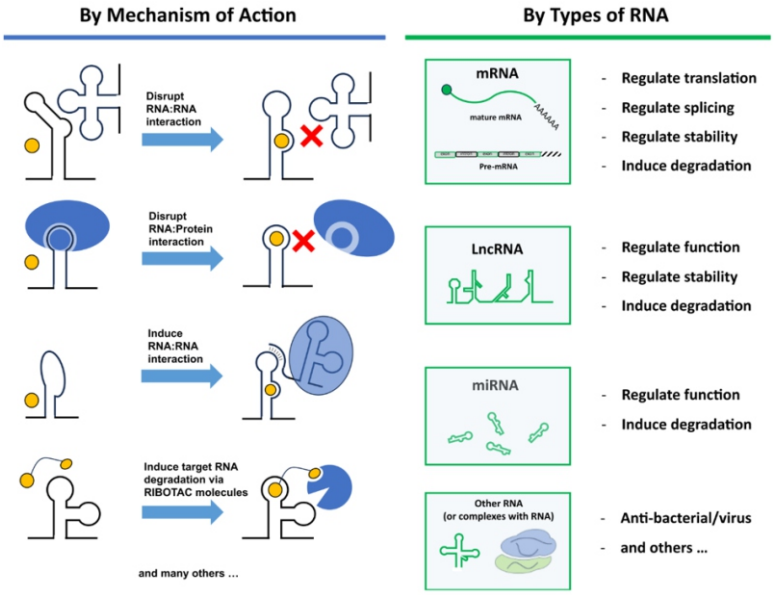


Figure 1. Small molecules targeting RNA (by mechanism of action and by RNA types).

DEL for RNA: Current challenges

Dynamic nature of RNA structures: RNA molecules are known for their dynamic nature, often forming complex secondary and tertiary structures. These structures can be vulnerable to certain DEL selection buffers, which can stabilize one conformation over others. This can lead to the enrichment of features in the DEL selection based on a single conformation, complicating downstream off-DNA compound confirmation due to the dynamic nature of RNA in physiological conditions.

Interactions between DNA tags and RNA targets: DELs rely on affinity-based methods for ligand discovery. However, interactions between the DNA tags of the DELs and RNA targets can introduce a significant number of false positive signals. This makes it akin to finding a needle (true positive) in a haystack (false positive signals), adding a layer of complexity to the discovery process.

Underexplored chemical space for RNA ligands: The knowledge about RNA binders is relatively limited

when compared to protein binders. The chemical space for RNA ligands remains largely underexplored, posing a challenge for the identification of effective small molecule ligands for RNA targets.

Pioneering small molecule ligand discovery for RNA targets at HitGen

At HitGen, we endeavor to overcome the above-mentioned challenges and to deliver robust small molecule ligands for RNA targets. Our approach includes:

Assessment of RNA conformation under various selection conditions beforehand: Prior to screening experiments, we conduct extensive bioinformatic analyses of RNA structures under different selection conditions. This allows us to identify and select the conditions that do not or only minimally impact RNA folding, ensuring the integrity and functionality of the RNA targets during the screening process.

Innovative screening methods and informatics approaches to control the interference of the DNA: RNA interaction: To minimize false positive signals during DEL screening, we deploy a variety of strategies such as the addition of competing oligonucleotides during both incubation and elution steps. In addition, proprietary algorithms were developed at HitGen to decrease the impact of the DNA:RNA interaction.

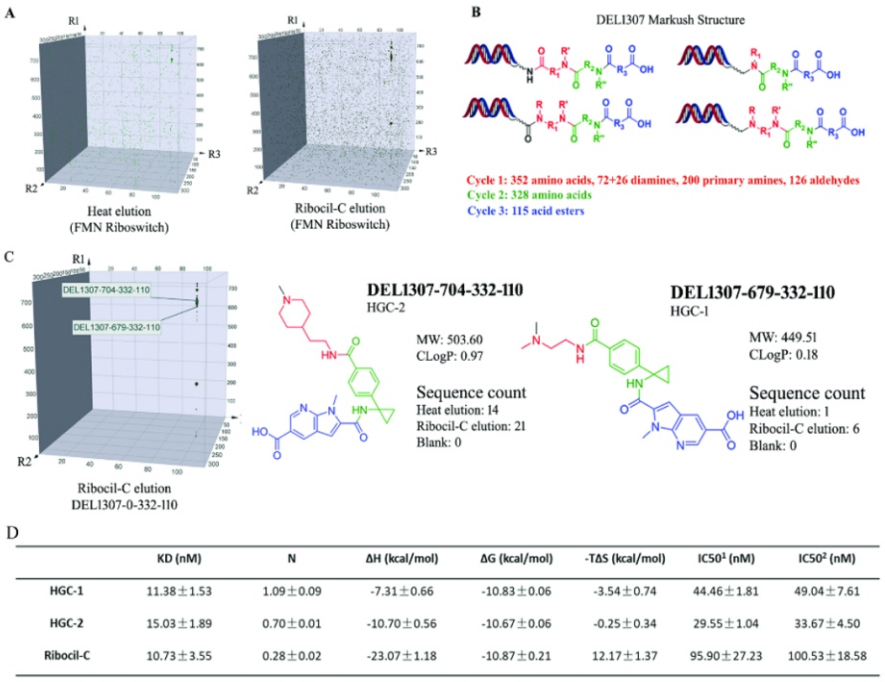
Exploring new frontiers with HitGen's DEL library of one trillion compounds: Our newest DEL library with over one trillion compounds provides an expanded repertoire to identify RNA ligands that may not fit into the traditional chemical space, which significantly increases the likelihood to discover robust small molecule ligands for RNA targets.

Collaborative and transparent partnership: At HitGen, we believe in the power of collaboration, and we work closely with our partners to ensure the success of their discovery projects. Our commitment to transparency means we share data openly and completely, but exclusively with our collaborators. Therefore, we not only deliver successful scientific findings, but also warrant the safety of their intelligence properties.

Showcasing success in small molecule ligand discovery for RNA targets

Our platform has already shown promising results in small molecule ligand discovery for RNA targets. We also published the detailed methods for applying DEL on RNA targets in Nucleic Acids Research (Nucleic Acids Res. 2022 Jul 8;50(12):e67.). Here are a few examples of our successful projects:

Novel inhibitors for E. coli FMN riboswitch: The FMN riboswitch is a class of RNA element that plays a crucial role in controlling bacterial growth by regulating gene expression in response to fluctuations in the cellular environment. Our platform has successfully identified novel inhibitors for the E. coli FMN riboswitch that are comparable the reference compound, ribocil C (Figure 2). These findings not only validate our approach but also open up new avenues for the development of effective antibacterial agents.



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	KD (nM)	N	ΔH (kcal/mol)	ΔG (kcal/mol)	-TΔS (kcal/mol)	$IC50^1$ (nM)	$IC50^2$ (nM)
HGC-1	11.38±1.53	1.09±0.09	-7.31±0.66	-10.83±0.06	-3.54±0.74	44.46±1.81	49.04±7.61
HGC-2	15.03±1.89	0.70±0.01	-10.70±0.56	-10.67±0.06	-0.25±0.34	29.55±1.04	33.67±4.50
Ribocil-C	10.73±3.55	0.28±0.02	-23.07±1.18	-10.87±0.21	12.17±1.37	95.90±27.23	100.53±18.58

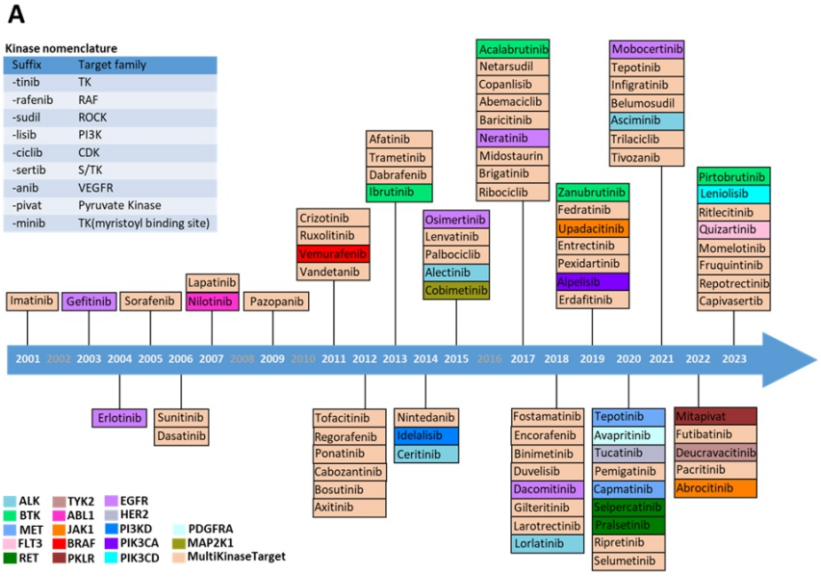
Figure 2. A-C) Representative features and compounds identified from HitGen's DEL for RNA selection.
D) ITC binding parameters and FMN inhibition potency.

Allele-specific RNA binders: In a significant breakthrough, our platform has been utilized to identify RNA ligands that demonstrate selective binding to a specific RNA motif, while effectively discriminating against a mutant allele with only a single nucleotide difference. This level of precision is achieved through careful design and incorporation of counter targets, which guide the selection of ligands with the desired specificity. This project is a testament to the power of our platform in addressing complex challenges in RNA-targeted drug discovery. As this project is a collaboration, the detailed data are confidential at this time. However, the success of this endeavor underscores our ability to work in partnership with other entities, respecting confidentiality and data security, while still achieving remarkable results.

With HitGen's DEL technology, the possibilities for small molecule ligand discovery for RNA targets are virtually limitless. Contact us and let our team of experts help you unlock the full potential of RNA targets for your drug discovery programs.

As one of the largest and most functionally diverse gene families, kinases have been recognized as key regulators/mediators of biological signaling networks. More than 30% of drug development efforts target these enzymes in the past two decades. As of January 2024, more than 90 small molecule kinase drugs have been approved by the US Food and Drug Administration (FDA), with approximately 200 orally available protein kinase inhibitors in clinical trials. These compounds have had a significant impact on the treatment of both oncological and non-oncological conditions.

Among the small molecule kinase drugs approved by the FDA, more than 50 are classified as multi-target drugs, representing the majority on the market (Figure 1 A). While the multi-target approach offers a broader therapeutic spectrum and enhances treatment flexibility and efficiency by acting on multiple related pathways, it also presents potential drawbacks. These may include increased risks of off-target effects and toxicity due to the broader activity profile, which can complicate the management of side effects and patient care. Only less than 40 of the FDA approved drugs are single-target kinase drugs, focusing on crucial kinase targets such as EGFR (Epidermal Growth Factor Receptor), BTK (Bruton's Tyrosine Kinase), BCR-ABL (Breakpoint Cluster Region-Abelson). These drugs demonstrate precision in targeting specific disease pathways, offering targeted therapies with potentially fewer side effects due to their specific mechanism of action. According to Cortellis CCI, 15 kinase drugs achieved sales exceeding 1 billion dollars in 2023, including 2 BCR-ABL inhibitors, 3 CDK4/6 (Cyclin-dependent Kinase 4/6) inhibitors, 2 BTK inhibitors, 3 JAK (Janus Kinase) inhibitors, and 5 other inhibitors targeting EGFR, BRAF (Rapidly Accelerated Fibrosarcoma B-type), ALK (Anaplastic Lymphoma Kinase), MET (Mesenchymal to Epithelial Transition factor) and VEGFR (Vascular Endothelial Growth Factor Receptor), respectively (Figure 1 B). The top-selling drugs, Ibrutinib, Palbociclib, and Ruxolitinib, are all first-in-class medications. Moreover, Alectinib, approved in 2015, has become the best-in-class drug for treating ALK-positive non-small cell lung cancer (NSCLC); Abemaciclib, approved in 2017, is believed to have the potential to surpass Palbociclib as the best-in-class treatment for ER-/HER2- (Estrogen Receptor-/Human Epidermal growth factor Receptor 2-)breast cancer.



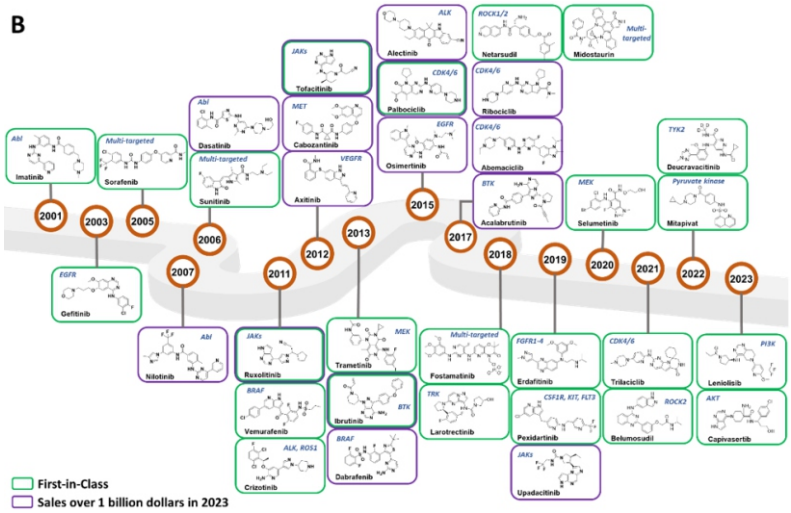


Figure 1. FDA approved small molecule kinase drugs since 2001. A) Drugs categorized by target types. Drugs are ordered by the year of approval and drug targets are represented by boxes with different colors. B) Structures of representative drugs. The first-in-class drugs are in green boxes and drugs with sales over $1 billion in 2023 are in purple boxes, the main targets are labeled.

Key challenges for kinase inhibitor discovery

Selectivity

Due to the high degree of homology in the ATP-binding sites, achieving selectivity and circumventing off-target interactions in kinase inhibitor design has been a significant challenge. The simultaneous inhibition of several protein kinases may bring potential advantages by targeting more than one target, however, this is more often related to toxicity and unwanted side effects. For example, the first-generation JAK inhibitors (Tofacitinib, Incyte's Ruxolitinib, Baricitinib, Peficitinib, and Delgocitinib) are pan-JAK inhibitors, exhibiting similar interaction patterns with JAK1, JAK2, JAK3, and TYK2. They bind within a hydrophobic pocket composed of residues L881, A906, V938, L959, and L1010 (based on JAK1 numbering) and form hydrogen bonds with hinge region residues (Figure 2 A). These non-selective inhibitors target multiple JAKs and block several associated signaling pathways, which leads to severe side effects including infections and anemia. However, through continuous research and development, several second-generation JAK inhibitors have been approved up to date (Figure 2 B), they selectively inhibit JAK family members, thereby suppressing specific disease-related signaling pathways while preserving the function of other cytokines.

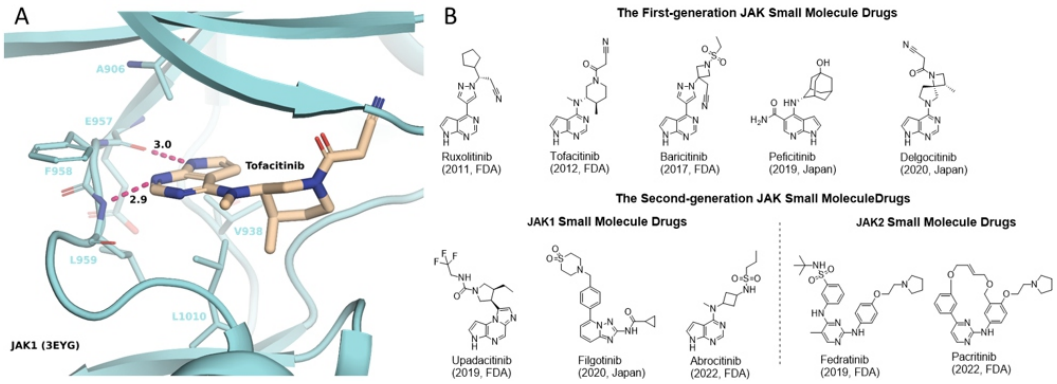


Figure 2. A) Tofacitinib forms two hydrogen bonds with hinge region residues of JAK1 (PDB: 3EYG). B) Chemical structures of representative JAK Small Molecule Drugs.

Resistance

With the emergence of kinase mutants, the kinase inhibitors become less efficacious and newer generation inhibitors are needed. Next-generation sequencing is utilized to detect the driver mutations and structural analysis of the mutant sheds light on potential drug design strategies. In the case of EGFR, first and second generation inhibitors were approved for the treatment of non-small cell lung cancer. However, the T790M mutation, with increased affinity for ATP, exhibited resistance and made the inhibitors ineffective, the inefficacy of the first and second generation inhibitors necessitated the development of the third generation covalent inhibitors, specifically targeting the 797 Cysteine (Figure 3). Nevertheless, the emergence of new resistance mutant C797S abolished the covalent formation. Currently, there is a pressing need for fourth-generation EGFR inhibitors that can overcome the C797S mutation, which is still in the developmental stage. Therefore, the requirement for effective hit-finding tools for the discovery and development of kinase inhibitors against the mutants, meanwhile keeping good selectivity over kinase panel, is paramount.

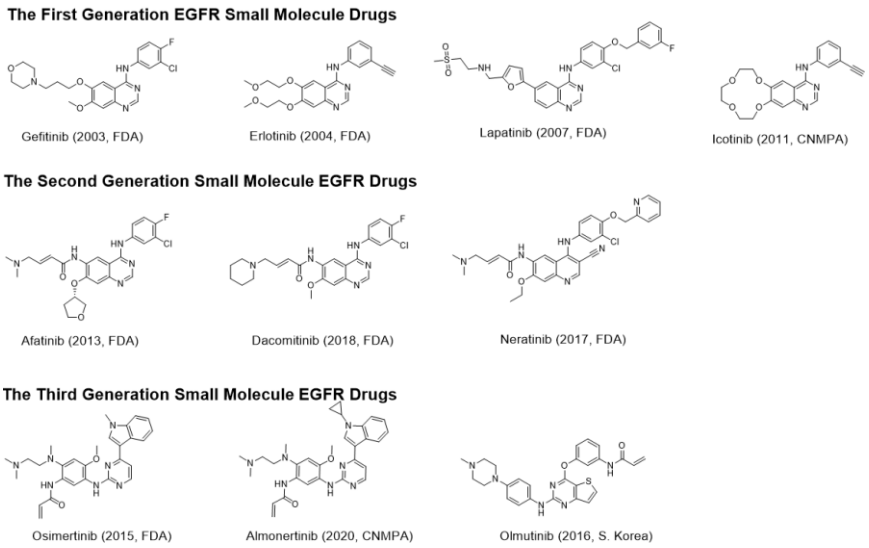


Figure 3. Chemical structures of representative EGFR Small Molecule Drugs.

DNA encoded library (DEL) technology, as a structural hypothesis free approach, is capable of screening billion to trillion diverse molecules and potentially identify compounds selectively interacting with the target kinase. This is exemplified by the discovery of the Receptor Interacting Protein 1 (RIP1) Kinase inhibitor, with minor optimization of the original hit from DEL, the candidate GSK2982772 progressed into clinical trial (J. Med. Chem. 2017, 60, 4, 1247–1261) (Figure 4). Actually, kinase inhibitor discovery via DEL screening has gained a lot of traction recently, which is evidenced by many HitGen partners' kinase inhibitor discovery projects. Our comprehensive understanding of kinase biology and rich screening data provide a very effective way of identifying potent and selective inhibitors for more kinases.

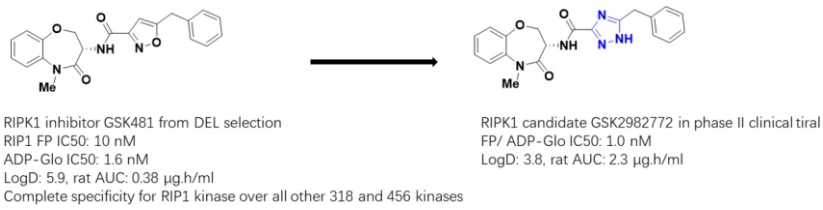


Figure 4. RIPK1 inhibitor discovered from DEL selection and the optimization to clinical candidate.

» "Smart Selection Campaign" for inhibitor selectivity

As one of the key advantages for DEL technology, multiple selection experiments could be conducted in parallel. With our "Smart Selection Campaign" strategy, the selectivity of DEL compounds against different kinases can be defined at the very beginning. For example, a tyrosine kinase target has multiple family siblings who share a highly conserved carboxyl-terminal kinase domain and a relatively unique amino-terminal domain. In order to achieve good selectivity of the compounds against this particular kinase, four closely related kinases (A-D) were included in the DEL selection campaign as counter-screen samples. Upon filtering out all overlapped binders, specific binders can be selected for synthesis and validation. The selection results of these 5 samples and compound validation confirming good potency and selectivity are shown in Figure 5.

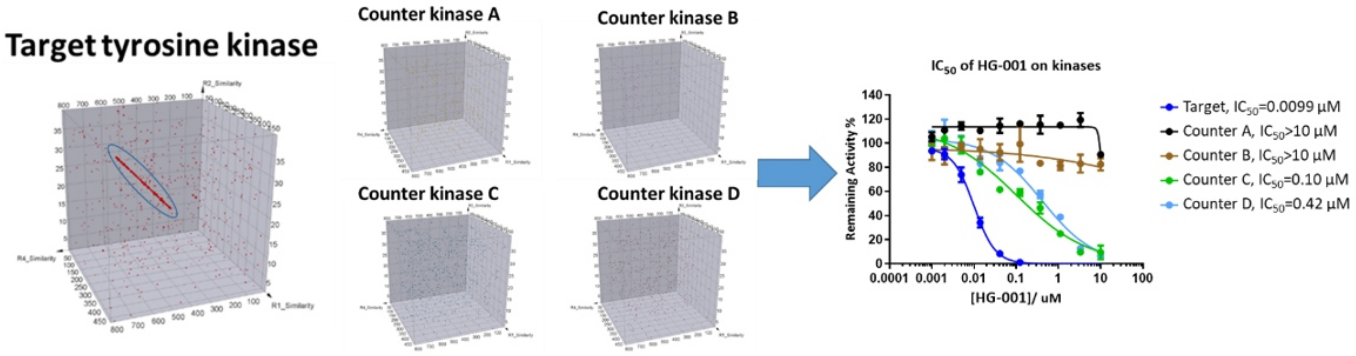


Figure 5. DEL selection results including target kinase as well as four count-screen kinase targets and compound validation for potency and selectivity.

» Identification of kinase inhibitors with different MoA and mutant selectivity

As a mature target class of drug discovery, kinase inhibitors for the treatment of diverse types of diseases have proven successful. The most recent effort has also been extended to exploration of unique MoA (Mechanisms of Action) with kinase inhibitors. Tool compound(s) properly conjugating with DNA tag(s) could be used as spike-in reference(s) in the DEL selection to assess the pocket accessibility. With potent tool compound(s) occupying pocket(s) of interest, we are able to fine tune the pocket/binding site mapping for DEL selection (Figure 6A). By incorporating the mutants and wild type proteins in the same selection campaign and using the known selective inhibitor to differentiate potential binding sites, mutant selective kinase inhibitors were identified (Figure 6B).

A

Compound ID	Representative hit from DEL series-1, ATP site binder	Representative hit from DEL series-2, allosteric binder	Representative hit from DEL series-3, allosteric binder
Target 1, Ki	23nM	10nM	36nM
Counter Target 2, Ki	163nM	>686nM	No inhibition
Selectivity ratio	7	>68	Absolute selectivity

B

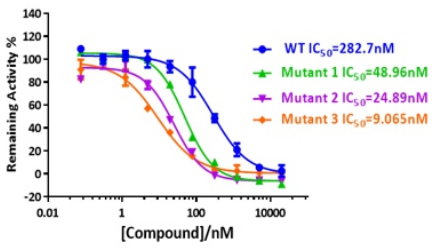


Figure 6. Multiple hits with different MoA (A) and mutant selectivity (B) have been identified against different kinases.

Targeting different kinase subfamilies with catalytic domain and/or regulatory domain

Kinases can be further classified according to their capability to phosphorylate different residues of the substrates, the kinases screened with HitGen DELs are summarized in Figure 7A. Regardless of their subtypes, almost all kinases share a similar catalytic domain and many kinases also contain regulatory domains. Therefore, a fundamental question to discover a kinase inhibitor is whether to target the catalytic domain or the regulatory (non-kinase) region.

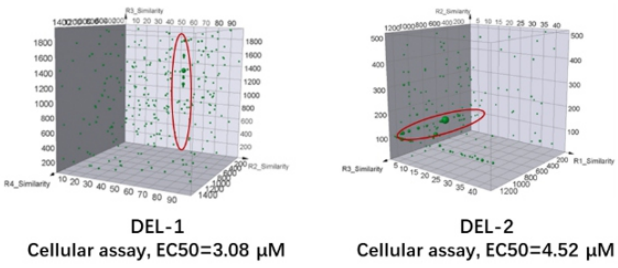
Lipid kinases: The intrinsic structural differences of lipid kinases with protein kinases provide possibilities to identify selective inhibitors. The regulatory subunits of lipid kinases usually harbor binding pockets for potential small molecule inhibitors. This offers a good opportunity to identify novel kinase inhibitors, not only because of their pocket accessibility, but also the potential better selectivity by avoiding targeting the more conserved catalytic domains.

Pseudokinases: Pseudokinases usually harbor similar 3D structures as their catalytically active siblings, making them potentially attractive targets for DEL selection. Indeed, occupancy by competitive ligands of the ATP binding sites could result in potential interruption of the downstream pathway, whereas the conformation changes introduced by the allosteric ligands would likely lead to regulatory disruption, and these binders could be further developed into targeted degraders. By focusing on the non-catalytic pseudokinase domain, HitGen has identified multiple series of hits that have been later confirmed to be direct regulatory interrupters (Figure 7B).

A

Kinase class	Percentage	Potency of representative hits on the protein
Serine/threonine kinase	56%	1.9 nM
Tyrosine kinase	21%	5.4 nM
Lipid kinase	15%	4.9 uM
Serine/threonine and tyrosine kinase	5%	1.4 nM
Pseudokinase	3%	4.8 nM

B



DEL-1
Cellular assay, EC50=3.08 μM

DEL-2
Cellular assay, EC50=4.52 μM

Figure 7. A) Distribution of kinase subtypes screened at HitGen and potency of representative hits.
B) Pseudokinase domain-focused DEL selection for kinase subfamilies with hits identified.

Discover your kinase inhibitors at HitGen

Discovery of kinase inhibitors is about compound potency, selectivity, novelty and patentability. With our proprietary fine-tuned trillion libraries, rich DEL selection experiences, utilization of accumulated selection data as filters, the identification of kinase inhibitors using HitGen DELs has proven to be fast, cost effective and most importantly guarantees success rate.

DEL for Enzymes with Specific MOA

Enzymes are arguably the most ideal targets for small molecule drugs, owing to their involvement in chemical reaction catalysis and the better ligandability of the binding pockets. Although high-throughput screening (HTS) of inhibitors for enzyme targets has been well established, DNA encoded library (DEL) selection holds unique advantages, especially for those enzyme targets with multi-binding pockets. This is because the different conformations and binding pockets of those enzymes could be investigated at the same time, making the identification of truly differentiated ligands with desired mode of action (MoA) possible. In our previous series, DEL selection for enzymes like kinases and DNA binding proteins has already been reviewed, and therefore in this chapter we mainly focus on enzymes with multi-substrates and unique reaction mechanisms, as well as the ones that bind to specific undesired moieties.

DEL selection for enzymes with compulsory ordered reaction mechanism

While most activity-based screenings yield functional hits, the affinity-based DEL selection presents unique advantages by providing ligands with desired MoA and therefore higher likelihood of successful drug efficacies. This is particularly beneficial for those targets with multiple substrates. For example, Naa50, an enzyme that utilizes a compulsory ordered reaction pathway, in which acetyl-CoA binds first, revealing a binding pocket for the recognition by a second substrate. The detailed understanding of this reaction mechanism helped us design a delicate DEL selection plan, leading to the identification of hits with single digit nM potency (ACS Med Chem Lett.2020 Apr 10;11(6):1175-1184, Figure 1).

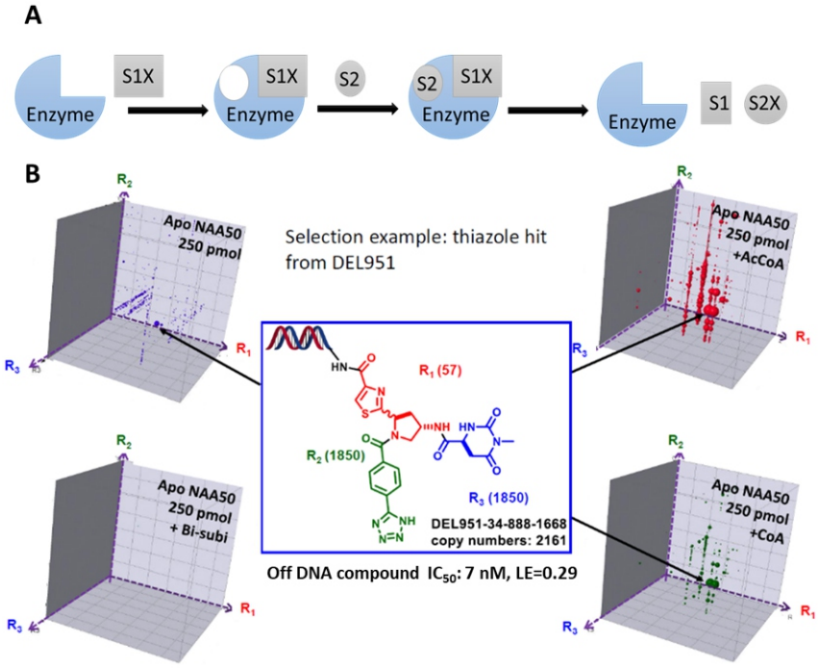


Figure 1. A) Compulsory ordered reaction pathway. B) Naa50 inhibitor identified from DEL selection with a unique binding mechanism.

DEL selection for enzymes with ping-pong reaction mechanism

Ping-pong mechanism is another typical enzymatic reaction pathway that proceeds in two sequential steps. During such a reaction, the enzyme first catalyzes the turnover of one substrate to form an enzyme-X intermediate complex, and then releases the first product; only after that the second substrate could bind to the enzyme and proceed to generate and release the second product. At HitGen, DEL selections are carefully designed to include the intermediate forms of interested targets in addition to the apo enzyme, which offers unique opportunities to investigate the target structures that represent distinct conformations. Therefore, we significantly increase the chance to identify hits with desired MoA (Figure 2).

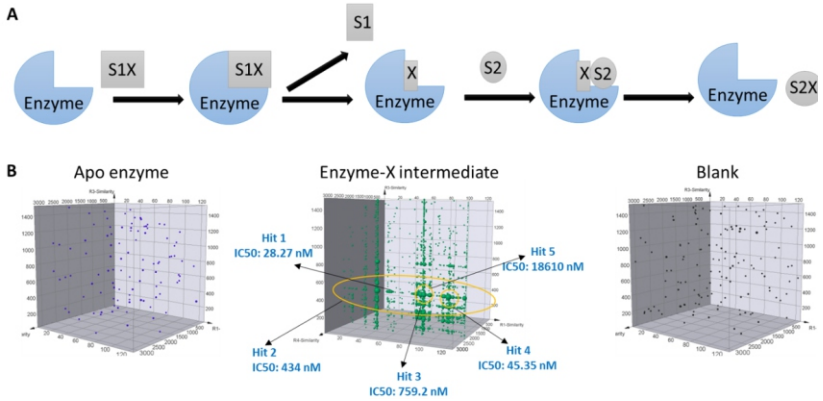


Figure 2. A) Ping-pong reaction pathway. B) DEL selection signal comparison for different forms of target, and multiple inhibitors were identified from DEL selection for an enzyme target with ping-pong mechanism.

DEL selection for enzymes with desired competition mechanism

Competitive, non-competitive, and uncompetitive inhibitions are major mechanisms of reversible inhibitors. A selection that includes a physiological substrate or a well-characterized ligand of the interested enzyme could help identify hits with different competition MoAs. On the other hand, some targets such as tyrosine phosphatases might have basic or acidic binding pockets or interaction moieties. Although it is relatively easy to identify binders for these targets, further optimization from these hits for pharmaceutically acceptable ligands is usually challenging due to their poor physical/chemical properties. Incorporating a catalytic site binder during DEL selection could help rule out identification of more active site binders, but to allow the identification of binders to potential allosteric sites. With such a design, a DEL selection offers opportunities to identify hits with more desired pharmaceutical properties. Figure 3 illustrates an example that utilizes active site blocker to identify ligands binding to allosteric sites.

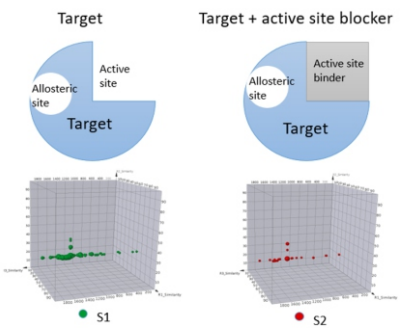


Figure 3. Schematic of including active site binders to differentiate allosteric site binders.

DEL for Enzymes with Specific MOA

Enzymes are the primary effectors in physiological conditions and account for nearly half of drug targets, and therefore identification of ligands targeting enzymes provide opportunities to cure different diseases. At HitGen, we offer DEL selections that help your exploration of enzyme targets with quick turn-around time, low cost and unique advantages:

- Investigation of enzymes at specific conformations or distinct structures.
- Convenient exploitation of enzyme ligands at orthosteric and allosteric sites.
- Quick inclusion of counter targets at low expenses.

DEL for Covalent Ligand Discovery

Although many covalent drugs were serendipitously discovered historically, covalent inhibitors were in general discouraged due to the concerns over their interference with biological assays and potential lack of selectivity. Over the past decades, rational design of covalent inhibitors has gained traction and garnered increased interest, particularly due to their stronger potency, prolonged target engagement, increased selectivity, and effectiveness to some drug-resistance mutants to reversible inhibitors.

Covalent Ligand Discovery Approaches

Reversible ligands can be rationally designed to covalent inhibitors by incorporating reactive warheads to proper positions. The reversible portion offers binding affinity (Ki) while the electrophile reacts with specific amino acids (cysteine, serine, lysine, etc.) and offers covalent interaction (Kinact). The discovery of EGFR (Epidermal Growth Factor Receptor) and BTK (Bruton's Tyrosine Kinase) covalent inhibitors shared this strategy with ligand discovered first and Michael acceptor electrophiles incorporated later (Figure 1).

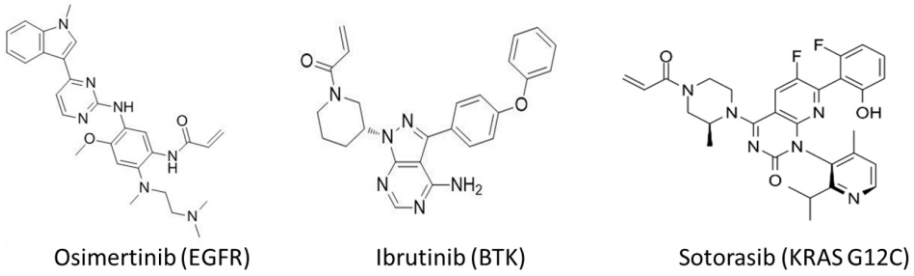


Figure 1. Examples of covalent drugs.

Direct screening for covalent libraries also provides an effective strategy for covalent ligand discovery, which is nicely exemplified by the discovery of KRAS G12C initial hits that led to the ultimate approval of Sotorasib (AMG510, Figure 1). Mass spectrometry, activity-based protein profiling (ABPP), as well as covalent DNA encoded libraries (DELs) represent the main experimental approaches. Although the first two approaches are straightforward and offer good selectivity, the throughput is relatively low, whereas the covalent DEL selection stands out with unique advantages of high throughput, low cost and fast investigation of large chemical space. On this front, HitGen has applied comprehensive exploration on covalent selection spanning DEL design, selection optimization, and compound characterization, leading to multiple successful covalent DEL screenings.

HitGen Covalent DELs

HitGen covalent DELs are composed of hundreds of millions of proprietary, drug-like binding motifs conjugated to tens of diverse covalent warheads (Figure 2, library design; Figure 3, examples of off-DNA structures). Specifically, binding motifs are constructed with several hundreds of proprietary

scaffolds and more than 30,000 building blocks, resulting in over hundreds of millions of expandable compounds for covalent warheads to attach to. Different sets of covalent warheads are conjugated to these binding motifs based on the specific type of targets. For example, Michael acceptors and similar Cys-targeting electrophiles are used for cysteine-containing proteins. The expanded covalent warheads increase the probabilities of direct discovery of covalent inhibitors when the binding motifs are not strong enough, also the extended functionality on warheads may potentially pick up interactions therefore generating additional specificity. Similarly, the covalent DELs have specific sets of covalent warheads for Lys-, Ser- targeting proteins.

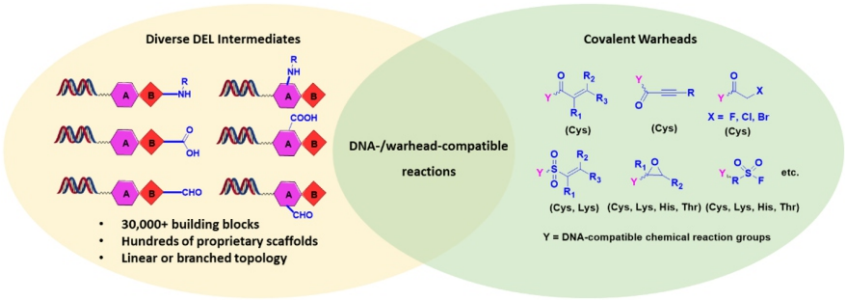


Figure 2. Covalent DEL design at HitGen.

At HitGen, the reactivities of the warheads are carefully examined and only the warheads with proper reactivity are chosen for covalent DEL synthesis, furthermore, the stabilities of these warheads are also carefully evaluated to ensure they are stable enough in the harsh conditions of DEL purification and DEL selection. The qualities of our covalent DELs, especially their warhead activities, are also periodically examined against representative targets.

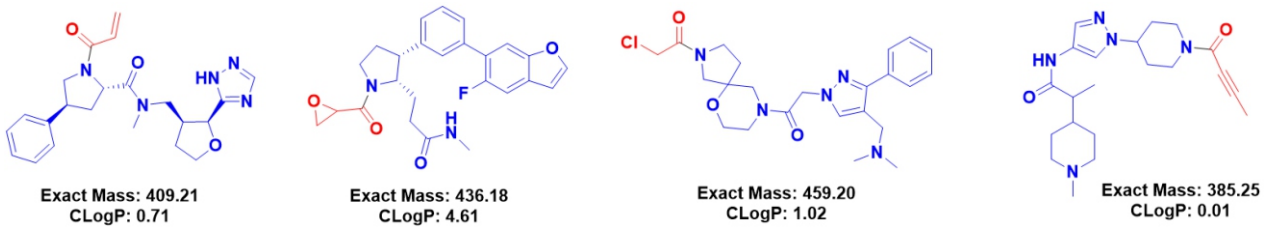


Figure 3. Examples of off-DNA structures of some HitGen covalent DEL compounds (warheads are shown in red).

Suitable Targets for Covalent Ligands

- Targeting kinases with non-conserved cysteine(s) near the ATP binding site to improve potency, and in some cases, to overcome drug resistance due to mutations (e.g. EGFR, BTK, JAK3).
- Targeting proteinases with crucial catalytic nucleophilic amino acid(s) (e.g. deubiquitinases, SARS-CoV-2 main proteases).
- Targeting enzymes with cysteine-ubiquitin conjugate formation during the catalysis (e.g. E2s, HECT and RBR E3s).
- Targeting other proteins with Cys, Ser, Lys, or Thr in or near the binding pocket for either inhibitor development or conversion to degraders.

Covalent DEL Selection

Given the "Bind and React" nature of covalent compounds, covalent DEL selections are performed with multiple parallel conditions to differentiate the compounds that bind and react with specific nucleophilic amino acid(s). Several DEL selection parameters including reducing agents, DEL input, DEL size and incubation time should be optimized before the selection. In addition, the positive control - DNA conjugate, mutants of nucleophilic residues and covalent tool compound blockings are frequently used to increase the confidence of finding positive hits. All these practices have been extensively explored at HitGen (Figure 4), leading to our comprehensive understanding of the covalent DEL selection process as well as demonstrated successful selection cases.

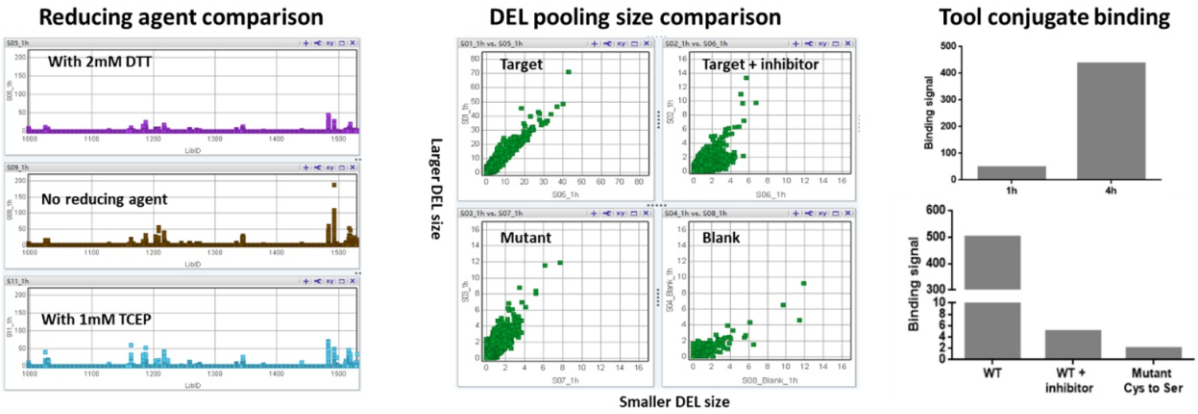
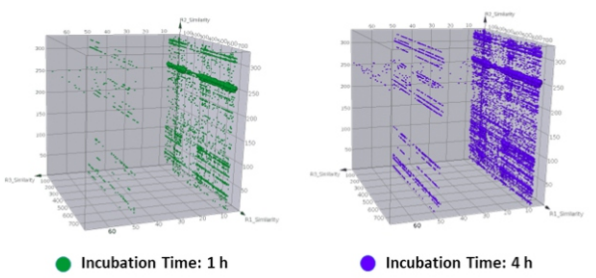


Figure 4. Covalent DEL selection exploration (selection of reducing agents, DEL pooling size comparison, selection of incubation times, inhibitor blocking and mutant comparison).

Case Study: identification of cysteine protease inhibitors

Selection Plan

No	Target	Compounds	Incubation	Purpose
1	Target-Cys	Covalent DELs	1h	Sample 1 vs 2 comparison to Identify compounds showing increasing signals in selection results
2	Target-Cys	Covalent DELs	4h	
3	Target-Cys	Covalent DELs + Inhibitor	1h	Sample 3 vs 1 & Sample 4 vs 2 comparison to Identify compounds competing with reference inhibitor
4	Target-Cys	Covalent DELs + Inhibitor	4h	
5	No Target	Covalent DELs	4h	Identify binders being captured by matrix



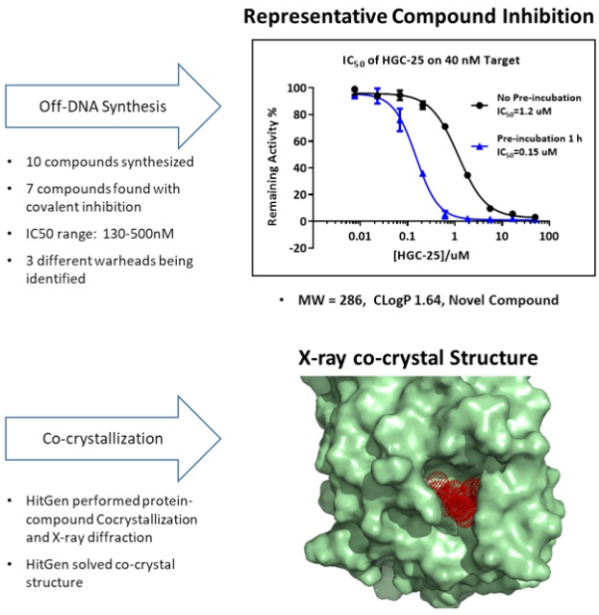


Figure 5. Covalent DEL selection plan, selection results, time-dependent inhibition and structure confirmation of covalent attachment to Cys.

Characterization of Covalent Ligands

Compared to classical reversible inhibitors, covalent compounds hold some unique features. First of all, the irreversibility reflects a time-dependent inhibition and a non-linear progression curve, compromising the accuracy of using IC50 for covalent compound profiling and ranking. Indeed, the Kinact/Ki represents a more meaningful parameter and has become the key metric for covalent compound characterization and the structure-activity-relationship (SAR) analysis. In addition, the irreversibility of the binding can also be validated by mass spectrometry, the jump-dilution experiment, dialysis, and the utilization of mutant proteins (Figure 6).

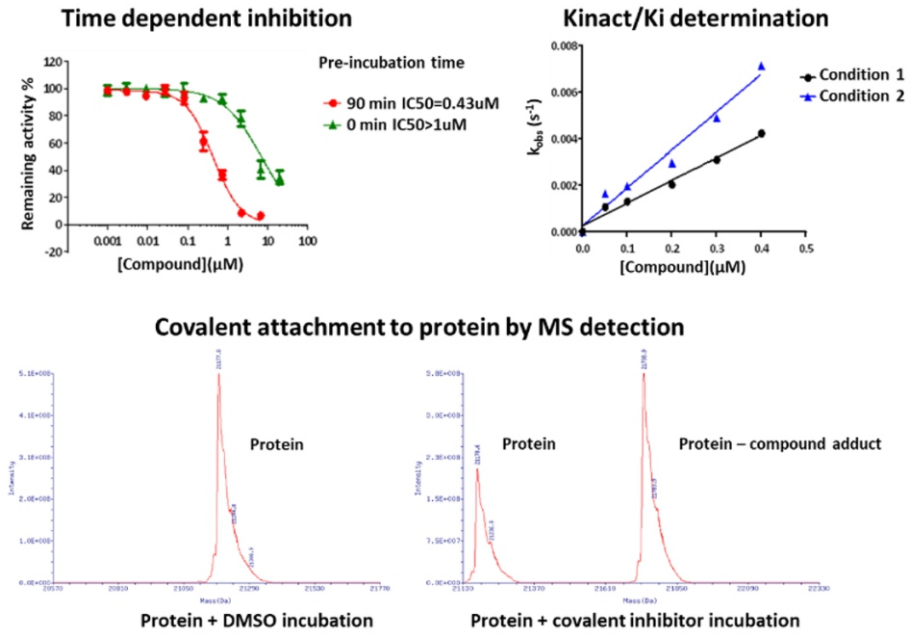


Figure 6. Representative covalent compound validation methods.

Outlook

Exploration of covalent ligands for direct inhibition of challenging targets has emerged as a very attractive approach for modern drug discovery. Covalent DEL screening, with its inherited large chemical space accessibility, less material consumption and the excellent screening efficiency, will be a very powerful approach to advance the field. With HitGen's billion-scale, well-validated covalent DELs, rich experiences in DEL selection and comprehensive covalent profiling capabilities, your covalent ligand discovery effort will be significantly accelerated.

DEL for Macrocycles

Macrocycles[1] are generally defined as organic compounds with a ring of at least 12 atoms and hold in a chemical space between small molecules and biologics (Figure 1). With the constrained but somewhat flexible conformation, macrocycles often show higher affinity and selectivity to targets compared with their linear analogues. Therefore, macrocycles constitute an excellent tool for the targets which are difficult to modulate by traditional small molecules. With the enormous structural diversity, many of macrocycles display a wide range of functional capability and remarkable biological activities, which makes them as an important modality in drug discovery. More than 60 macrocycles have been approved as drugs by the U.S. Food and Drug Administration. Most of the macrocyclic drugs are natural products or their derivatives. Infectious disease, oncology, autoimmune disorders, and immunosuppressants are the major therapeutic indications of macrocyclic drugs.

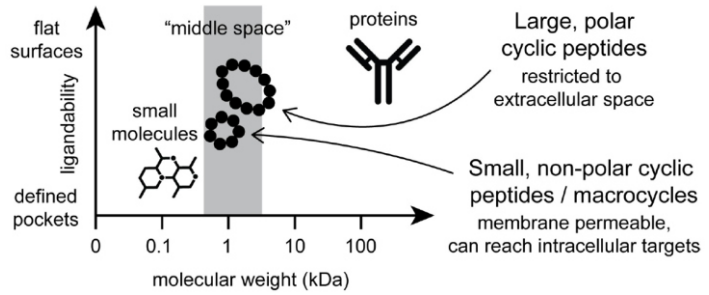


Figure 1. Comparison of macrocycles to small molecules and biologics.[1b]

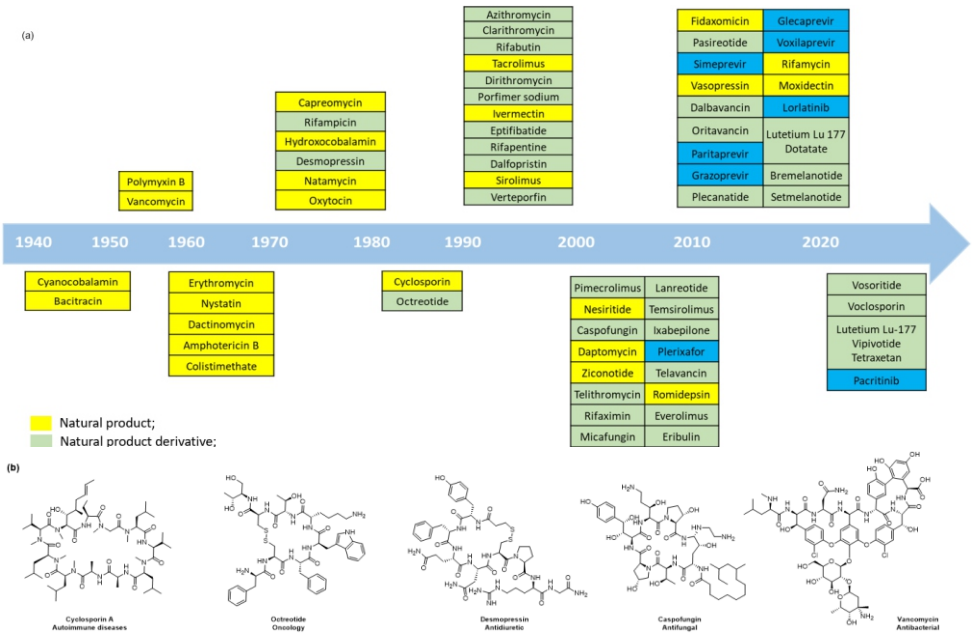


Figure 2. (a) Macrocyclic drugs approved by FDA[1a]; (b) Examples of macrocycle drug structures.

In addition to being used as drugs, macrocycles have also been widely applied in disease diagnosis and drug delivery[2]. There are several kinds of payloads, including: (1) Radionucleotides: Lutathera® ([^{177}Lu] Lu-DOTA-TATE) was approved by FDA in 2018 for treating somatostatin inhibitor receptor-positive gastroenteropancreatic neuroendocrine tumors;[2c] (2) Organic fluorophores: Combination of organic fluorophores and cyclic peptides could generate useful probes for biological assays targeting specific proteins;[2d] (3) Peptides: Qiu et al. reported cyclic RGD-peptide-functionalized polylipopeptide micelles for enhanced loading and targeted delivery of monomethyl auristatin E;[2e] (4) Nucleic acids: Hirano et al. reported magainin 2-derived stapled peptides for intracellular delivery of nucleic acids such as pDNA, mRNA, and siRNA.[2f]

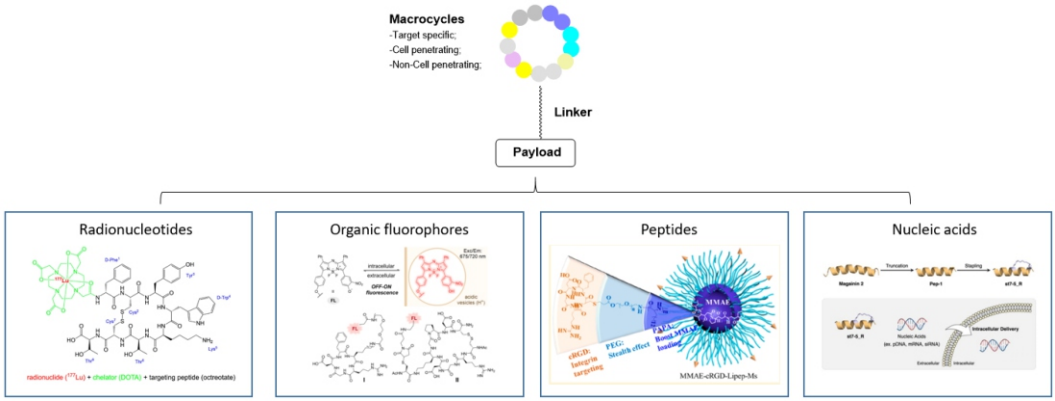


Figure 3. Conjugates with macrocycles.[2]

Properties of macrocycle drugs

Properties of FDA-approved macrocyclic drugs and clinical candidates have been analyzed.[1a] Macrocycles usually occupy in chemical space beyond the Rule of 5 (bRo5), but 30-40% of the drugs and clinical candidates are orally bioavailable. Simple models (HBD ≤ 7 in combination with either MW < 1000 Da or cLogP > 2.5) could be used as a first filter to assess whether a macrocycle is oral or parenteral (Figure 4). This analysis will provide the guidance for the design of macrocycles in the future.

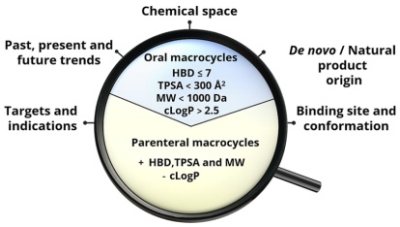


Figure 4. Properties of macrocycle drugs.[1a]

Methodologies for macrocyclic drug discovery

The discovery of macrocyclic drugs is predominantly stemmed from natural products or their derivatives. However, some of the natural products have the complex structures and are difficult to be synthesized by traditional small molecule chemistry. Therefore, de novo design and synthesis of macrocycles have attracted much attention. With the development of macrocycle synthesis and screening technology, phage display and mRNA display have enabled the de novo discovery of macrocycles. However, both technologies are limited to mostly natural amino acids as building blocks and the restricted monomers limits the overall macrocycle diversity.

As compared to biological display methods, DNA-encoded library technology (DELT)[3] can easily incorporate a wide range of unnatural amino acids as building blocks. The combination of unnatural elements and various macrocyclization methods could expand the chemical space and structural diversity of macrocycles. Rational design and building blocks selection could modify the skeleton and side chain of macrocycles, which have the potential to improve the properties of macrocycle molecules generated. In addition to finding ligands to the targets directly, DEL selection can afford much information of enriched macrocycles, which might be a good starting point for pharmacokinetic property optimization to achieve membrane permeation or oral bioavailability. With billions of DNA-tagged macrocycles and high throughput screening, macrocyclic DELs stands out as a practical technology for the discovery of macrocyclic hits. Many macrocyclic binders against various targets have been discovered by DELT (Figure 5).

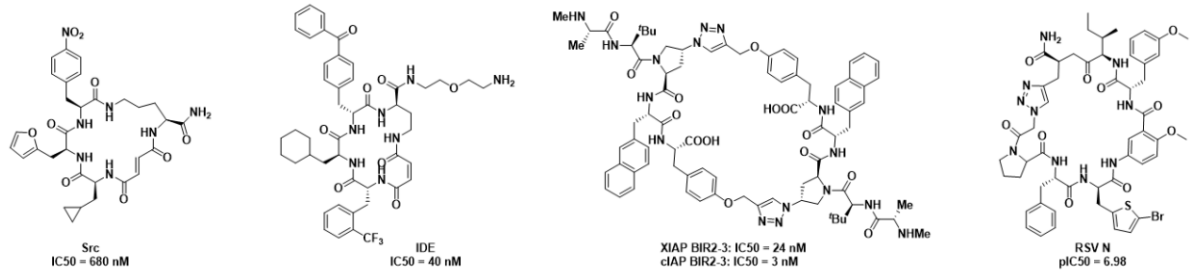


Figure 5. Examples of discovered macrocyclic hits from DELT.[3]

HitGen Macrocyclic DELs

High-quality of macrocyclic DELs with different ring sizes have been designed and synthesized at HitGen (Figure 6) and this ready-made macrocyclic DELs collection can be directly used for target screening. Key features of macrocyclic DELs at HitGen are:

- Library size: >40 Bn compounds.
- Diversity of building blocks: >500 natural and unnatural amino acids and >300 di/tri/tetra-peptides.
- Peptide length within rings: 2-13 amino acids.
- Introducing of special amino acids including N-methyl building blocks and lipophilic side chain to modify properties of macrocycles such as MW, LogP, HBA/HBD etc.
- Macrocyclization strategy: click chemistry, thioether formation, disulfide formation and other novel cyclization methods.

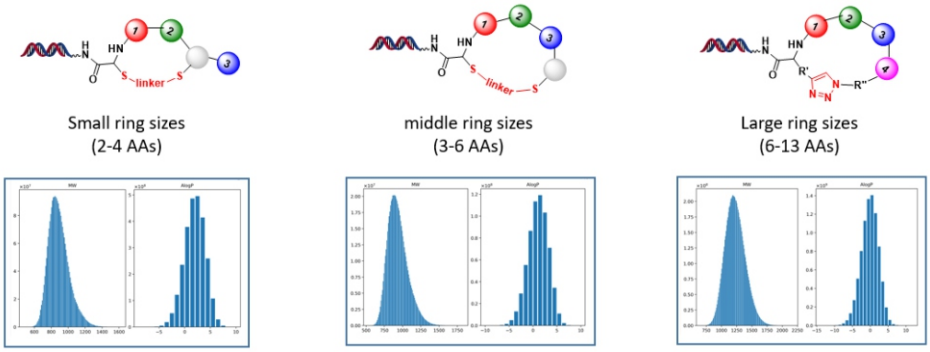


Figure 6. Macrocyclic DELs at HitGen.

Application of macrocyclic DELs

HitGen has rich experiences in macrocyclic DEL selection. Our macrocyclic DELs has been used for various targets screening. A successful selection case of macrocyclic DELs are showed in Figure 7.

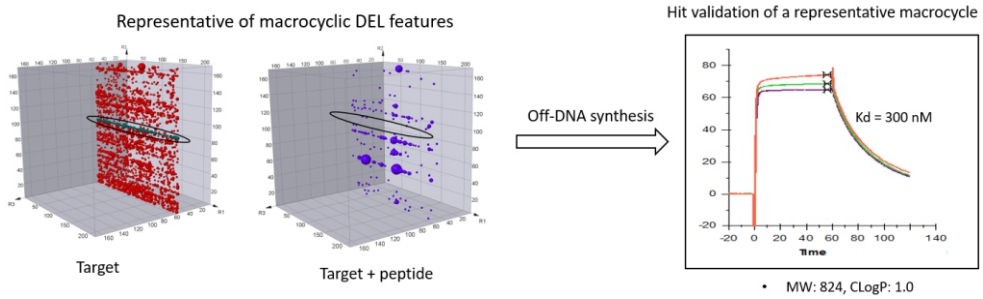


Figure 7. Macrocyclic DEL selection results and hit validation.

In addition to finding macrocyclic lead compounds directly, the macrocyclic DEL could be a good starting point for transporting cargos including small molecules, antibody, nucleic acids etc. (Figure 8).

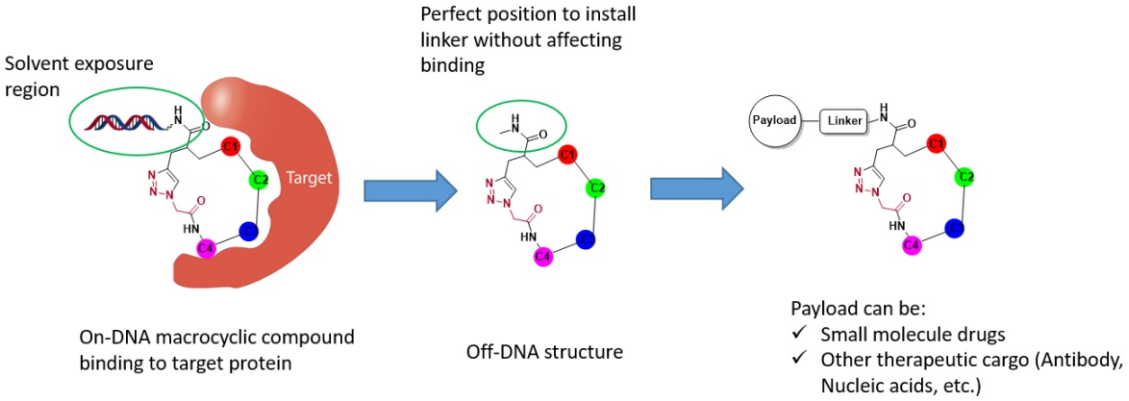


Figure 8. Application of macrocyclic DELs for delivery system.

HitGen PCP Platform

By integration of parallel peptide synthesis, peptide DEL construction and screening etc., HitGen has established a comprehensive peptide-conjugate platform (PCP) (Figure 9). This platform can provide the synthesis of specific amino acids/peptides (used for DEL library design and build), peptide conjugates synthesis (validation of activity before/after DEL selection), peptide DEL design and construction, DEL selection and high throughput off-DNA synthesis of peptides.

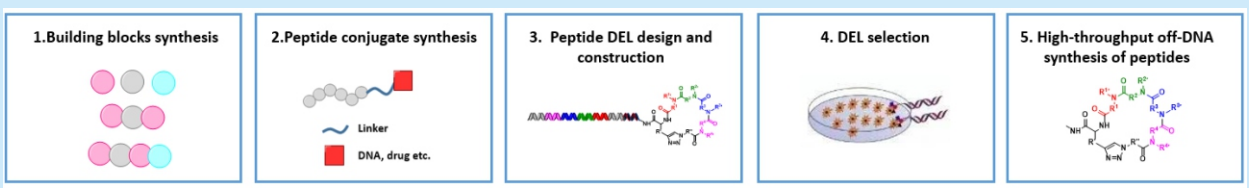


Figure 9. HitGen PCP Platform.

HitGen services include:

- Custom macrocyclic/linear peptide DEL build.
- >40 Bn macrocyclic DELs for selection of targets.
- High throughput off-DNA synthesis of peptides.
- Synthesis of macrocyclic/linear peptide conjugates.

Reference:

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[2] (a) Gong, L.; Zhao, H.; Liu, Y.; Wu, H.; Liu, C.; Chang, S.; Chen, L.; Jin, M.; Wang, Q.; Gao, Z.; Huang, W. Research advances in peptide–drug conjugates. Acta Pharmaceutica Sinica B, 2023, 13, 3659. (b) Cooper, B. M.; Iegre, J.; O' Donovan, D. H.; Halvarsson, M. Ö.; Spring, D. R. Peptides as a platform for targeted therapeutics for cancer: peptide–drug conjugates (PDCs). Chem. Soc. Rev. 2021, 50, 1480. (c) Hennrich, U.; Kopka, K. Lutathera®: The First FDA- and EMA-Approved Radiopharmaceutical for Peptide Receptor Radionuclide Therapy. Pharmaceuticals 2019, 12, 114. (d) Mendive-Tapia, L.; Wang, J.; Vendrell, M. Fluorescent cyclic peptides for cell imaging. Peptide Science 2021, 113, e24181. (e) Qiu, M.; Wang, X.; Sun, H.; Zhang, J.; Deng, C.; Zhong, Z. Cyclic RGD-Peptide-Functionalized Polylipopeptide Micelles for Enhanced Loading and Targeted Delivery of Monomethyl Auristatin E. Mol. Pharmaceutics 2018, 15, 4854. (f) Hirano, M.; Yokoo, H.; Goto, C.; Oba, M.; Misawa, T.; Demizu, Y. Magainin 2-derived stapled peptides derived with the ability to deliver pDNA, mRNA, and siRNA into cells. Chem. Sci. 2023, 14, 10403.

[3] Plais, L.; Scheuermann, J. Macrocyclic DNA-encoded chemical libraries: a historical perspective. RSC Chem. Biol. 2022, 3, 7.

DEL for Compound Optimization & Expansion

DNA-encoded library (DEL) technology represents a revolutionary method in drug discovery. Unlike conventional approaches, DEL enables unparalleled exploration of chemical space, leading to the rapid identification of novel potent compounds. Although the importance of DEL screening in hit discovery has been well recognized, the power of DEL technology in compound optimization has only been appreciated recently. Researchers can systematically explore chemical space around existing compounds using DEL technology, generating diverse analogs and derivatives (Figure 1). This approach is particularly powerful in identifying analogs with enhanced properties, such as increased potency, selectivity, or reduced toxicity. The ability to utilize DELs for compound optimization highlights their versatility and impact in drug discovery endeavors.

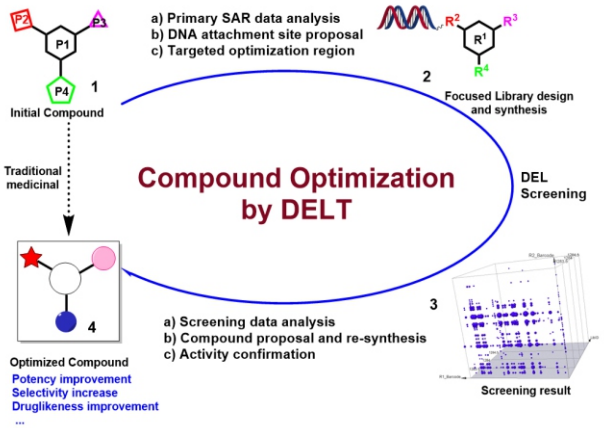


Figure 1. DELs for compound optimization.

Fragment expansion using focused DELs

Fragment-based drug discovery (FBDD) is one of the most well-developed approaches for drug discovery starting from small, low-affinity compounds. These low-affinity fragments pose a challenge in evolving them into compounds with desirable affinities. The most widely accepted approach for enhancing the potency of fragments involves iterative fragment growing, a process that can be intricate and time-consuming.

In 2022, a collaboration between HitGen and Vernalis teams has led to the development of PAC-FragmentDEL, a novel approach involving the photoactivated covalent capture of DNA-encoded fragments (RSC Med. Chem, 2022, 13, 1341-1349).This method facilitates the rapid identification of fragments binding to specific targets. This approach only requires a small amount of tagged protein (250 pmol per sample), therefore reducing the quantity of biological targets necessary for screening. Moreover, the approach is less time-consuming, allowing the identification of hits within a few weeks including library selection, sequencing, and comprehensive analysis. Importantly, PAC-FragmentDEL can incorporate fragments with lower solubility, expanding the range of compounds compared to traditional methods. Additionally, the fragments identified through PAC-FragmentDEL have clear DNA-tagging points, providing valuable insights into the interactions between the fragments and their respective target proteins.

Recently, an innovative strategy for fragment hit optimization utilizing poised DNA-encoded chemical libraries was proposed (Chem. Sci., 2023, 14, 8288-8294). These poised libraries allow researchers to systemically investigate chemical space, facilitating the swift transformation of fragments into potent hit/lead compounds. In contrast to this method, the fragments identified through PAC-FragmentDEL possess established DNA-tagging points and functional groups, serving as reaction headers for the construction of fragment-focused DNA-encoded libraries (Figure 2).

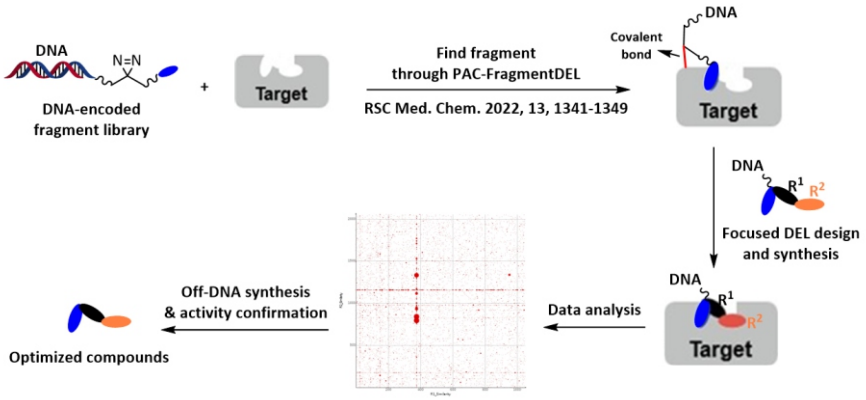


Figure 2. PAC-FragmentDEL selections and fragment expansion using focused DELs.

Aiming to generate potent protein kinase PAK4 inhibitors, we incorporated diverse in-house DEL intermediates into the fragments of PAK4 identified through PAC-FragmentDEL (RSC Med. Chem, 2022, 13, 1341-1349), resulting in fragment-focused DELs containing over 100 million encoded compounds within two weeks. These resultant libraries then underwent screening against PAK4 utilizing traditional affinity-based selection. Following comprehensive data analysis, off-DNA synthesis, and activity confirmation, multiple series of hit compounds exhibiting low hundreds of nM activity were identified. The entire process spanning from library design and synthesis, library selection and data analysis, as well as off-DNA compound synthesis and subsequent validation only took 4-5 weeks (Figure 3).

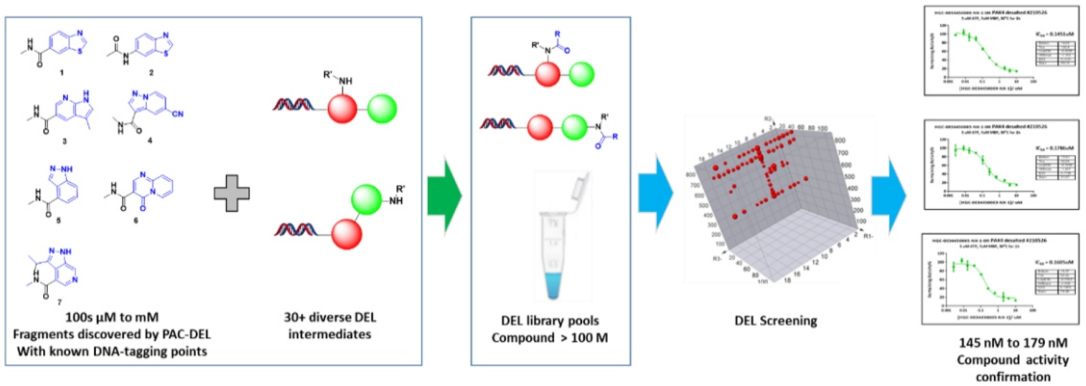


Figure 3. fragment expansion using focused DELs.

Optimization of known hit/lead compounds using focused DELs

DEL technology enables systematic exploration of the chemical space surrounding known hit/lead compounds, facilitating the generation and screening of extensive and diverse focused compound libraries. This approach aims to enhance the potency, selectivity, or druglikeness of these compounds.

DEL for Compound Optimization & Expansion

At HitGen, DEL technology has been employed in various projects for compound optimization. For instance, to enhance the selectivity of a reported compound, we designed two focused DNA-encoded libraries comprising millions of compounds, which involved confirming DNA-tagging points, designing and synthesizing core scaffolds, and controlling physicochemical properties (Figure 4). This whole process could be facilitated by automated instrumentation that allowed multiple DEL selection experiments to run simultaneously. Furthermore, the "Smart Selection Campaign" strategy was utilized, prioritizing DEL screening selectivity for proteins (wild type, mutations, etc.) from the starting point. By filtering out overlapping binders, specific inhibitor series were identified and subsequently validated, demonstrating exceptional selectivity.

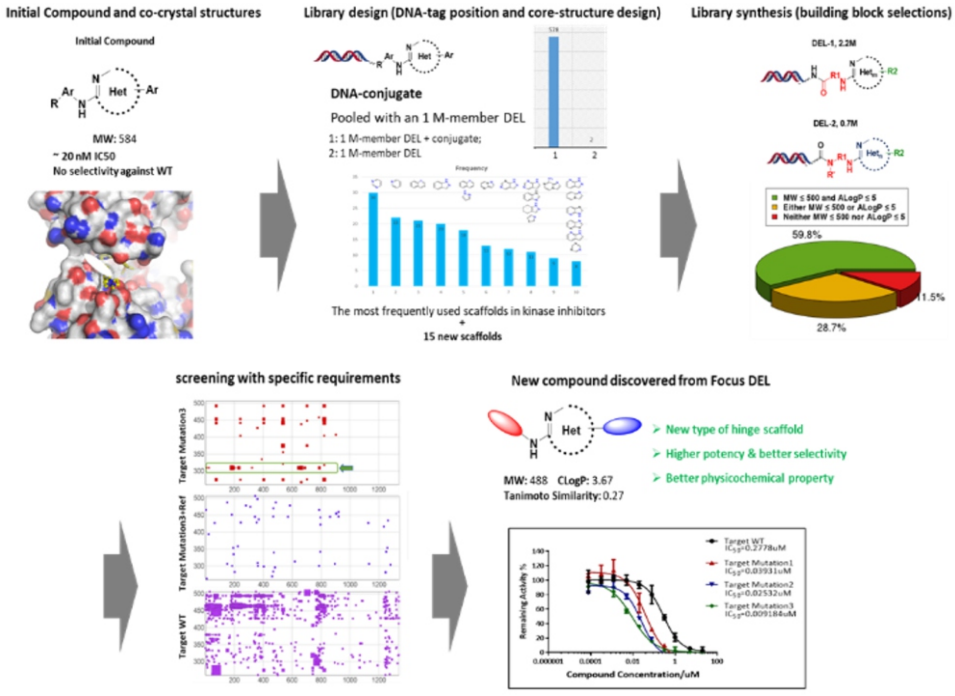


Figure 4. hit/lead compound optimization using focused DELs.

DEL for compound optimization at HitGen

DEL technology exhibit significant potential applications beyond the well-established novel hit discovery, proving high values for compound optimization. Unlike traditional methods, this approach avoids the iterative cycles of Design-Make-Test-Analyze (DMTA), streamlining the optimization process significantly. Through the design and synthesis of focused DELs, HitGen achieves the following objectives:

- Supporting fragment-to-lead optimization: HitGen combines PAC-FragmentDEL with subsequent focused fragment-expansion DEL techniques to facilitate the optimization process from fragments to lead compounds.
- Optimizing known hit or lead compounds: HitGen employs focused DELs to optimize existing hit or lead compounds, enhancing their potency, selectivity or druglikeness.

DEL for Protein Degradation

Protein degraders (most popularly known as PROTAC or Molecular Glue) utilize an event-driven process, recruit protein of interest (POI, or targeted protein) and E3 ligase directly or indirectly, induce poly-ubiquitination of POI and subsequent proteasome-mediated degradation. Unlike protein inhibitors, protein degraders do not require inhibitory activity to the protein, therefore, this modality of therapeutics applies to broader target classes including undruggable proteins, scaffold proteins, mutant proteins and etc.

PROTAC

Proteolysis Targeting Chimera (PROTAC) is a bivalent molecule consisting of a POI binder, an E3 ligase binder, and a linker tethering the two binders. Transferring an inhibitor and known E3 ligase binders (most commonly used ones are CRBN and VHL binders) with suitable linkers has been very popular in the past couple of years and yielded several clinical PROTAC programs (Figure 1). The efficacy against mutations, lower dosage and long-lasting effect highlights the PROTAC advantages over direct inhibitors.

Molecular Glue

Molecular glue (MG) is a monovalent molecule that brings two proteins in proximity as if they are glued together. This mechanism of action has been reported in different contexts. In protein degradation case, it induces POI:MG:E3 complex formation with the following several possible events: 1) MG significantly enhances POI:E3 interaction; 2) MG binds to POI, followed by recognition of E3; 3) MG binds to E3 ligase to allow the recognition of neosubstrate. Only proper binding pose(s) between POI and E3 allow ubiquitination and proteasome degradation. Molecular glue is a desired protein degrader, but it's much more challenging for discovery or design, which is evidenced with less targets and programs in the clinical.

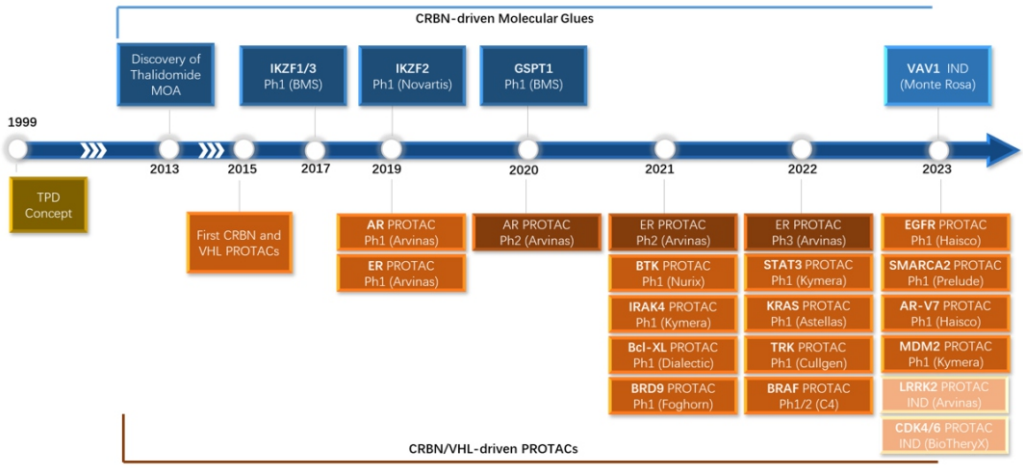


Figure 1. Protein degrader discovery history.

DEL for PROTAC based selection

DNA Encoded Library (DEL) is a very powerful technology for protein binder discovery because of its ability to access vast number of chemical compounds (HitGen full DEL contains more than one trillion compounds) with efficient protein usage, rapid turn around and low cost. Multiple selection experiments and deep data analysis ensure that the binders identified are selective to the POI. Also, due to the nature of DEL compounds, the binders discovered by DEL screening have a natural attachment site for building linkers of a PROTAC molecule. We have recently developed a method to optimize POI:degrader:E3 complex formation with POI, E3 dual protein selection, the PROTAC DEL and corresponding selection are published in ACS Chem. Biol. 2023, 18, 25-33 (ACS Editors' Choice, figure 2).

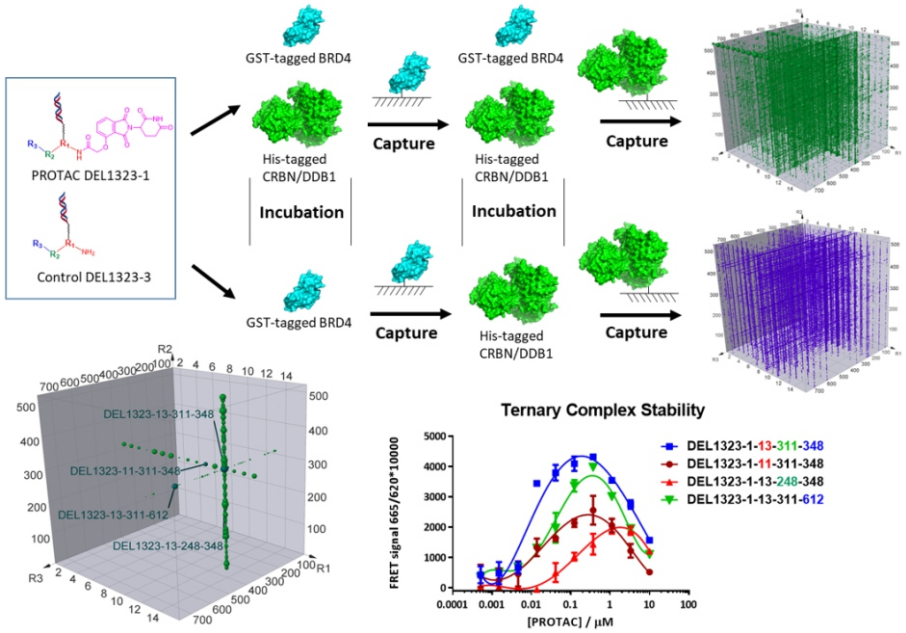


Figure 2. DEL technology for ternary complex optimization.

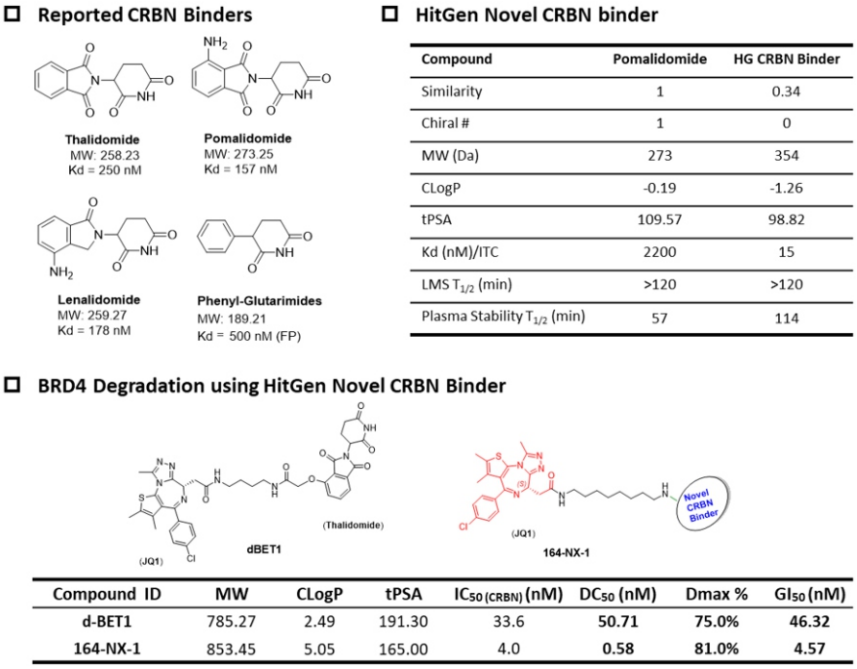
PROTAC and MG development from DEL

PROTAC discovery and development are now beyond oncology disease area hence tissue/cell restricted E3 ligase expression has become a very useful way for differentiation. As mentioned above, DEL technology is very effective for binder discovery, even in the situation where protein function is not well understood like in the case of most of E3s. Several dozens of purified E3s have been expressed, purified and screened against HitGen DELs, yielding many specific binders especially for those E3s without any binders reported. These binders are gradually approved for their PROTAC functions for important proteins of interest. During the exploration of PROTAC degradation, some of these E3 binders have been found with potential molecular glue function (More details will be given upon request).

PROTAC development from DEL derived CRBN binders

Emerging from the reported CRBN binders, novel CRBN binders discovered from DEL selection showed

higher binding affinity, more potent degradation with its PROTACs against many popular targets, and no IMiD (immunomodulatory drug) function (Figure 3).



Compound	Pomalidomide	HG CRBN Binder
Similarity	1	0.34
Chiral #	1	0
MW (Da)	273	354
CLogP	-0.19	-1.26
tPSA	109.57	98.82
Kd (nM)/ITC	2200	15
LMS $T_{1/2}$ (min)	>120	>120
Plasma Stability $T_{1/2}$ (min)	57	114

Compound ID	MW	CLogP	tPSA	$IC_{50\,(CRBN)}$ (nM)	DC_{50} (nM)	Dmax %	GI_{50} (nM)
d-BET1	785.27	2.49	191.30	33.6	50.71	75.0%	46.32
164-NX-1	853.45	5.05	165.00	4.0	0.58	81.0%	4.57

Figure 3. Novel DEL-derived CRBN PROTACs for potent POI degradation.

PROTAC development from DEL derived BIRC7 binders

BIRC7 (specifically expressed in cancer cells) ligand is the very first molecule identified from HitGen 1T DEL screening and its PROTAC shows selective degradation in BIRC7 high expression cell line (Figure 4).

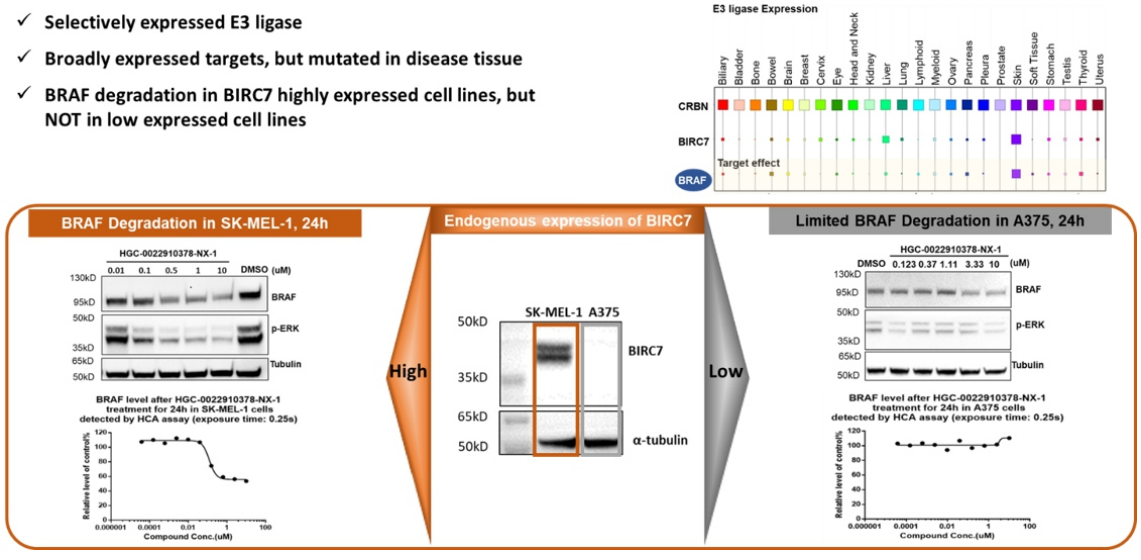


Figure 4. BIRC7 based PROTAC affords selective target degradation.

PROTAC development from DEL derived TRIM21 binders

TRIM21 (broad expression but different from CRBN) binders discovered from HitGen DEL screening have been developed into PROTACs and found with very potent BRD4 degradation (pM DC50, 8000X more potent than CRBN based PROTAC), differentiated kinase degradation comparing to CRBN based PROTACs, more importantly, TRIM21 ligands have potential Molecular Glue function (Figure 5).

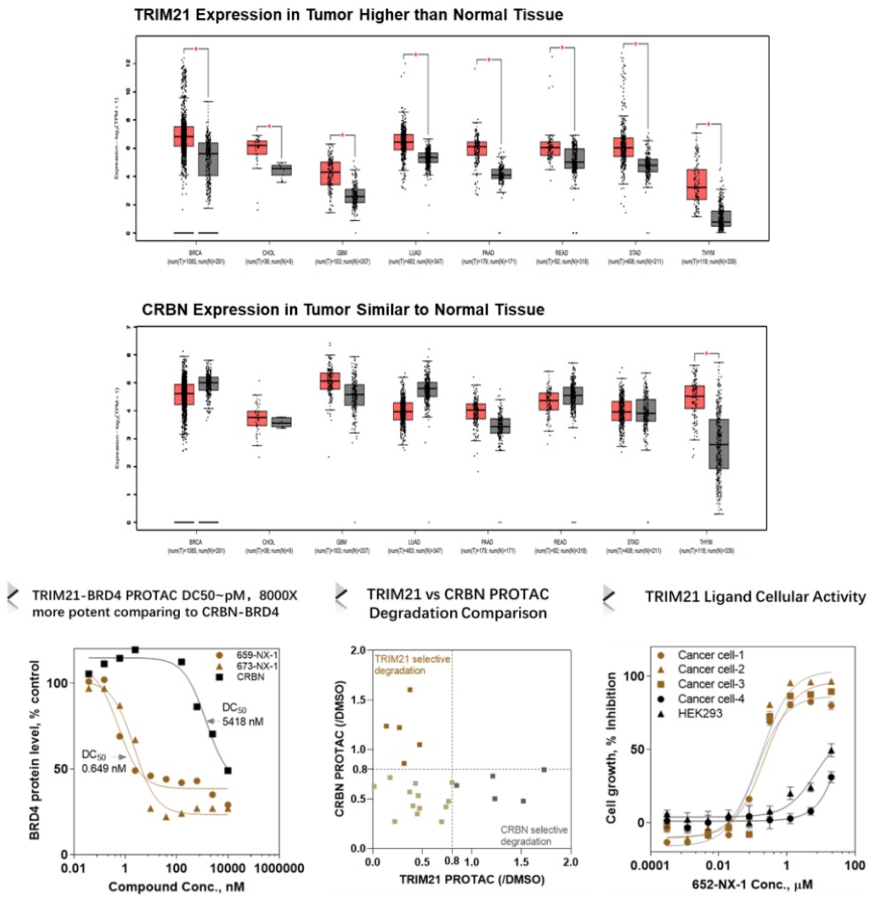


Figure 5. TRIM21 based PROTAC offers more potent and different degradation profile.

DEL selection for protein degraders at HitGen

Along with our trillion size DEL and PROTAC DEL, HitGen provides customized selection strategies to ensure a successful screen for protein degrader discovery:

- HitGen trillion DEL offers large chemical space for binder identification, independent of protein activity.
- PROTAC DEL provides ternary complex based selection.
- Multiple E3 ligases incorporated in one selection give high throughput and selectivity.
- Linker position is known for PROTAC development.
- Track record of successful selection cases with novel ligands identified.

DEL for Unconventional Applications

The advancement of DNA Encoded Library (DEL) technology has been significant in recent decades. Beyond its conventional role in small molecule drug discovery, its integration into emerging fields has begun to garner increased interest. This can be attributed to its large chemical space, abundant Structure-Signal Relationship (SSR) data, and inherent DNA conjugation properties. In this context, we will explore alternative applications of DEL technology. It is postulated that with the continuous expansion of the DEL field and technological progression, more applications are yet to be unveiled.

DEL for probe development

Thanks to the vast diversity of DEL, the role of DEL in obtaining new starting points for novel drug development has been recognized well. The rapid on-DNA and off-DNA chemical synthesis methodology established at HitGen not only allows fast DEL design and constructing, but also allowed quick affinity-based assay development in terms of screening and optimization. Compound series identified by DEL screening would guarantee both good potency and known attachment points that have been used for DNA tagging without extra validation effort. For challenging targets with no reported positive binders/inhibitors, any potential binders would be useful not restricted to direct drug development candidate but also can serve as probes for target validation and assay development. With ensured good proximity between DEL compound series as results of DEL screening and combination with various labelling techniques allowed by the known linker attaching points, probe development based on DEL results became promising and efficient.

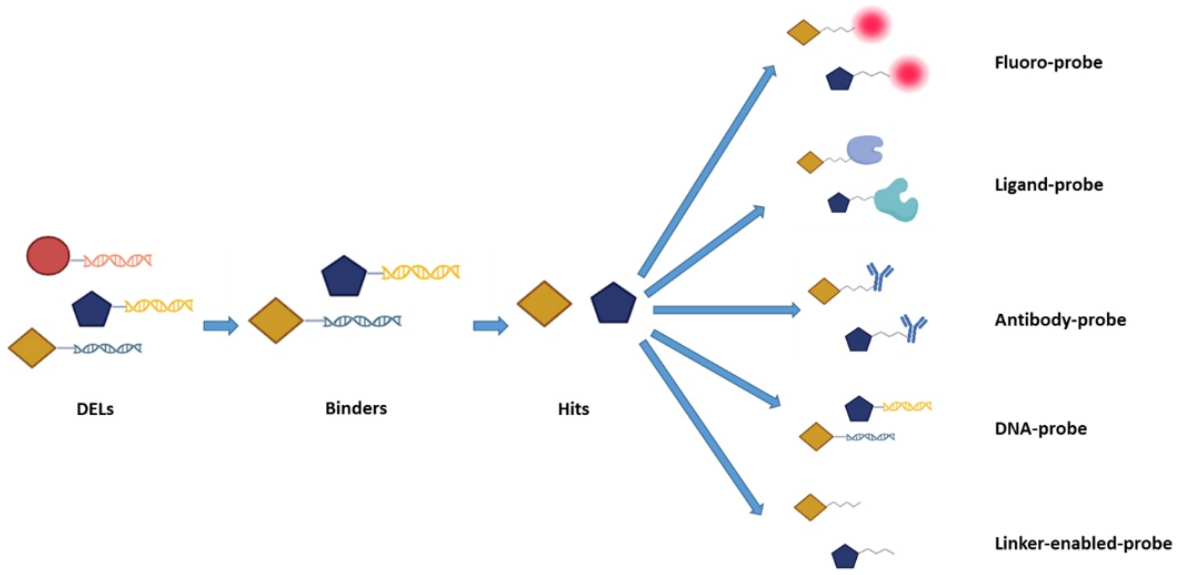


Figure 1. A schematic workflow for probe development based on DEL screening. Different probes can be developed and used in FRET (Fluorescence Resonance Energy Transfer), BRET (Bioluminescence Resonance Energy Transfer), target fishing, pull down, qPCR (quantitative Polymerase Chain Reaction) assays and etc.

DEL for sensor and diagnostic reagent development

Aptamers have been developed as recognition element for binding and monitoring the change of specific biological molecules. The capacity of DEL to investigate an extensive array of chemical spaces is indisputable, thereby facilitating their contribution to such applications through the identification of molecules that can sense alterations in proteins, peptides, and even small molecules. In addition, disease progression is usually associated with the change of multiple biomarkers or malfunction of various targets, with the rapid and efficient screening of a vast chemical space, DEL is a promising tool to play a significant role in the future of diagnostic reagent development. This can be achieved in the traditional setting of screening against the targets of interest, or more importantly, screen against biological samples, tissues or even in vivo models to identify multiple molecules responding to various targets or a specific disease pattern and phenotype.

DEL for nucleic acid delivery

Nucleic acid delivery on one hand relies on nanoparticles, on the other hand, the utilization of specific receptor mediated internalization represents a delicate strategy. DEL not only provides vast chemical space for the identification of small molecule ligand to these receptors in traditional selection format, the DNA conjugation nature of DEL molecules also offers the benefit of screening DNA delivery directly. The DNA tag of the DEL molecule can serve as the cargo in nucleic acid delivery, and direct cell based internalization selection could help identify receptor mediated nucleic acid delivery reagent directly from the selection.

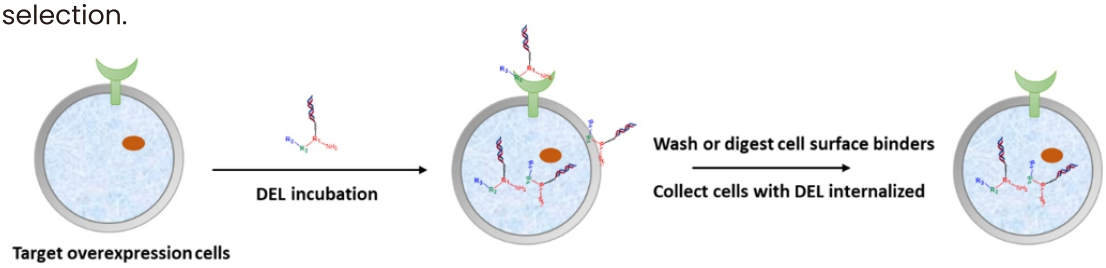


Figure 2. A schematic example for identifying nucleic acid delivery reagents from DEL selection.

DEL for functional based selection

Currently, the predominant selection method for DELs is based on affinity, however, the functional-based selection of DELs is being recognized as a prospective approach. The One Bead One Compound (OBOC) DEL strategy has been employed in functional-based selection, identifying hits that directly modulate target activity. The application of DEL selection in diverse cell-based methodologies, either by screening against the cell surface target or introducing DELs into the cells, yields hits that exert a biased effect on specific cellular outcomes. By conducting DEL screenings against a distinct phenotype, cellular function, or specific reporting system, investigators can discern compounds that regulate these functions or unveil potential novel targets for pharmacological discovery.

DEL for pesticide development

The use of DEL in pesticide development offers the similar advantages in drug discovery. It allows for the screening of large compound libraries with different conditions investigated in parallel and enables the identification of hits with diverse chemical structures and potentially novel modes of action. Upon rigorous evaluation against human target selection, DEL confers supplementary benefits for attaining selectivity over human homologs in pesticide discovery. The vast chemical space presents the potential for pinpointing unique enzymes in insects or fungi, thereby, mitigating potential environmental pollution issues. By leveraging the power of DEL, we efficiently identified developable hits and optimized the chemical property with improved efficacy, these compounds were further selected as candidates for the pesticide discovery (Figure 3).

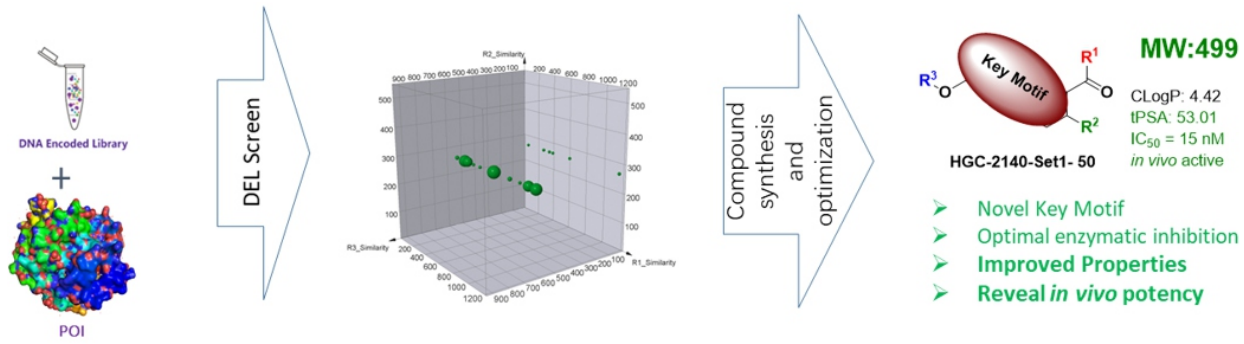


Figure 3. Identification of pesticide candidates from DEL selection.

Outlook

With the advancement in scientific research, the trajectory of DEL technology extends beyond the aforementioned applications and the list can go on and on. For example, utilizing the inherent properties of DEL molecules such as DNA attachment, DELs hold potential utility in the development of Antibody-Drug Conjugates (ADCs). This utility applies not only in the identification of novel cytotoxic agents, but also in offering supplementary data pertaining to linking and conjugation. Moreover, DELs serve as a robust approach for profiling target ligandibility. It facilitates the acquisition of critical information concerning the nature of molecules interacting with targets and the mapping of various target binding sites. Concurrently, the extensive datasets derived from DEL selection can be employed in the training of Artificial Intelligence (AI) models, thereby assisting in the design of innovative molecules. It is anticipated that numerous novel applications will emerge in the near future and we are excited to witness the forthcoming applications of DEL.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