

An overview of DNA-encoded libraries: A versatile tool for drug discovery

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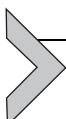
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1. Introduction

A DNA-encoded library (DEL) is a collection of small molecules covalently attached to DNA carrying unique information about the identity and structure of each library member. The small molecules are often prepared by combinatorial assembly of smaller building blocks (BBs), thus giving rise to a high number of members in the final library.

The discovery of small molecules from screening large chemical libraries towards biological targets has over the years provided tools for basic scientific research and assisted identification of lead compounds [1]. The protein world is complex and often challenges are faced in the identification of chemical entities able to bind and potentially modulate the function of a given protein. A study conducted on the first-in-class drugs approved by the Federal Drugs Administration (FDA) between 1999 and 2013 shows that 78 out of 113 come from target-based approaches [2]. The integration of the target-based approaches with phenotypic screening can potentiate the power of the two distinct methods [3]. The same holds for high-throughput screening (HTS), fragment-based approaches, and DELs. These three approaches have great powers and high potentials, but their level of success is often case-specific for each protein. Today, the importance of the integration of the different methodologies to increase the rate of success of different drug discovery projects is well-known [4].

Discovery platforms, such as phage-display [5], yeast-display [6], and mRNA-display technologies [7], take advantage of the biological machinery to produce large encoded libraries of peptides, antibodies, and proteins that are screened simultaneously towards a biological target of interest. The binders are then separated from the non-binders and identified by exploiting

the biosynthetic linkage between proteins (chemotype) and nucleic acids (genotype). Inspired by this, Brenner and Lerner proposed the concept of DNA-encoded combinatorial libraries [8]. In this seminal paper, the basic concepts currently used in DELs were outlined, including a combinatorial synthesis approach to generate large DNA-tagged libraries where affinity-based protocols were employed to select the strongest binders. This was followed by PCR amplification and DNA sequencing to identify the enriched binders and off-DNA resynthesis to validate the hits. In the following year, Janda *et al.* [9] and Gallop *et al.* [10] put this concept into practice and reported the generation of DNA-encoded peptide libraries on solid support containing a DNA tag and a small peptide, which were used for the selection of antibody epitopes. After the initial breakthrough, it took approximately a decade until several research groups reported the synthesis of DELs in solution for the identification of bioactive small molecules [11–15]. The progression of the field has in recent years made the DEL screening platform an accepted technique for early drug discovery campaigns in pharmaceutical companies [16], and compounds discovered from DELs have now entered clinical testing [17,18]. The synthesis and selections of DELs require only standard laboratory infrastructure, with no need for tedious screening assays and advanced robotics. Several libraries containing hundreds of millions to billions of members have been reported and a single library can be used for hundreds of selections [19–24]. Furthermore, only a minute amount of each tagged library member is required during screening, and with the advent of high-throughput DNA sequencing, this methodology offers a rapid and cost-effective approach for screening a variety of biological targets towards small molecule ligands.

Several approaches have been reported for the development of DELs. They all rely on common aspects where BBs are assembled in a combinatorial manner and chemically synthesized oligonucleotides are added to a tag and encode the successive chemical steps. The chemistry going into the preparation of DELs is restricted by the stability of the DNA tag during the synthesis. Therefore, low pH, strong Lewis acids, oxidizing agents, and anhydrous conditions are avoided in order to ensure integrity of the DNA. Despite these limitations, several DNA compatible reactions have been described for the synthesis of DELs [25]. Especially in recent years, the focus on this area has increased from both academia [26–30] and industry [31–35], ultimately expanding the structural diversity of DELs. In addition, the chemical and structural complexity of the BBs going into the library synthesis have been improved by novel approaches for their synthesis and incorporation [36–41].

In the following sections, we aim to highlight the developments and achievements in methods for DNA encoding of small molecules, selection and enrichment of target binders, DNA compatible chemistries, and biologically active compounds discovered from DELs. In the latter part, representative paradigmatic examples for several target families will be described, elaborating on the type of compounds a DEL screen can provide and how the physicochemical properties are optimized in subsequent lead structure development.



2. Encoding methods

Several methods for the encoding of DELs have been developed in which unique DNA tags are formed and covalently connected to a small molecule [42,43]. The principle of tagging combinatorial libraries with DNA was realized in the early 90s [8]. Here, libraries of peptides were being synthesized on a solid support from beads containing two orthogonally protected linkers. Alternating oligonucleotide and peptide synthesis was carried out and thus the DNA coding was done chemically [9,10]. Exploiting the split-and-pool approach, DELs of up to 10^6 members were synthesized on solid support. A few years later, an enzymatic ligation approach for the introduction of DNA codes in a combinatorial setting was realized [44]. Consequently, the compound synthesis was no longer required to be compatible with the subsequent oligonucleotide synthesis and the DEL synthesis was not restricted to solid support. Hence, the encoding could be created by iterative ligations, and the process of alternating chemical modification and enzymatic ligation in solution has been widely used for the synthesis of DELs [19,20,45–49].

2.1 DNA-recorded libraries

DNA-recorded library synthesis is a widely used approach for the synthesis of DELs [19,50]. As presented in Fig. 1, these libraries are prepared by split-and-pool synthesis where a DNA strand specific for a given BB is ligated after a chemical reaction has taken place. The starting point for library assembly is typically an amino-modified double stranded DNA (dsDNA) construct, and the codon ligation is carried out using ds codon/anti-codon duplexes with overhangs [19], which are ligated enzymatically by either polymerases [51] or ligases [19]. Alternatively, splint ligation procedures have been used for DNA-recorded libraries with single stranded DNA (ssDNA), where the

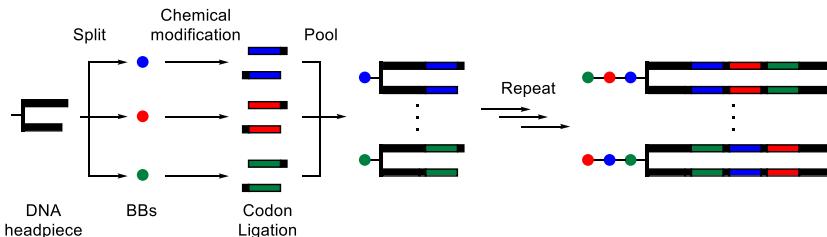


Fig. 1 DNA-recorded library synthesis. During each split, the synthetic modification is recorded by ligation of a DNA tag. The intermediates are pooled, and the process is repeated until the final library of DNA-tethered small molecules is assembled.

final dsDNA construct is formed *via* a Klenow polymerization step [52,53]. The chemical reactions in DNA-recorded libraries are often driven by a large excess of reactants and reagents. As enzymatic ligations are sensitive to contaminants from the previous synthetic step, buffer exchanges are often required to remove these. Indeed, the enzymatic encoding process is a crucial step, and it should be nearly quantitative in order to ensure fidelity and correct encoding of the BBs. In contrast to enzymatic ligations, alternative chemical ligation methods have also been developed using the CuAAC reaction [22] or photo-crosslinking [54]. High fidelity in both the chemical modification and encoding are crucial factors for achieving a high quality and reliable library. During each cycle of synthetic modification and encoding, the pooled mixture can be purified by reverse phase-HPLC or ethanol precipitation to remove unreacted amino-modified DNA and excess reagents. Alternatively, ‘Cap-and-Catch’ procedures have been developed where unreacted amino-modified DNA is capped with biotin and subsequently removed by affinity capture on streptavidin-coated sepharose [55].

2.2 DNA-directed libraries

In contrast to DNA-recorded libraries, DNA-directed chemistry is a concept where the encoding DNA strand not only serves to track the synthetic history of the attached small molecule, but it also has an additional role of directing the chemistry of each step. Thus, the power of DNA-hybridization is used to achieve a high proximity effect of the building blocks and allow the reaction to take place in low DNA concentrations, much like an intramolecular reaction.

The field of DNA-templated synthesis (DTS) was pioneered by Liu and co-workers [56,57], where libraries were developed using pools of pre-encoded DNA templates (Fig. 2A) [11,58]. In this technique, and prior

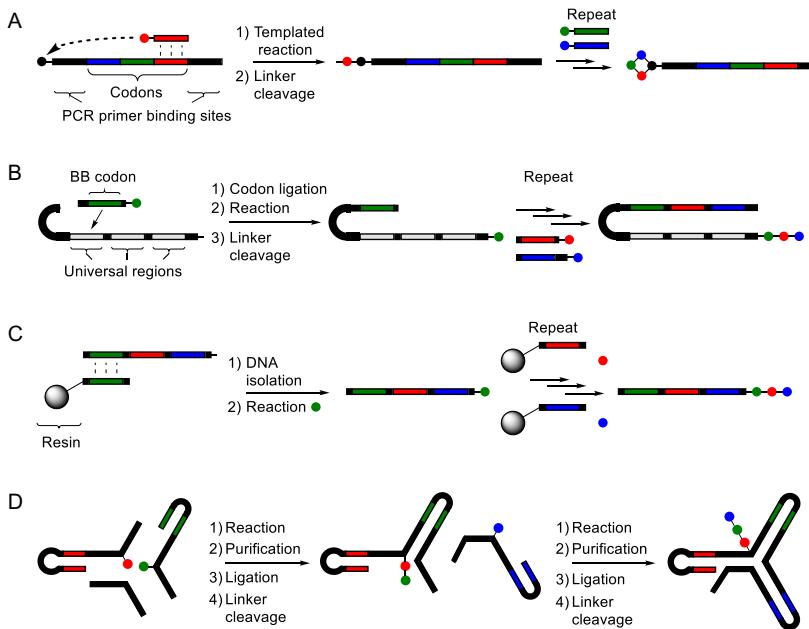


Fig. 2 DNA-directed library synthesis. (A) DNA-templated synthesis. Code-specific annealing brings reacting building blocks into proximity with the modified DNA-template and the reaction is performed specifically. (B) Universal template strategy. A template DNA strand with poly-inosine sequences allows annealing of BB codons with various sequences. Encoding is ensured *via* ligation and the BB is transferred to the template DNA. (C) DNA routing. Template pools containing various codon combinations are passed through a series of resins with immobilized complementary oligonucleotides. The separated sub-pools are chemically modified. (D) The YoctoReactor[®] approach. Upon annealing of conjugated BBs, DNA junctions are formed during library synthesis. The chemical reaction takes place in the centre, and the linker attaching the BB is cleaved after purification and ligation.

to the library synthesis, the bifunctional BBs are conjugated to DNA with sequences complementary to the codes on the templates. Upon hybridization, a key duplex formation between the template and the complementary DNA, brings the reacting BBs into proximity and establish a high effective molarity, which ensures that the reaction will only occur between BBs with complementary tags [59]. Since all BBs need to be conjugated to DNA prior to the DEL synthesis, libraries developed from DTS are limited to bifunctional BBs. Upon complete chemical reaction, the linker connecting the BB to the codon oligo is cleaved, which releases a functional group that can be used for the following round of chemical modification. This process is iterated until the final library is created. This approach has been

successfully used for the synthesis of DELs containing macrocyclic library members [11,58,60,61].

In contrast to the procedure described above, where the templates carrying the codes for the BBs are prepared prior to the library synthesis, a different approach to the DTS technology involves the usage of universal templates (Fig. 2B) [62]. In this system, the template DNA contains sequences of deoxyinosine, which can hybridize with all four of the canonical nucleobases. Since the BBs conjugated to their respective DNA codes hybridize to the template strand unspecifically, encoding is achieved by ligation.

DNA routing is a DNA-directed technology which is not limited to the use of bifunctional BBs [12–14]. In this setup, a pool of DNA templates containing all combinations of codes is passed through resin beads coated with complementary oligonucleotides to the individual codons on the template DNA strand (Fig. 2C). Hereby, the template DNA is captured and separated into sub-pools, where a chemical reaction is carried out. This process can be repeated for all subsequent codon regions until the full library is synthesized. The DNA routing technology has been further developed to work in parallel in a 384-well plate format [63,64]. Strictly speaking, this technique does not employ DNA-directing on the molecular level. However, it still has a tighter integration of sequence and chemical structure compared to the DNA-recorded techniques.

In DTS and DNA routing, a proper sequence and codon design is vital in order to prevent mis-hybridization and potentially wrong encoding. In addition, these types of libraries require preparation of DNA templates or solid-supported anti-codons prior to the synthesis of the library. A DNA-directed library design where the reaction is facilitated by hybridization of complementary DNA strands, independently of the attached codons, is known as the ‘YoctoReactor®’, [65–67]. In this setup, BBs are conjugated to amino-modified oligonucleotides that are designed to form three- or four-way DNA junctions during library synthesis (Fig. 2D). Upon annealing, the building blocks are guided into the centre of the DNA junction, generating a volume in the yoctolitre (10^{-24} L) range. The reacting BBs are brought into very close proximity, giving a high local concentration (mM to M) of the DNA conjugated BBs. The setup is applicable for both appendage reactions [65,67] and multicomponent reactions, such as the Hantzsch dihydropyridine synthesis [66], where encoding of the individual BBs occurs simultaneously as the product is being formed. Another unique feature of the YoctoReactor® library design is the high

fidelity that is accomplished by purification between each step in the library synthesis. Thus, all incomplete intermediates are removed and truncations are eliminated from the combinatorial library. This is in clear contrast with DNA-recorded synthesis, which require high-yielding reactions, since truncation, incomplete reactions, or synthetic side-product formation can produce false positives in the selection [68].

Similar to the YoctoReactor®, Li and co-workers developed an encoding method where the BBs were brought into proximity *via* a double-hairpin duplex [69]. The authors showed that they could use the setup for multicomponent reactions and on appendage reactions using orthogonal chemistries. In this setup, a Holmes-type photo linker [70] was used to photolytically release the primary amides from their attached DNA conjugate using 365 nm UV irradiation [69]. Alternative linker systems in DNA-directed DEL synthesis include sulphonyl based linkers, such as the bis-[2-(succinimidooxycarbonyloxy)ethyl] sulphone (BSOCOES) linker [11,71] and the heterobifunctional di-(*N*-succinimidyl) carbonate (SVEC linker) [65,72]. Both of these linker systems have similar modes-of-action and release a linked BB amine upon treatment with an aqueous buffer at pH 11.6.

2.3 Encoded self-assembling combinatorial libraries

A unique encoding and screening approach was developed in 2004 by Neri and co-workers, known as encoded self-assembling combinatorial (ESAC) libraries [15]. These libraries are often referred to as dual-pharmacophore chemical libraries. The libraries are developed in a radically different approach, by hybridizing complementary sub-libraries of 5'-modified conjugates with 3'-modified conjugates each containing distinctive codons [73]. The screening approach is comparable to fragment-based drug discovery and the selection identifies pairs of BBs that bind to the target protein with a relatively high degree of flexibility, which allows them to reach adjacent binding sites of the protein. The initial ESAC libraries identified individual fragments, rather than fragment pairs [15,74]. This setup was later optimized by adding a *d*-spacer into one of the two sub-libraries, which allowed hybridization with no base pair mismatches (Fig. 3A) [75]. A subsequent Klenow fill-in facilitated a code transfer, and enabled both the identification of fragment pairs and creation of large ESAC libraries. Furthermore, high purity DELs are often associated with this method since all BB conjugates can be purified before the library is developed, and no reaction between

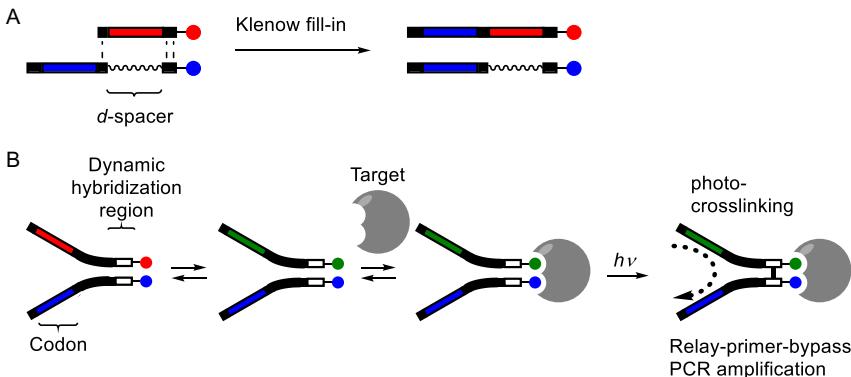


Fig. 3 (A) Encoded self-assembling combinatorial (ESAC) library. Partially complementary sub-libraries are assembled and a Klenow fill-in facilitate a code transfer, thus encoding the fragment pairs of the library. (B) DNA-encoded dynamic library. DNA strands with short complementary dynamic hybridization regions are mixed. The equilibrium is shifted towards the high-affinity fragment pairs upon addition of the target, and subsequently locked *via* photo-crosslinking.

the two sets of BBs is taking place. Inevitably, a challenging task emerges during post-selection hit resynthesis, as a subsequent connection of the identified fragment pairs *via* a suitable linker is required. This process was optimized by connecting the two identified fragment pairs on ssDNA using a set of predefined bifunctional scaffolds [76]. The small molecule DNA conjugate was hereafter hybridized with a fluorescently labelled complementary locked nucleic acid (LNA) strand and used in a fluorescence polarization setup to measure the dissociation constant. From this screen, the best binders were identified and selected for off-DNA synthesis.

Dynamic analogues of the ESAC-type library have been prepared. In comparison with ESAC, the sub-libraries have a relatively short complementary DNA strand which has enabled a heat-induced DNA-encoded dynamic combinatorial chemical library [77]. After a round of selection with an immobilized target, the non-binding library pairs can be shuffled by heating and melting the DNA pairs, resulting in new sets of library members that can undergo a second round of selection. Enrichment is hereby accomplished by reforming and selecting high-affinity binding fragments [78]. The concept of photo-crosslinking has been applied to lock the equilibrium of DNA-encoded dynamic chemical libraries [79,80]. In a recent study, library DNA strands with short complementary dynamic hybridization regions were mixed in an ESAC approach (Fig. 3B), and upon addition of the target the equilibrium was shifted towards the high-affinity duplexes. Each pair of

library DNA strand carries a *p*-stilbazole unit in the hybridization region and upon UV irradiation, a [2 + 2] cycloaddition locks the two DNA strands of the binding pairs. The resulting duplexes were isolated, PCR amplified using a relay-primer-bypass, and sequenced to read the DNA codes [80].

2.4 PNA-encoded libraries

An alternative library encoding strategy makes use of peptide nucleic acids (PNAs) [81,82]. Encoding using PNA is achieved by standard solid-phase peptide synthesis (SPPS). The peptidic backbone is composed of *N*-(2-aminomethyl)-glycine repeating units, which makes it chemically stable towards reaction conditions that would lead to DNA degradation. This expands the scope of compatible reactions employed in PNA-encoded chemistry and enable synthesis of a more diverse set of structural scaffolds on the final library compounds [83–85]. The codes of PNA are not amplifiable, hence unlike DNA, enzymatic encoding cannot be used and the template is not recognized by polymerases. However, PNA forms stable double-helical complexes with DNA, RNA, and itself [86], and thus selections of PNA-encoded libraries often involves an intermediate hybridization step. A microarray is the typical choice for decoding such libraries [87–91]. Using microarrays, the library is screened in solution and the tagged ligands hybridize to the complementary microarray spot. The target protein is interrogated against the library, and the binders can then be identified using a fluorescent readout [92]. The method has been shown to be useful for libraries of fewer than 10^5 members, but unsuitable for larger libraries [93].



3. Selection methods

In traditional HTS, each individual compound in the library is discretely screened under predefined assay conditions. DEL hits, on the contrary, are identified from mixtures of compounds and selected out of a pool by an affinity selection. Furthermore, DEL screenings are more amenable to testing several binding conditions in parallel as each experiment takes place in only a single tube. After isolating the DNA from the selection, this is PCR amplified and upon high-throughput DNA sequencing, the target binders are identified from the counted sequences. The structure of the hits can then be deconvoluted through translation of the corresponding DNA ‘barcode’ and compounds are subsequently synthesized *off*-DNA to confirm their activity in biological assays. The technique enables the screening of compound collections containing more than a billion distinct members [20], and successful screens are routinely carried out with only atto-

femtomoles of each tagged library member [68]. Hence, very small quantities of both library and target are required which, in comparison to the widely used HTS, offers a more rapid and simple screening approach with significantly reduced cost and time investments [94].

3.1 Immobilized targets

A commonly used selection method for DELs relies on immobilized targets on solid support, allowing enrichment of binding library members by removal of the non-binding through stringent washing steps (Fig. 4) [19,50,53,95–100]. Supports for capture of the target include magnetic or sepharose beads, and protein immobilization can be accomplished by covalent capture [101] or directed *via* His-tag [19,97], biotin-tag [15,48,75,96,100], or FLAG-tag [21]. The enriched binders on the solid support are subsequently eluted, for example, through heat denaturation, and 2–3 selections are typically performed to increase the stringency of the selection process. Automation of the washing steps [95] and screening process has improved the efficiency and throughput of the method, and enabled screening of multiple targets simultaneously in a single setup [16]. However, precautions must be taken during the selection, since target immobilization can disturb the natural folding of the protein, causing the target to display altered properties, which impacts the binding of library compounds. Thus, real binders and attractive library compounds can be lost during the washing step, leading to lower enrichment factors, which complicates interpretation of the data [102]. Furthermore, non-specific binding of library members to the matrix can lead to false positives [103]. To minimize the latter issue, sheared salmon sperm DNA and bovine serum albumin have been used as blocking agents in the washing step [19]. Despite the drawbacks associated with the immobilized target approach, its simplicity and throughput has made this selection method attractive for many screening campaigns, and facilitated the identification of numerous biological active small molecules (*vide infra*).

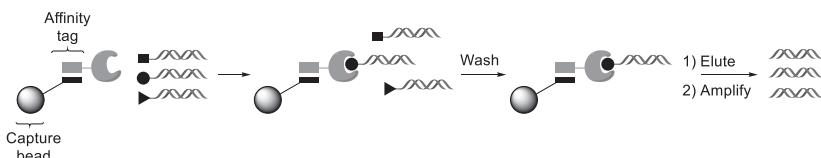


Fig. 4 Selection using immobilized targets. Tagged target proteins are either directly or indirectly immobilized on solid support. After incubation of the DEL, non-binding members of the library are removed during washing. Binding members of the selection are subsequently eluted, amplified by PCR, and decoded using high-throughput DNA sequencing.

3.2 Non-immobilized targets

In contrast to the widely used immobilized target approach, several homogeneous selection methods have been developed. Their intent is to overcome the background noise coming from the matrix on immobilized proteins and to abolish the need of washing steps. In 2010, Liu and co-workers reported a homogeneous selecting method designated Interaction Dependent PCR (IDPCR), where a target protein is covalently conjugated with a piece of ssDNA, which can hybridize with interacting members of the DEL to form a pseudo-hairpin structure (Fig. 5A) [104]. This enables primer extension and PCR amplification, which cannot be done for the non-binding ligands due to the lack of a priming site. The selection approach was further expanded to work with unpurified targets in complex biological samples, a methodology known as Interaction Determination using Unpurified Proteins (IDUP) [105]. In this case, fused protein targets of interest are connected with the primer binding site non-covalently *via* an antibody or a SNAP-tag, using a DNA-linked probe (Fig. 5B). The method is especially advantageous for targets that are difficult to purify, poorly soluble, intrinsically unstable, or prone to aggregate. In addition, the targets can undergo post-translational modifications and interact with accessory proteins and metabolites in ways that reflect their biological environment.

Researchers from Vipergen developed a selection method for DELs, known as Binder Trap Enrichment (BTE)[®], where a DNA-tagged target protein is trapped with bound members of the library in water-in-oil emulsion droplets (Fig. 5C) [67]. In this procedure, an equilibrium is established after incubation of the DEL with the target. Subsequently, the sample is diluted to disturb the equilibrium, causing stronger binders to remain bound, and the aqueous phase is then trapped inside separate micelles formed from a water-in-oil emulsion. Thus, library members still bound to the protein will end up in the same droplet and an enzymatic ligation of the DNA tags records the co-trapping event. PCR amplification allows the identification of binders since only ligated products contain two PCR priming sites.

DNA-programmed Photo-Affinity Labelling (DPAL) is a method developed to select binders from a DEL using unmodified target protein without the need of tagging or immobilizing the protein prior to the screen [106–108]. A short oligonucleotide modified with a phenylazide photo-crosslinking moiety positioned on the 5'-end, hybridizes with the complementary primer binding site of the DNA strand of a tagged library member. Upon interaction between the target and the DNA-encoded small

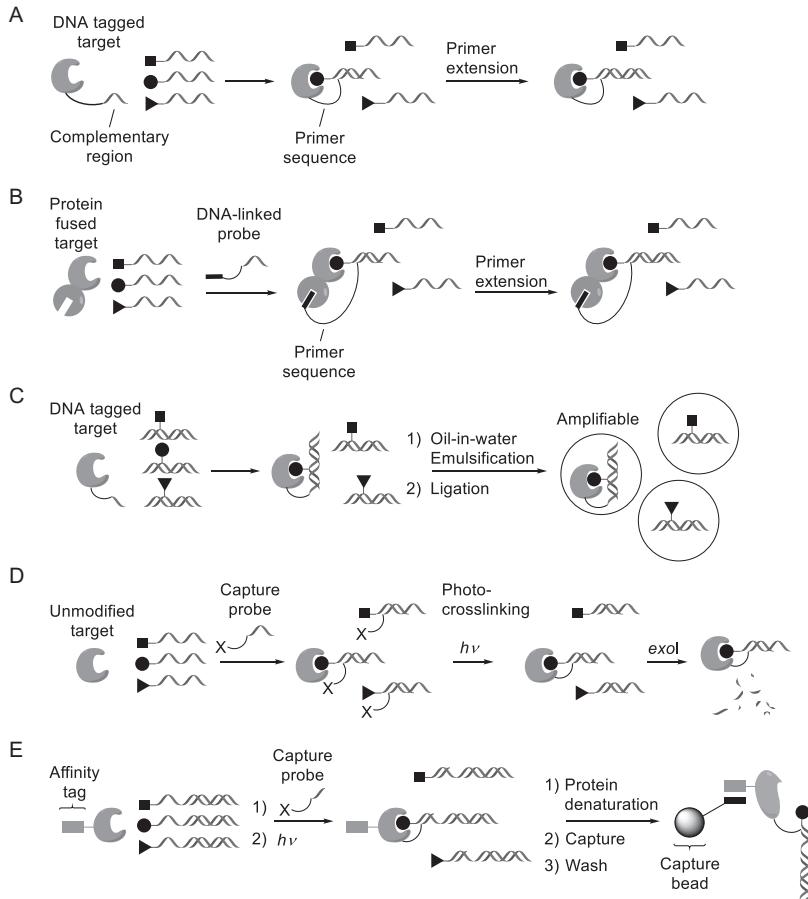


Fig. 5 Selection methods using soluble target protein. (A) Interaction dependent PCR (IDPCR). A target protein tagged with a primer binding site is incubated with a DEL. Enrichment of the binders is accomplished since PCR amplification is only possible upon hybridization of the interacting members of the DEL. (B) Interaction determination using unpurified proteins (IDUP). Similar to (A), but in this setup, the primer binding site is introduced to the target *via* a DNA-linked probe such as a SNAP-tag. (C) Binder Trap Enrichment (BTE)[®]. A target protein DNA-tagged with a primer binding site is trapped in water-in-oil emulsions with binding members of the library. A subsequent ligation encodes the co-trapping event and enable PCR amplification. (D) DNA-programmed photo-affinity labelling (DPAL). Photo-crosslinking upon irradiation creates a covalent bond between the unmodified target protein and a probe that hybridize with binding members of the DEL. Subsequent addition of *exo*/ nuclease digests the non-crosslinked library members leading to enrichment of target binders. (E) DPAL with affinity-tagged target. Similar to (D), however, an affinity tag enables immobilization of the target onto solid support, and enrichment is attained by a subsequent washing procedure.

molecule, the photo-crosslinker is brought into proximity with the protein and irradiation will trigger a covalent bond formation between the protein and the modified DNA (Fig. 5D). Subsequent addition of *exoI* nuclease will digest the non-crosslinked library members, whereas the tag-binding DNA sequences are protected and preserved for PCR amplification and sequencing.

Alternative methods using photo-crosslinking in DEL selections involve affinity-tagged proteins [109]. After photo-crosslinking, the protein is denatured without impairing hybridization between the photo-crosslinking DNA and the DNA of the DEL member. Subsequently, the DEL member and the denatured protein is immobilized onto solid support *via* the affinity tag (Fig. 5E). This procedure allows stringent washing to remove non-binding library members and improves the enrichment and recovery of target binders. The authors recently showed that they were able to apply this screening technology to live cells, targeting proteins within the cytosol and on the cell surface [110]. In this study, access of the DEL to the cell interior was facilitated by conjugation of the DEL to a cyclic cell-penetrating peptide [111].

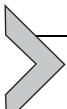
Membrane proteins and ion channels are major target classes in drug discovery. The expression and purification of such protein targets can be difficult and as a result, screening on these types of targets is usually made directly on cells. The advantage of performing selections inside or on the surface of cells is that the screening takes place in its natural biological environment, in such a way that the native structure and function of the proteins are maintained. Moreover, small molecules identified from cell-based selections are believed to have higher rate of success for the subsequent *in vivo* experiments. However, many other proteins are present within or on the cell and can potentially interact with the DEL, hence overexpression of the target protein in the selection experiments is often required. Additional studies of *on-cell* target selections include the screening of PNA libraries against cells overexpressing surface proteins [112,113], and a DEL selection towards the G protein-coupled receptor, NK3, reported by researchers from GSK [114].

For screenings in general, minimal structural manipulation of the target protein is preferred in order to avoid loss of activity. Tagging or immobilization of a target protein can potentially alter its properties and ultimately lead to unsuccessful or terminated screening campaigns. Selection methods with unmodified target protein [106–108], or screening in complex

biological samples [105,108,110,112–114] have only been reported in more recent years and these methods can potentially provide biologically active compounds from DELs, which would not be possible by conventional techniques. Nonetheless, the simplicity and throughput of the immobilized and non-immobilized target methods makes them attractive approaches for many targets. Indeed, these methods emphasize the advantages associated with the DEL approach, including rapid screening of massive compound collections at low cost using a simple instrumental setup.

3.3 DNA-encoded solid-phase synthesis

As described in the previous section, the early days of DEL synthesis comprised compatible reactions on solid support and primarily involved synthesis of peptides [8–10]. Until recent years, the field of DNA-encoded solid-phase synthesis (DESPS) [115] has gained little attention, since DEL synthesis has been dominated by the development of DNA-recorded or DNA-directed libraries in solution. The solid-phase system allows conditions to be used for small molecule synthesis, which are not believed to be compatible with solution based DEL synthesis, and great efforts have gone into the expansion of the chemical and structural diversity of compounds synthesized by this methodology [116–118]. One-bead one-compound (OBOC) libraries were initially screened using fluorescently labelled targets subjected to a solid-supported DNA-encoded library, followed by isolation of beads containing hit compounds [10]. Recently more advanced activity-based screening methods have been described from DNA-encoded OBOC libraries [119]. In DEL selections, the inevitable covalent attachment of the library member to the DNA tag may cause unfavourable interactions with the target, and the activity of the unbound compound can differ, which can ultimately influence the selection outcome. For OBOC libraries several off-bead screening formats have previously been reported [120–122], where library compounds are released from the solid support, and the interaction between the target and the small molecule is studied in solution. In connection to DELs, Paegel and co-workers recently reported an *off*-DNA screening format, where a 67,100 member OBOC DEL was screened in a droplet-based microfluidic setup. Upon irradiation, a photo-labile linker was cleaved and the solid-supported DEL compounds were liberated. Subsequently, the off-DNA target interaction was detected *via* in-droplet laser-induced fluorescence polarization [119].



4. Building blocks in DELs

Designing DELs that conform to the criteria associated with a higher probability of oral bioavailability [123], limits the number of BBs that can go into the synthesis of such libraries. Molecular weight in particular is a limiting factor for the design of a library [124]. For DELs with two splits, BBs with a molecular weight below 200 Da are preferred, and with careful selection and design, libraries containing three BBs can be formed where the average molecular weight is below 500 Da. Hence, most DNA-encoded small molecule libraries are synthesized with either two or three diversity components in branched or linear formats (Fig. 6). However, successful studies on DELs that go beyond the rule of 5 have been reported, including screening of macrocyclic structures [47,52,60,125].

The availability of BBs applicable for DELs drastically decreases when the number of synthetic handles increases. For split-and-pool based libraries with a linear design, at least one bifunctional BB is required, and branched libraries often rely on trifunctional compounds. Typically, monofunctional BBs are used in one or more of the varying positions. More than 40,000 amines, 20,000 acids, 5000 aldehydes, 2000 boronic acids, and 1400 aryl halides with a molecular weight below 250 Da are commercially available as monofunctional BBs for a price below \$150 per gram [126]. The exact numbers for the corresponding bifunctional compounds are uncertain, but are significantly lower, and they are typically more expensive. The quality of the BBs going into the library synthesis is a crucial factor for the screening outcome [68]. Schreiber *et al.* described in 2019 the development of a DEL, where a set of trifunctional 2,3-disubstituted azetidines and pyrrolidines were used to induce stereochemical diversity and complexity [39]. The BBs were synthesized *via* a Pd-mediated C—H arylation using DNA non-compatible conditions prior to the library development [127]. This study emphasizes how modern organic synthesis can assist the development of new scaffolds, which ultimately enhance the structural diversity of DEL members. The scope of a DNA compatible reaction and preparation of

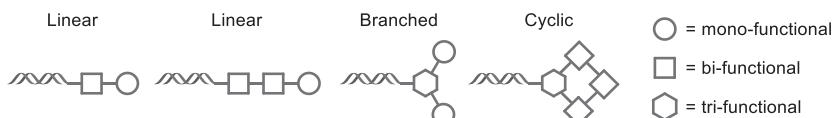


Fig. 6 Examples of library designs in DELs illustrating assemblies of mono-, bi-, and trifunctional BBs.

a DEL is restricted by the number of BBs that go into the synthesis thereof. Large sets of structurally diverse compounds which react to give high yields are essential for the quality and fidelity of the DEL. In order to increase the number of BBs for a certain chemical modification, *on-* or *off-*DNA functional group modifications have been performed prior to introduction of the BB. This include diazotransfers [128,129], azide substitutions [130], Seydel–Gilbert homologations [25], nitro reductions [45,131,132], azide reductions [133], carboxylations [134], and sodium periodate diol cleavages [11,58].



5. DNA compatible chemistries

The choice of chemical methods for library synthesis is severely restricted by the need of preserving the DNA tag. This limitation has led to a relatively low variation in assembly of the BBs during synthesis, and the construction of libraries is often biased towards assemblage *via* appendage reactions. Structural diversity of the libraries arises from assembly geometry, BB selections, and synthetic methodologies [124]. Research going into the area of expanding and optimizing DNA compatible chemistries has gained a lot of focus in recent years and is continuously progressing in order to increase the size and diversity of DELs, while ensuring synthesis of compounds with physicochemical properties that predict oral bioavailability. However, the available chemical toolbox still only covers a small fraction of known reactions when compared to classical organic synthesis. The synthetic modifications allowed are restricted by conditions that need to be compatible with any potential competing side-reactions that can alter or destroy the polyfunctional nucleic acids. Thus, oxidative reagents, Brønsted acids, Lewis acids, many transition metal ions, long reaction times at elevated temperatures, or anhydrous conditions, are to be avoided or controlled in order to minimize loss of generic information. Since purification after each synthetic modification is typically not allowed, high-yielding reactions with minor side-product formation are important for developing high-fidelity DELs. A few robust chemical steps are often more productive, since low yields in library assembly will lead to a reduced signal-to-noise ratio, ultimately increasing the rate of false negatives [102,135].

5.1 Appendage diversity in DEL synthesis

The highest contributor to structural diversity in most DELs arises from introduction of different appendages to a common molecular scaffold.

The most common appendage reactions in DEL constructions are acylations ([Table 1](#), entry 1) [19,57,96,136], reductive alkylations ([Table 1](#), entries 2 and 3) [20,27,46,137,138] nucleophilic aromatic substitutions ([Table 1](#), entries 4 and 5) [19–21,45,131,132,139], and Suzuki cross-couplings ([Table 1](#), entry 6) [22,46,48,140–146]. For the acylation reactions in DELs, the most commonly used coupling reagent is the 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM), however optimization studies suggested that the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/1-hydroxy-7-azabenzotriazole (EDC/HOAt) combination is a superior choice [136]. The most straightforward approach for reductive alkylation is by having the aldehyde connected to the DNA and the amine in solution, in order to avoid double alkylation ([Table 1](#), entry 2) [137,138]. This setup has been used in the development of a 3.5 million member DEL [46]. However, with the amino group being dominantly present in intermediates of DEL development, and several protection groups are available for this functional group, examples of the opposite approach have also been explored and used in library synthesis ([Table 1](#), entry 3) [20,46]. In addition, this modification has been optimized to work with ketones as well [27].

In DEL synthesis, nucleophilic aromatic substitution has primarily been used in stepwise substitution of cyanuric chloride ([Table 1](#), entry 4) [19,21], and in connection to the synthesis of benzimidazoles, where primary amines have been used as nucleophiles towards *ortho*-fluoro nitrobenzenes ([Table 1](#), entry 5) [45,131]. The former approach has led to the development of a library consisting of a 1,3,5-triazine core with more than 4 billion members [20].

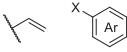
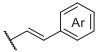
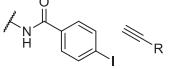
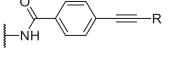
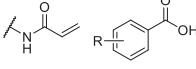
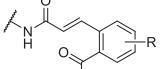
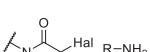
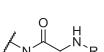
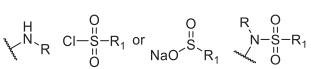
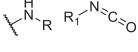
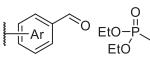
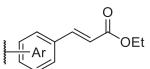
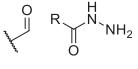
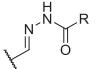
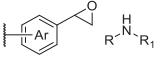
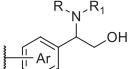
The Suzuki reaction was initially published to work with $\text{Pd}(\text{PPh}_3)_4$ at 80 °C and good to excellent conversion was observed for a large set of boronic acids and boronic esters ([Table 1](#), entry 6) [141]. Further investigation of these conditions revealed that a significant amount of the DNA was damaged under these conditions [143]. Waring and co-workers recently reported a micelle approach for Suzuki cross-couplings on DNA-tagged compounds using commercial surfactants [145]. Here, the reaction was taking place in the centre of the micelle, while the DNA tag remained located in the aqueous phase. The authors were able to perform Suzuki cross-couplings with no detectable DNA damage and it is speculated to be a general tool for expanding the chemistry available for DEL synthesis. Optimization of the reaction conditions for Suzuki cross-couplings permitted challenging aryl halides to be used with a diverse set of boronates, which broadened the chemical space obtained from this reaction [142,144].

Table 1 Reported examples of chemistries in DEL synthesis for the introductions of appendage diversity.

#	Reaction	Reactants ^a	Product	References
1	Acylation			[19 ^b , 57 ^c , 96 ^b , 136]
2	Reductive amination			[46 ^b , 137, 138]
3	Reductive amination			[20 ^b , 27 ^d , 46]
4	S _N Ar ^e			[19 ^b , 20 ^b , 21 ^b]
5	S _N Ar			[45 ^b , 131, 132, 139]
6	Suzuki			[22 ^b , 46 ^b , 48 ^b , 140, 141, 142, 143, 144, 145, 146]
7	Buchwald-Hartwig; Ullmann-type; Electrochemical amination			[27 ^f , 147, 148, 149 ^b]
8	Decarboxylative alkylation ^g			[150, 151]
9	Decarboxylative arylation ^h			[26, 27 ^f , 32 ⁱ]
10	Defluorinative alkylation ^j			[26]
11	CuAAC ^k			[22, 25, 47 ^b , 69 ^c , 125 ^b , 130 ^b , 133, 146, 152]
12	C3 alkylation of indoles			[31 ^l]

Continued

Table 1 Reported examples of chemistries in DEL synthesis for the introductions of appendage diversity.—cont'd

#	Reaction	Reactants ^a	Product	References
13	Heck-type reaction ^m			[33 ⁿ , 57 ^c , 153 ^c]
14	Sonogashira			[25, 146]
15	CH activation			[154]
16	S_N2 reaction			[25, 138 ^b , 155 ^b]
17	Sulphonylation			[20 ^b , 156 ^o]
18	Urea formation			[20 ^b , 157]
19	Horner-Wadsworth-Emmons			[25]
20	Hydrazone ligation			[137]
21	Epoxide opening			[49 ^{b,p}]

^aWavy lines indicate DNA conjugation site.

^bChemistry included in DEL synthesis.

^cDNA-templated reaction.

^dStudy also included reductive amination of ketones.

^eY and Z=CH or N.

^fDNA was adsorbed to ion exchange resin prior to the reaction.

^gZ=Ar or carbonyl. Y=OH if R₂=NBoc, otherwise Y=NHPI.

^hY=COOH if R1=NBoc, otherwise Y=DHP, bis(catecholato)silicates, or N-hydroxyphthalimide ester.

ⁱA few examples of alternative radical precursors for the photoredox transformation were presented, including desulfinative, deborylative, and cross-electrophile coupling reactions.

^jY=COOH if R1=NBoc or NFmoc, otherwise Y=DHP or bis(catecholato)silicates.

^kThe DNA can be positioned on either or both ends of the two reactants.

^lExamples of having the aldehyde conjugated to DNA and the indole in solution is also reported.

^mX=I, Br, B(OH)₂, or Bpin.

ⁿExamples of DNA conjugated aryl iodides towards styrenes in solution was also reported.

^oExamples of having the sulphonic acid conjugated to DNA and the amine in solution is also presented.

^pExamples of epoxides positioned on non-aromatic compounds is also presented.

Suzuki couplings have been vastly explored in DEL synthesis, and three different libraries have been reported using this reaction [22,46,48].

The formation of *N*-aryl bonds is commonly used in medicinal chemistry and is present in many bioactive molecules [158,159]. Since 2017 several C—N cross-coupling reactions have been reported, including Buchwald-Hartwig aminations [147,149], Ullmann-type couplings [147,148], and electrochemical aminations (Table 1, entry 7) [27]. This modification is attractive for construction of DELs, not only for the C—N bond formation, but also due to the availability of amine and aryl halide BBs.

A major challenge in developing DNA compatible reactions is bringing the classic organic reactions into an aqueous medium. Extensive optimization studies have been reported, as in the case of the development of an $\text{sp}^3\text{-sp}^3$ coupling *via* a Giese-type reaction, where more than 100 optimization experiments were required to identify conditions that were compatible with on-DNA synthesis (Table 1, entry 8) [151]. An alternative to optimize reactions for compatibility with water, is bringing the DNA into organic solvents. Researchers from Scripps research institute introduced in 2019 a concept known as Reversible Adsorption to Solid Support (RASS) [27]. A strong anion exchange resin based on quaternary ammonium moieties was identified and used for adsorption of the DNA on to the resin. Hereafter, various chemistries were carried out on the amino-modified DNA, followed by removal of excess reagents by washing. The polyvalent DNA molecule remained adsorbed onto the solid support and was finally eluted using a high salt concentration buffer. A variety of organic solvents were allowed, and the setup was applicable for decarboxylative $\text{sp}^2\text{-sp}^3$ cross-couplings (Table 1, entry 9), electrochemical amination of DNA conjugated aryl halides (Table 1, entry 7), and reductive alkylations (Table 1, entry 3) [27].

Photoredox chemistries have in recent years expanded the choice of appendage reactions for DNA-encoded chemistry. To this end, formation of $\text{C}(\text{sp}^3)\text{-C}(\text{sp}^3)$ (Table 1, entry 8) [150] and $\text{C}(\text{sp}^2)\text{-C}(\text{sp}^3)$ (Table 1, entry 9) [26,32] bonds on DNA conjugated BBs have been widely explored. These modifications allow installation of sp^3 carbons in DEL synthesis, which previously have been known to be lacking in libraries. Various functional groups have been used as radical precursors for both $\text{C}(\text{sp}^3)\text{-C}(\text{sp}^3)$ and $\text{C}(\text{sp}^2)\text{-C}(\text{sp}^3)$ cross-couplings. In cases of free carboxylic acids, bond formation was only feasible for the ubiquitous *N*-protected α -alpha amino acids [26,32,150]. However, introducing other radical precursors, such as bis(catecholato)-silicates, dihydropyridines, or *N*-hydroxyphthalimide esters, enabled similar transformations without the limitation of having a

radical stabilizing heteroatom present on the α -position [26,27,151]. Using the same radical precursors as described above, Molander and co-workers showed that they were able to use photoredox catalysis in defluorinative alkylation reactions, forming gem-difluoroalkenes from trifluoromethyl alkene precursors (Table 1, entry 10) [26]. This motif is an attractive functional group, as it serves as a metabolic stable carbonyl isostere.

The CuAAC click reaction is known to be highly biocompatible, and several examples of its usage to form 1,2,3-triazoles in connection to DEL synthesis have been reported (Table 1, entry 11) [22,25, 47,69,125,130,133,146,152]. However, a high amount of Cu(I) during prolonged reaction times can lead to oxidative damage of the DNA [152]. In order to minimize this, the reaction has been handled with short reaction times (5–10 min) [133,160], addition of complexing agents such as Tris((1-benzyl-4-triazolyl)methyl)amine (TBTA) or Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) to stabilize the catalytic active Cu(I) [22,25,47,130], and/or addition of DMSO as an oxygen radical scavenger [152].

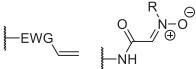
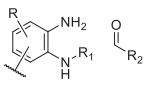
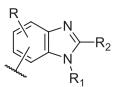
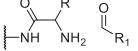
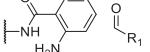
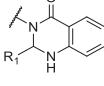
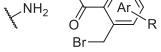
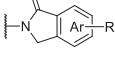
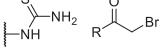
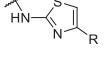
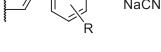
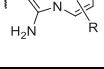
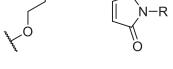
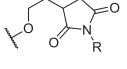
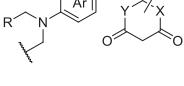
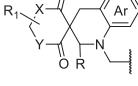
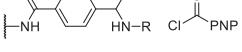
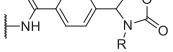
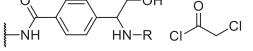
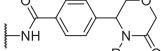
On-DNA C3 functionalization of indoles was reported in 2019 by Researchers from HitGen (Table 1, entry 12) [31]. Here, a two-step procedure was employed by using aldehydes as electrophiles to form the corresponding indolyl alcohols with DNA-appended indoles. This intermediate was subsequently reduced using the Hantzsch ester under slightly acidic conditions (pH 4.8). The overall reaction provided alkylated products in good to excellent yields with a broad scope of aryl and alkyl aldehydes.

Other known appendage reactions which have been reported in connection to DEL include Heck couplings (Table 1, entry 13) [33,153], Sonogashira couplings (Table 1, entry 14) [25,146], C—H activations (Table 1, entry 15) [154], S_N2 reactions (Table 1, entry 16) [25,138,155], sulphonylations (Table 1, entry 17) [20,156], urea formations (Table 1, entry 18) [20,157] Horner-Wadsworth-Emmons reactions (Table 1, entry 19) [25], hydrazone ligations (Table 1, entry 20) [25], and Henry reactions [57].

5.2 Formation of heterocycles in DELs

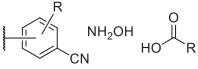
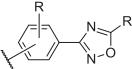
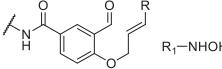
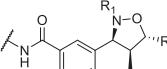
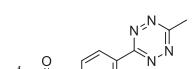
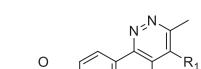
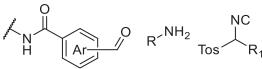
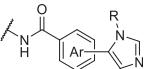
Diversely substituted heterocycles are structural components in many bioactive compounds including synthetic and natural product-like molecules [161]. Hence, bringing heterocyclic structure motifs into DELs has been a focus since the early work of *on-DNA* synthesis. Liu and co-workers described in 2002 the synthesis of isoxazolidines *via* a 1,3-dipolar cycloaddition using DNA-templated chemistry (Table 2, entry 1) [57]. Researchers

Table 2 Reported examples of heterocycles in connection to DEL synthesis.

#	Reaction	Reactants ^a	Product	References
1	1,3-Dipolar cycloaddition			[57 ^b]
2	Benzimidazole synthesis			[25,45 ^c ,131]
3	Imidazolidinone synthesis			[25]
4	Quinazolinone synthesis			[25]
5	Isoindolinone synthesis			[25]
6	Thiazole synthesis			[25]
7	Imidazopyridine synthesis			[25]
8	Diels-Alder			[51 ^c ,162 ^c]
9	T-reaction ^d			[139]
10	Oxazolidine-2-one synthesis			[49 ^c]
11	Morpholin-3-one synthesis			[49 ^c]

Continued

Table 2 Reported examples of heterocycles in connection to DEL synthesis.—cont'd

#	Reaction	Reactants ^a	Product	References
12	1,2,4-Oxadiazole synthesis			[29]
13	Isoxazolidine synthesis			[28]
14	Inverse-electron-demand Diels-Alder reaction			[34 ^e]
15	Van Leusen imidazole synthesis			[35]
16	Ring-closing metathesis			[163,164]

^aWavy lines indicate DNA conjugation site.

^bDNA-templated reaction.

^cChemistry included in DEL synthesis.

^dX and or Y = NH, N(alkyl), CH₂, O.

^eProline was added in cases of ketones and aldehydes as alkene surrogates. A few examples of enol ethers were also reported.

from Roche reported in 2015 the synthesis of a variety of heterocycles, including benzimidazoles (Table 2, entry 2), imidazolidinones (Table 2, entry 3), quinazolinones (Table 2, entry 4), isoindolinones (Table 2, entry 5), thiazoles (Table 2, entry 6), imidazopyridines (Table 2, entry 7), and triazoles (Table 1, entry 11) [25]. From this impressive selection, the benzimidazoles have especially gained a lot of attention, and various procedures for preparing this compound class on DNA have been reported and used in the preparation of a 320 million member DEL [23,45,131]. Neri and co-workers described in 2008 the synthesis of bicyclic tetrahydroisoindolinones using unactivated dienes and maleimide modified BBs in a Diels-Alder reaction (Table 2, entry 8) [51]. This methodology was employed for the preparation of a 1 million member library and used in a successful selection of ligands for carbonic anhydrase IX [162].

In more recent years, DNA compatible methodologies for the synthesis of heterocyclic compounds have expanded. A method for preparing diversely substituted spirocycles from *ortho*-dialkylaminoaryl aldehydes and activated methylenes has been carried out using the ‘T-reaction’ ([Table 2](#), entry 9) [139]. Using relatively mild conditions, several examples of complex polycyclic compounds were prepared on-DNA with good to excellent conversion. In 2017, a method for ring opening epoxides using Lewis acid catalysed aminolysis was described ([Table 1](#), entry 21) [49]. The reaction performed well towards a broad spectrum of aliphatic and aromatic amines affording the corresponding β -amino alcohols. After formation of the amino alcohol, oxazolidin-2-one ([Table 2](#), entry 10) and morpholin-3-one ([Table 2](#), entry 11) heterocyclic motifs were introduced using 4-nitrophenyl chloroformate and chloroacetyl chloride, respectively. The synthetic design was used for the development of a 136 million member DEL. On-DNA synthesis of a selection of 1,2,4-oxadiazoles using a multistep synthetic approach has been described ([Table 2](#), entry 12) [29]. Here, DNA conjugated aryl nitriles are converted to amidoximes using hydroxylamine. Subsequently, an *o*-acylation was performed where the *in situ* coupling reagent, PyAOP, was found to be a superior choice. Finally, a cyclodehydration reaction provided the product oxadiazoles. Schreiber *et al.* reported in 2019 an on-DNA synthesis of sp^3 -rich polycyclic isoxazolidines *via* a [3 + 2] cycloaddition using olefin-containing salicylaldehydes and *N*-alkylhydroxylamines ([Table 2](#), entry 13) [28]. The methodology was applicable to a broad spectrum of hydroxylamines and the olefins could readily be prepared in one step from the corresponding phenol.

Having both tetrazines in solution or conjugated to DNA, researchers at Amgen described the synthesis of the corresponding pyridazines from a one-pot two-step synthesis involving an inverse-electron-demand Diels-Alder reaction and subsequent oxidation ([Table 2](#), entry 14) [34]. The authors showed that the reaction was applicable for a range of alkenes, styrenes, enol ethers, ketones, and aldehydes. For the two latter reactants, proline was added to promote product formation.

In 2019 researchers from Roche presented an elegant method for synthesizing highly functionalized imidazoles on-DNA *via* a Van Leusen multicomponent reaction ([Table 2](#), entry 15) [35]. The reaction proceeded in high conversion for a large set of DNA-appended aldehydes and amines, but was limited to a small subset of aryl-functionalized toluenesulphonylmethyl isocyanides. Despite the potential of creating

interesting structural motifs from DNA-linked multicomponent reactions, the combinatorial library design and encoding process becomes challenging since two or more building blocks need to be encoded in one step. As an example, if a library is synthesized with three sets of 1000 different BBs in each position using single component appendage reactions, a total of 3000 splits are required for the synthesis and subsequent ligation. However, for library synthesis using a multicomponent reaction with the same sets of BBs, more than 10^6 splits are required: 1000 splits for conjugation of the first set, followed by 1000×1000 splits for the multicomponent reaction with two sets of BBs in solution, to ensure encoding of all BBs.

Macrocyclic compounds have the potential of interacting with challenging targets, such as disrupting protein–protein interactions, and a significant number of macrocyclic drugs are currently on the market [165]. Several DELs containing macrocyclic compounds have been described where the macrocyclization has been carried out using the Wittig reaction [11,58,60], acylation [47], *via* the CuAAC reaction [125], or synthesized using a constant macrocyclic scaffold [52]. Recently, researchers from GlaxoSmithKline (GSK) reported yet another method for the synthesis of on-DNA macrocycles using ring closing metathesis (RCM) (Table 2, entry 16) [163]. The procedure was applicable for DNA-tagged macrocycles of various sizes, but has yet to be described in DEL synthesis.

5.3 DNA incompatible reactions for DEL synthesis

A major limitation for access to drug-like structures in DELs is the inability to use Brønsted acids. To address this problem, several new approaches have been introduced in recent years. In 2017, a synthetic strategy known as oligothymidine-initiated DNA-encoded chemistry (TiDEC) was developed. In this strategy, a hexathymidine oligonucleotide (hexT) equipped with an amino-modified PEG-linker was synthesized on controlled pore glass solid support (Fig. 7) [36].

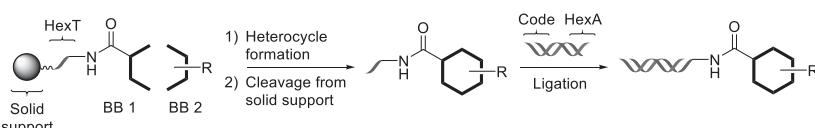


Fig. 7 Oligothymidine-initiated DNA-encoded chemistry. Illustration of the hexT approach for the synthesis of DNA-encoded small molecules using conditions that are known to lead to DNA degradation.

The hexT can tolerate a broad range of catalysts that are known to degrade DNA [38], and the authors showed that they could use the setup for the synthesis of a broad range of heterocycles. These include synthesis of substituted pyrazol(in)es *via* a Au(I)-mediated cascade reaction, Brønsted acid catalysed Pictet-Spengler reactions, an Yb(OTf)₃-mediated Castagnoli-Cushman reaction, an AgOAc-mediated (1,3)-cycloaddition forming substituted pyrrolidines, ZnCl₂-mediated aza-Diels-Alder reactions, the Povarov reaction, and the Biginelli reaction [36–38]. Furthermore, the setup was exploited to investigate the scope of several multicomponent reactions, including the Ugi four-component reaction, the Ugi-azide reaction, the Ugi/aza-Wittig reaction, and the Groebke-Blackburn-Bienaymé reaction [166]. In some cases, the reaction conditions of the multicomponent reactions allowed a DNA codon to be present on the solid support. After complete reaction, the heterocyclic compound conjugated to the hexT was cleaved from the solid support by treatment with NH₃/MeNH₂ and subsequently ligated with a complementary hex-adenine oligonucleotide bearing the codon for the attached heterocycle.

Researchers from Roche developed in 2019 a method to prepare thousands of BBs using DNA incompatible chemistries by using solid-phase synthesis [41]. The Petasis and Pictet-Spengler reaction was used to access new BBs. Upon release of the BBs from the solid support, an acid functional group was revealed, which could then be conjugated to an amino-modified oligonucleotide. These examples enable synthesis of large sets of BBs containing scaffolds that are otherwise inaccessible with the current synthetic methodologies applied to DEL, and hereby assist the expansion of structural diversity. However, since two or more reactants go into the synthesis of these BBs and only one encoding step is typically allowed, the molecular weight of the final BBs are often above 200Da, which compromises the number of possible splits in the final library.

The usage of micellar catalysis for organic synthesis in aqueous media is an approach where organic reagents can be kept inside the micelles, leading to high effective concentrations [167]. This principle was exploited by Brunschweiger *et al.* in an interesting technological development, which allows DNA conjugated compounds to undergo Brønsted acid catalysed chemical modifications using micelle-based acid catalysts (Fig. 8) [30]. Thereby, exposure of the DNA to reagents that would otherwise be harmful, was avoided. In this study, a sulphonic acid catalyst based on amphiphilic block copolymers was used to form nanometre-sized oil-in-water micelles. The chemical transformations occurred in the centre of the micelle, while the

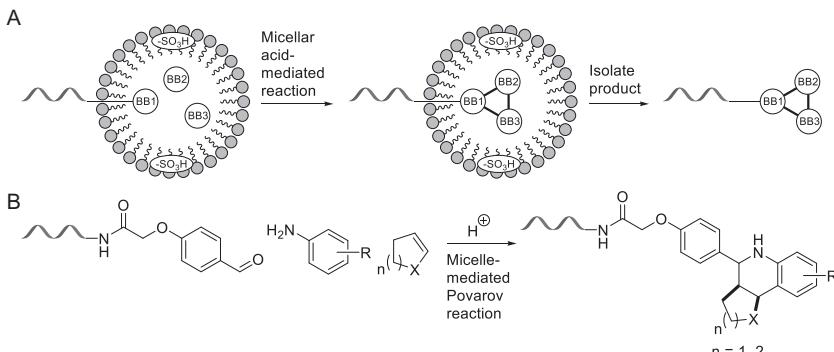


Fig. 8 (A) Illustration of acid catalysed micelle-mediated reactions of DNA conjugated intermediates. (B) Example of the Povarov multicomponent reaction carried out in micelles. X = O or NBoc.

DNA was localized in the surrounding aqueous media. The authors showed that they were able to form tetrahydroquinolines and aminoimidazopyridines using the Povarov and Groebke–Blackburn–Bienaymé reactions, respectively. Furthermore, upon the addition of MgCl₂ it was found that the system could be used to deprotect Boc groups from masked amines.

5.4 Functional group transformations and protecting groups in DEL synthesis

Several different protecting groups have been introduced to DEL synthesis and most of them belong to the protection of amines. The Boc group is a commonly used amine protection group in solution chemistry and is typically removed under acidic conditions. Since such conditions are not compatible with DNA, *on*-DNA Boc removal is typically performed using NaOAc/MgCl₂ [143] or a borate buffer [25] at elevated temperatures (80–90 °C) over long reaction periods. These conditions can be damaging to the DNA and alternative groups have been utilized for the protection of amines, including Fmoc [19,143], Alloc [25], Nvoc [25,52,53], trityl [14], 4-cyano-2-nitrophenyl (CNP) [14], Nosyl [14,47], and trifluoroacetamide [52]. Carboxylic acids are typically protected as esters, and deprotected by hydrolysis under alkaline conditions [25]. Liu *et al.* introduced an alternative procedure, where the azide of a 4-azidobenzyl ester was reduced photolytically in the presence of Ru(bpy)₃Cl₂, which led to exposure of the masked acid [133]. Several methods for reduction of nitro groups have been reported including the use of Raney nickel [25], Na₂S₂O₄ [131], FeSO₄ [45], and recently B₂(OH)₄ [132]. This transformation has primarily

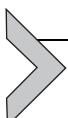
been used in connection with the synthesis of benzimidazoles. Other functional group transformations include oxidative cleavage of 1,2-diols using NaIO₄ [11,58], diazotransfers [128,129], azide reductions [133], and palladium catalysed hydroxycarbonylations [134].

5.5 Fidelity in DEL synthesis

There are three main aspects that contribute to the fidelity of a DEL: correspondence between code and molecule, robustness of the chemistry, and integrity of the DNA [168]. Damage or degradation of the DNA tag will lead to loss of structural information about the attached small molecule in post-selection sequencing, hence non-degrading reaction conditions are vital for creation of a high-fidelity DEL. Different approaches have been used to measure the DNA integrity, including Sanger sequencing [28,144,154], qPCR [152], and dsDNA-functionalized magnetic ‘sensor beads’ used as a model DNA tag for subsequent qPCR amplification [143]. Recently, an assay was developed where the *on*-DNA reaction yield, the ligation efficiency, and the DNA compatibility was measured [168]. However, despite the importance of this aspect of DEL synthesis, analysis of post-synthesis DNA integrity is not always provided in reported studies. Indeed, minimizing DNA degradation is an important aspect. Donahue and co-workers described that an excess of Mg²⁺ during *on*-DNA ruthenium catalysed ring closing metathesis reactions reduced DNA decomposition [163]. The authors suggested that this observation could be due to coordination of the Mg²⁺ to the DNA backbone, thereby preventing ligation of the ruthenium, thus leading to an increased amount of active catalyst and reduced DNA degradation.

The concept behind all DEL technologies relies on the right code-molecule association. Mismatches impose a high risk of identifying an increased number of false positives. A similar risk exists when unexpected side-reactions occur, making the robustness of each chemical transformation extremely important. Recently, the field has made a great progress in the exploration of new chemical synthesis with the aim of expanding the scope of DNA compatible reactions and it will be exciting to see where the field can extend its boundaries. However, the use of simple chemistry with complex building blocks remains the first-choice approach to ensure control of the chemical transformations and an adequate chemical complexity of the library members. As described in the previous sections, novel technological approaches, such as micelles [30,145], the hexT approach [36–38], and anion exchange resins [27] have been used to improve reaction yields and

reduce DNA decomposition. Furthermore, optimizations and improvements of known DNA compatible reactions in terms of reagents [27,129,132,136], catalysts [133,142,144,149], or solvent mixtures [152] assist to expand the substrate scope, minimizing side-products or truncations during library synthesis, while reducing degradation of the attached DNA tag. In addition to implementing fidelity in DELs through chemical procedures, DNA-directed library technologies such as the YoctoReactor[®], enable purification of the combinatorial library during each step [65,66]. Thereby, all failed reactions are removed before the library synthesis is progressed, and truncated or incomplete products are eliminated.



6. Hits identified from DELs

The DEL approach has come a long way since its initial concept, and the field has been maturing to make it a powerful tool for hit generation. The initial examples reported in literature were mainly focused on technological developments with many compounds being identified from libraries formed by assembling four BBs [11]. This generated huge libraries with hundreds of millions to billions of members dominated by large, flexible, and lipophilic molecules with poor physicochemical properties, halting their progression as clinical candidates. Instead of generating large libraries with billions of members, it is now commonly accepted to pool smaller libraries with 2–3 points of diversity. This approach has led to tractable hits capable of being developed into preclinical candidates or chemical probes that can be evaluated *in vivo*. The target scope has been increasing and there is a diverse set of target families where successful DEL screening campaigns have been reported. The following section will describe representative examples for each of the target families, with a focus on the most paradigmatic examples.

6.1 Kinases

Kinases are phosphotransferases involved in modulation of many cellular signalling cascades, with a key role in the development of neoplastic disease, vascular diseases, CNS disorders, inflammation, and chronic inflammation. Therefore, kinases are one of the most studied families of targets in drug discovery; at present, 39 kinase inhibitors have been approved by FDA and several hundred are currently in various stages of clinical development [169,170]. Kinases are the family of receptors where the largest number of examples of hits identified by DELs have been reported. In many cases, the DEL screens were able to provide non-optimized hits directly out of

the library with single digit nanomolar activity on both biochemical and biophysical assays, and selectivity across other kinases. The compound VPC00628 (Fig. 9, 1) was identified by researchers from Vipergen from a screen of a 12.6 million membered library against p38 α MAP kinase [67]. The library was prepared using the YoctoReactor® [65], and was then screened using the binder trap enrichment protocol [66]. Among several inhibitors identified, VPC00628 was the most potent compound with an IC₅₀ of 7 nM in a cellular assay, and proved to be selective against a panel of other kinases [67]. The p38 α MAP kinase was also screened by Praecis

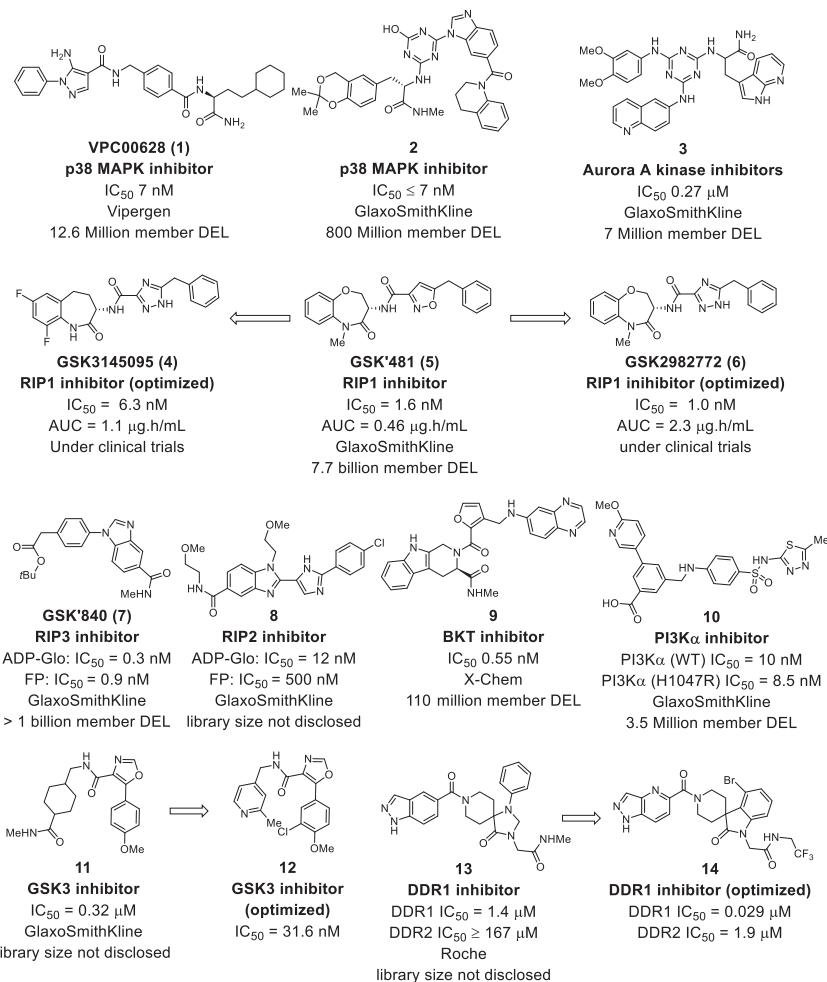


Fig. 9 Kinase inhibitors identified from DELs.

Pharmaceuticals (currently part of GSK), and inhibitors with an IC₅₀ lower than 7 nM were identified from a triazine DEL of 800 million members (2) [19]. In the same publication, Aurora A kinase inhibitors were reported and identified from a 7 million member triazine library, with the most potent compound (3) exhibiting an IC₅₀ of 0.27 μM.

Receptor Interacting Protein (RIP) kinases are a family of seven members containing a common serine-threonine kinase domain [171]. RIP1 was the first member to be identified, and it has been shown to be a critical regulator of inflammation and cell death [172,173]. As a result, it has emerged as a promising therapeutic target for inflammation, autoimmune, and neurodegenerative diseases. In 2016, Gough *et al.* reported the identification of a benzoxazepinone RIP1 inhibitor, GSK'481 (5) [24]. In the first attempts to identify potent and developable RIP1 inhibitors, two different screenings were run, including a kinase specific screening collection, and a HTS of about 2 million compounds. Inhibitors identified in both screening campaigns provided potent inhibitors, but their demanding developability led the researchers from GSK to run a DEL screening. The approach successfully allowed the identification of the GSK'481 hit series with high RIP1 potency, kinase selectivity, and a great potential to achieve oral compound administration. The discovery is a remarkable example, not only of the efficiency of DELs to deliver valuable and developable hits, but also a concrete evidence of the complementarity between different approaches. An intriguing medicinal chemistry work on this hit series delivered two new clinical candidates, GSK3145095 (4) and GSK2982772 (6), with a good oral bioavailability and high potency [18,174]. GSK3145095 is currently in Phase I clinical studies for pancreatic adenocarcinoma and other selected solid tumours, and GSK2982772 is currently in Phase IIa clinical studies for psoriasis, rheumatoid arthritis, and ulcerative colitis. In addition to RIP1, the identification of selective and potent binders for two additional kinases of the RIP family have been reported [175,176]. In 2014, researchers from GSK described the identification of three inhibitors of the RIP3 kinase, with the most potent compound coming from a 1 billion membered DEL screen. The most promising hit, GSK'840 (7), showed a potent inhibitory activity in the subnanomolar range and a good selectivity profile among 300 tested kinases. The crystallographic structure of compound 8 with RIP2, showed a type 1 inhibitor binding in the adenine and back pockets. This unusual binding mode led to a higher degree of selectivity among a panel of kinases, and it proved to be an attractive lead for the development of potent and selective RIP2 kinase inhibitors.

DELs have been used successfully for identifying new chemical matter with different binding modes in already established targets. Bruton's Tyrosine Kinase (BTK) is a validated anticancer target and a covalent inhibitor, Ibrutinib, is currently in use for the treatment of mantle cell lymphoma, chronic lymphocytic leukaemia, and Waldenstrom's macroglobulinaemia [177]. However, mutations at the binding site have caused the treatment to be ineffective in some cases, and demonstrated a need to identify novel inhibitors with a different binding mode. In 2017, researchers from X-Chem reported the identification of three potent inhibitors of BTK from a 110 million membered DEL [178]. Multiple parallel selection screens with different protein concentrations allowed the differentiation between strong and weak binders. Furthermore, upon addition of ATP, the allosteric binders could be distinguished from the ATP-competitive binders. This work highlights the potential of DEL screens and the binding information that can be obtained directly from multiple screens performed in parallel. Without any structural optimization, the most potent hit from this screen (**9**) was an ATP-competitive inhibitor with an IC₅₀ of 0.55 nM. Crystallographic studies revealed a novel hydrophobic binding pocket close to the ATP binding site, which could be helpful in designing selective BTK inhibitors.

Phosphatidylinositol-3-kinase (PI3K) is an attractive anticancer target. Currently there are drugs on the market targeting this kinase and several more are in clinical trials [179]. In an attempt to find new binding modes and scaffolds, researchers from GSK reported the identification of a selective inhibitor (**10**) for PI3K α wild-type and a mutant H1047R [180]. This hit was identified directly from a 3.5 million member DEL and crystal structure analysis revealed its unique binding to the ATP binding site.

Glycogen synthase kinase-3 (GSK3) is a serine/threonine kinase which is currently being investigated as a potential therapeutic target for neurodegenerative and neurological diseases. A team from GSK screened multiple DELs against this target and successfully managed to identify a potent hit (**11**) with a unique kinase inhibitor scaffold [181]. However, unfavourable physicochemical properties limited its CNS permeability and further optimization focused on reducing the polar surface area. Several analogues were synthesized and with structure-guided optimization, a potent and selective inhibitor was identified (**12**). This hit had an acceptable pharmacokinetic profile with good brain barrier penetration, demonstrating the possibility of using DELs for CNS targets.

Discoidin Domain Receptors, DDR1 and DDR2, are receptor tyrosine kinases (RTKs) involved in many key cellular processes, such as cell

differentiation, proliferation, adhesion, migration, and invasion [182]. DDR1 has emerged as a potential therapeutic target for several diseases such as pancreatic cancer and renal fibrosis [182], but identification of selective DDR1 inhibitors is recognized to be challenging. So far, most reported ligands lack selectivity across the kinase and this is partly due to the high degree of ATP binding pocket homology across all RTKs. Thus, most ligands targeting this pocket are recycled from other kinase projects [183]. Moreover, inhibitors for DDR1 need to be selective over its close analogue DDR2 since studies have suggested that DDR2 inhibition may promote chronic liver fibrosis [184]. Researchers from Roche Pharmaceuticals explored in a DEL screening campaign the interrogation of two library pools in parallel against DDR1 and DDR2. From the initial screen, 11 structurally distinct clusters were identified from 5 different libraries and the activity was confirmed for 16 of the resynthesized hits. The most promising series (**13**) was further optimized through structure-guided design to yield a potent DDR1 inhibitor (**14**) with high kinase selectivity, including 64-fold selectivity over DDR2, and a good pharmacokinetic profile. Subsequently, this compound was further tested in a mouse model for chronic kidney disease and showed a preservation of renal function with reduced tissue damage [183].

Liu *et al.* reported the identification of ethacrynic acid as a MAP2K6 covalent inhibitor [185]. This FDA-approved loop diuretic was identified from a DEL screen using unpurified proteins, where it was interrogated in a library of DNA-encoded bioactive compounds and DNA-tagged human kinases. In another study, Neri and co-workers reported the identification of a specific and covalent ligand of JNK-1 using an encoded self-assembling chemical (ESAC) library [186]. JNK-1 is a promising therapeutic target for cancer, inflammatory, and neurologic diseases [187]. A library of 148,135 members was screened, containing many BBs capable of covalent bond formation, and after an initial screen, with a large molar excess of glutathione to simulate the intracellular environment, a pair of two fragments was identified. These were joined by a short polyethylene glycol linker to give a ligand capable of covalently binding to JNK-1 with an apparent dissociation constant of $1\mu\text{M}$. Interestingly, when the two BBs were screened separately, no adduct was observed, indicating the synergic binding of the two BBs. Winssinger's group reported the identification of covalent inhibitors of MEK2 and ERBB2 by screening two PNA-encoded libraries of 10,000 members [90]. In this case, the covalent binders were distinguished from the non-covalent binders by a simple denaturing wash.

6.2 Soluble epoxide hydrolase (sEH)

One of the most advanced examples of DEL screenings has been in the identification of soluble epoxide hydrolase (sEH) inhibitors [188,189]. This enzyme is involved in the metabolism of biologically active epoxyeicosatrienoic acids (EETs) to less active metabolites dihydroxyeicosatrienoic acids (DHETs). EETs have been shown to be paracrine and autocrine effectors, modulating the endothelial function in the control of vasomotor tone, proliferation, and survival of endothelial cells. As a result, blocking metabolism of EETs by sEH has a potential therapeutic application in several conditions such as inflammation, cardiovascular disease, diabetes, respiratory disease, and neuropathy [190]. In 2013, researchers from GSK reported the identification of a hit from a DEL collection of triazine derivatives of 800 million members [188]. Upon identification of the initial hit (Fig. 10, 15) directly out of the DEL screen [17], subsequent structure optimization led to the discovery of GSK2256294A (16) [188]. This compound exhibited positive Phase I results, and it is currently undergoing Phase IIa clinical trials for diabetes. In 2015, researchers from X-Chem reported the identification of another sEH inhibitor from a 334-million member DEL constructed using a chemical ligation strategy [22]. The most potent compound (17) showed an IC_{50} of 2 nM and was 30,000-fold enriched after affinity-mediated selection.

6.3 Aminotransferases

Branched chain amino transferases (BCAT) are enzymes that catalyse transamination of branched chain amino acids. Of the two isozymes,

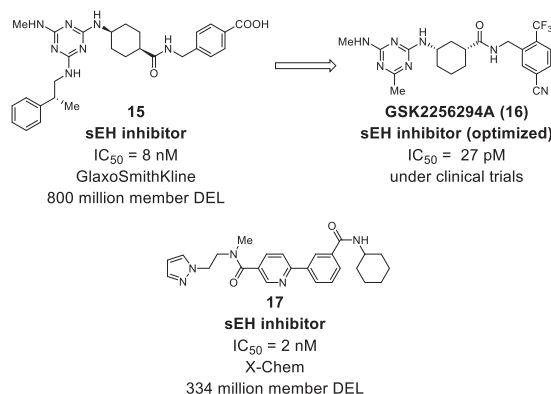


Fig. 10 Epoxide hydrolase inhibitors found from DEL screens.

mitochondrial (BCATm) and cytosolic (BCATc), the former is being studied as a potential target for metabolic diseases and cancer. In two different DEL screens, researchers from GSK reported the identification of BCATm inhibitors. In the first report, a hit (**Fig. 11, 18**) with an IC_{50} of $2\mu M$ was identified from a DEL of 34.7 million members [48], and in the second using a 117 million membered benzimidazole library, a hit with an IC_{50} of 251 nM was achieved (**19**) [23]. Hit **19** was further optimized to a lead (**20**) with improved pharmacokinetic properties. Both compounds **18** and **20** were crystallized with BCAT and were composed of comparable three-dimensional orientated moieties. However, it was suggested that the higher potency of compound **20** can be accounted by two additional hydrogen bonds.

6.4 Phosphatases

Wild-type p53-induced phosphatase (Wip1) is an enzyme involved in the regulation of key proteins responsible for the DNA damage-response pathway. This phosphatase is believed to be oncogenic by inactivation of tumour-suppressing pathways, and as a result, a potential therapeutic target [191]. Finding selective phosphatase inhibitors with appropriate bioavailability has proven challenging since the active binding site is conserved across phosphatases and is dense with positive charges [192]. Consequently, efforts have been made to search for allosteric inhibitors. In 2014, researchers from GSK reported the first allosteric Wip1 inhibitors identified by HTS and DEL screening campaigns [193]. Remarkably, the two hits contained an overlapping amino acid-like central core, which was referred to as capped amino acids, and were flanked by different groups that impacted the potency and bioavailability. The DEL hit showed a double-digit nanomolar IC_{50} value (**Fig. 12, 21**), while the HTS hit was 10-fold less potent in comparison.

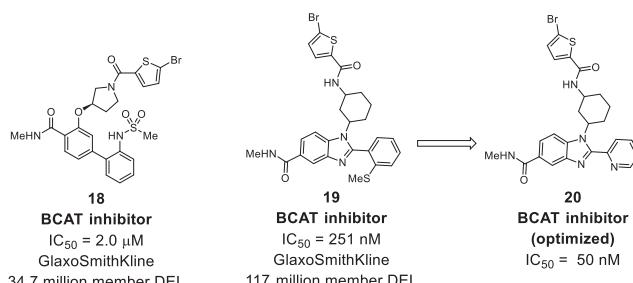


Fig. 11 BCATm inhibitors identified from DELs.

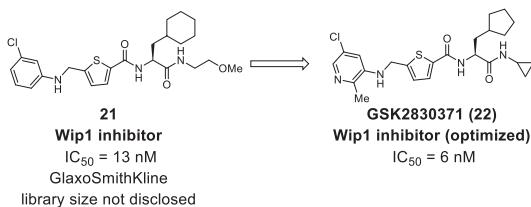


Fig. 12 Wip1 inhibitors identified from DEL screens.

Despite being a potent Wip1 inhibitor, the DEL hit showed a modest inhibition in cells assays, presumably due to poor permeability. This hit was further optimized into GSK2830371 (22), an inhibitor with improved physicochemical properties which could be translated into cellular activity. Further studies showed that this compound was selective for Wip1 over other phosphatases and interacted with the flap-subdomain.

6.5 Metalloproteases

Aggrecanases (a subset of the ADAMTS family of disintegrins) are a group of proteolytic enzymes involved in the degradation of aggrecan, which is one of the major components of the cartilage matrix. ADAMTS belong to the zinc-dependant metalloprotease family and ADAMTS-4 and ADAMTS-5 are currently being studied as therapeutic targets for osteoarthritis, which is a degenerative joint disease characterized by uncontrolled articular cartilage destruction [194]. In 2012, researchers from GSK reported the results from a screen of a 4 billion membered DEL against ADAMTS-5 [20]. Identified directly from the screen, the most potent hit (Fig. 13, 23) showed an IC_{50} of 30 nM. Contrary to many published metalloprotein inhibitors, this hit showed no zinc chelation and exhibited more than 10-fold and at least 88-fold selectivity over ADAMTS-4 and three other metalloproteins, respectively. In another study, researchers from GSK reported several ADAMTS-4 inhibitors, which were identified from screening a 802 million membered DEL [195]. In this study, the most potent hit showed an IC_{50} of 10 nM (24) with more than 1000-fold selectivity over ADAMTS-5 and -3 other metalloproteinases.

6.6 Protein arginine deiminases

Protein arginine deiminases (PADs) are enzymes capable of catalysing the citrullination of arginine residues and are involved in several physiological processes [196]. This family comprises five members (PAD1–4, PAD6)

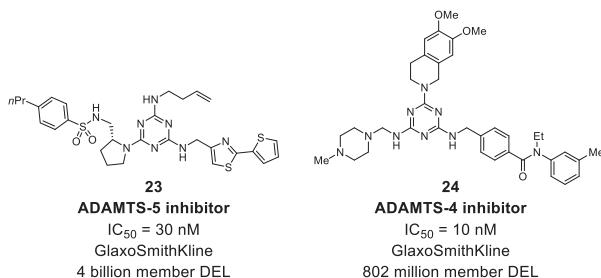


Fig. 13 ADAMTS hits identified from DEL screens.

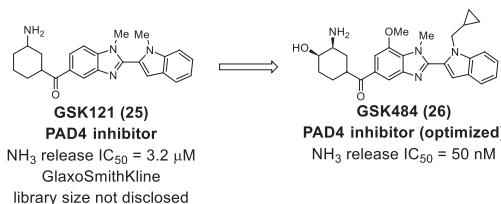


Fig. 14 PAD4 inhibitor identified from a DEL screen and its optimized structure.

and among them, PAD4 has been linked to many diseases including autoimmune and cardiovascular diseases and cancer. In 2015, researchers from GSK reported the identification of the first reversible PAD4-specific inhibitor, which was derived from a hit identified from a DEL screen and exhibited an IC_{50} of $3.2\text{ }\mu\text{M}$ (Fig. 14, 25). No details on the library size and chemical optimization were disclosed, however, structural improvements led to more than a 100-fold increase in potency (26), illustrating the feasibility for improvement of DEL hits with moderate potency [197].

6.7 NAD⁺ dependant enzymes

6.7.1 Poly(ADP-ribose) polymerases (PARPs)

Poly-ADP-ribose polymerases (PARPs) are a superfamily of enzymes that catalyse the poly-ADP-ribosylation of target proteins. This post-translation uses NAD⁺ as a substrate and is involved in DNA repair and the modulation of several other cellular processes [198]. There are 17 physiological human PARPs. PARP1 is already an established drug target, and currently four PARP1 inhibitors are in the market for ovarian and breast cancer [199]. Among other PARPs, Tankyrase1 (TNKS1 or PARP5) has received much attention for its regulatory role in the Wnt signalling pathway, telomere maintenance, and mitosis regulation [198]. Neri and collaborators reported

three different series of TNKS1 inhibitors identified by DEL screens [96,200,201]. The libraries were composed of small branched structures with two sets of diversity BBs, connected by a central linker. In the first report, a hit with an IC₅₀ of 290 nM (Fig. 15, 27) was identified from a DEL of 103,200 members [96]. In the same year, another series of TNKS1 inhibitors (28) was reported from a library of 76,230 members [200]. Remarkably, both series contained one fragment in common, indicating the potential of using this motif as a key element for the development of TNKS1 inhibitors. In a more recent study, an expansion of these libraries was reported where seven central scaffolds were used together with one set of diverse carboxylic acids and another of alkynes to generate a library of approximately 1 million members [201]. The geometry, stereochemistry, rigidity, and chemical nature of the central scaffold have a strong impact on the affinity as exemplified by the TNKS1 inhibitor, where a central linker derived from L-lysine led to a binder with an IC₅₀ of 15 nM (Fig. 15, 29), while the D-lysine counterpart proved to be inactive.

Franzini *et al.* reported the identification of a series of PARP inhibitors from a focused library of 58,302 members targeting NAD⁺-dependent enzymes [202]. This new DEL concept intended to expand the scope of DELs to target-class focused libraries, while taking advantage of the DEL capability to produce large combinatorial libraries in a short period of time [203]. The library named NADEL (NAD⁺ DNA-Encoded Library) was generated by attaching two sets of fragments in a 2,3-diaminopropionate

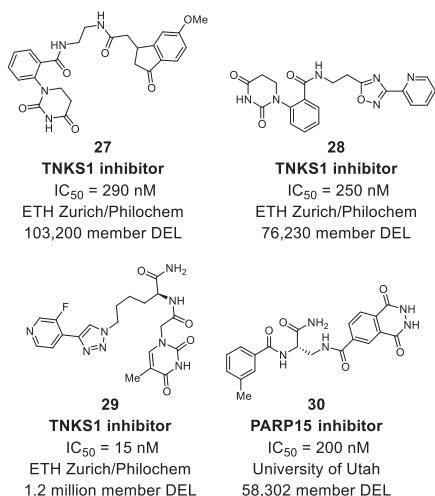


Fig. 15 PARP inhibitors identified from DEL screens.

linker. The chemical space of the NAD⁺ binding pocket was explored by joining 158 fragments that targeted the nicotinamide binding pocket together with 369 fragments of known ADP-ribosyl transferases binding motifs. This approach led to the discovery of PARP1, PARP2, PARP10, PARP12, PARP14, and PARP15 inhibitors, including the most potent PARP15 inhibitor reported to date, displaying an IC₅₀ of 200 nM (30). In addition, this library was further expanded to other NAD⁺-dependent enzymes, and one of the most potent Sirt6 inhibitor reported to date was also identified [202].

6.7.2 Histone deacetylases

Sirtuins (Sirt) are a class of NAD⁺-dependent histone deacetylases that have attracted much interest from the scientific community in the past decades for their key role in metabolism, lifespan, and epigenetics. Their therapeutic potential and biological role are not yet fully understood and there is a need to identify potent and selective modulators for each of the seven Sirt isoforms [204]. In a DEL screening campaign, researchers from GSK targeted Sirt3 and managed to identify several potent inhibitors directly from a library of 1.2 million members with nanomolar binding affinity [21]. These hits were not only potent Sirt3 inhibitors, but also showed to inhibit Sirt1 and Sirt2. The most promising hit (Fig. 16, 31) was further optimized (32) with an emphasis on reducing the molecular weight. From a DNA-encoded dynamic chemical library of 10,000 members, Li *et al.* described, as a proof-of-principle, the identification of an inhibitor of Sirt3 with low micromolar binding affinity (33) [80].

6.8 Protein-protein interactions

Inhibition of protein-protein interactions (PPIs) with small molecules has been viewed as challenging, since PPIs orthosteric binding sites are typically

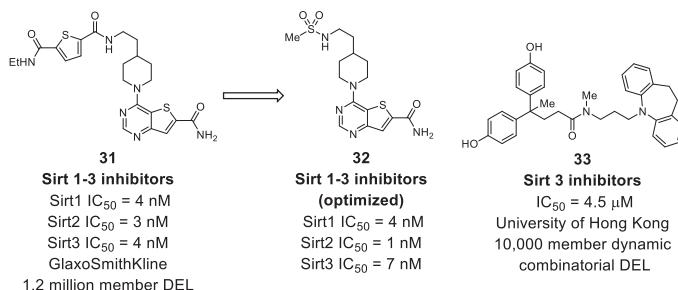


Fig. 16 Sirt1–3 inhibitors identified from DEL screens.

large and flat, which is very distinct from classical binding pockets targeted by small molecules [205]. However, the ability of small molecules to modulate certain classes of PPIs has been demonstrated by several reaching clinical trials [206]. DELs as a tool have also proven successful in identifying hits for PPIs. Among these, researchers from GSK reported the identification of sub-micromolar allosteric inhibitors of the integrin lymphocyte function-associated antigen 1 (LFA1), which is an established therapeutic target for the treatment of inflammatory and autoimmune diseases [207,208]. In this study, the small molecules were identified from a 4.1 billion member DEL containing a central triazine core. The most potent hit showed an IC_{50} of 16 nM (Fig. 17, 34). The attachment point of the DNA was used to introduce a fluorophore on the identified hits and confirm its binding to LFA1 expressed on cell membranes. Directly from DEL screenings, researchers from Neri's group have reported the identification of low micromolar inhibitors for IL2, Bcl-xL, and TNF, using libraries of a few thousand members [53,209,210]. In another study, a collaboration between researchers from AstraZeneca and X-Chem reported the identification of a single digit nanomolar inhibitor of Mcl1, which is a pro-apoptotic protein that is being

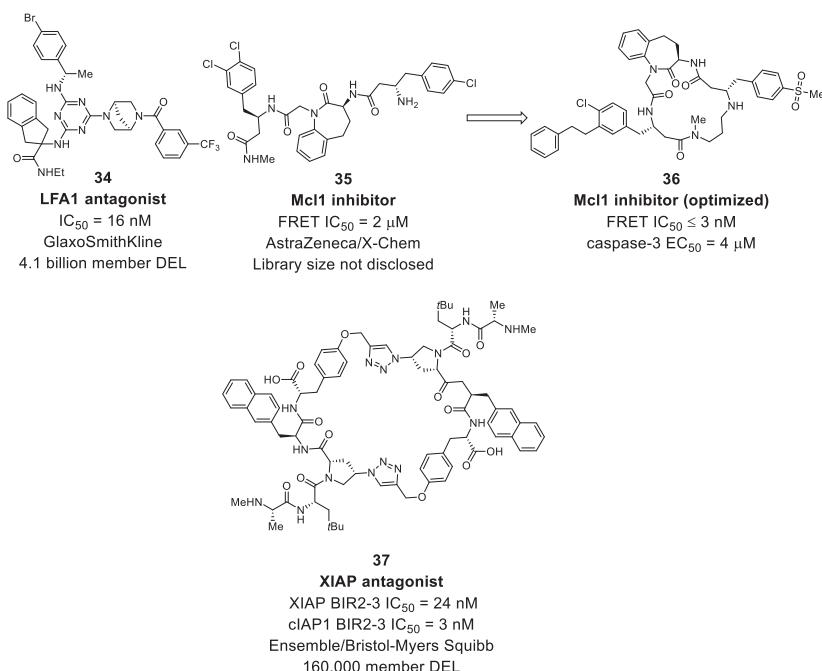


Fig. 17 PPI inhibitors identified from DEL screens.

studied as a potential anticancer target [211]. The initial hit (**35**) was identified through a DEL screen and after co-crystallization with Mcl1, it proved to bind to the target in a β -turn conformation, bringing the two ends of the hit into close proximity. Structure optimization, including macrocyclization of the hit, led to an approximate 500-fold increase in the potency (**36**).

Macrocycles are valuable motifs in drug discovery and have proved to be able to modulate targets where traditional small molecule methods have failed, particularly within PPIs [212]. In contrast to their linear counterparts, macrocycles are generally more stable *in vivo*, with a more rigid structure, and a lower polar surface area. Consequently, this leads to a decrease in entropic penalty, making them ideal tools for targeting large and superficial binding pockets [213]. A collaboration between Ensemble and Bristol-Myers Squibb (BMS) described the identification and optimization of a macrocyclic antagonist with nanomolar binding affinity (**37**). This compound targeted both the BIR2 and BIR3 domains of XIAP, which is a promising antitumoral therapeutic target [214]. The peptidomimetic macrocyclic DELs were generated by DNA-directed chemistry and optimization was accomplished using an iterative process where the most potent hits guided a following library, together with structure-based drug design [125].

6.9 Chromobox

The Chromobox homologue (CBX) subunit is part of the polycomb repressive complex 1 (PRC1), which is involved in gene expression during development [215]. The CBX subunit comprises five paralogues and genetic studies highlighted CBX8 as a potential oncogenic target [216]. However, identifying selective and potent CBX8 inhibitors has been challenging, since CBXs have high sequence similarity, high flexibility, and a shallow binding site. Krusemark and collaborators reported an iterative on-DNA medicinal chemistry optimization to develop cell-permeable and selective peptide-based CBX8 inhibitors [217,218]. In this study, a small-focused DEL based on known CBX ligands was synthesized and tested against a panel of different CBXs. Depending on the enrichment, immediate SAR studies could be inferred for both potency and selectivity. Subsequently, this information was used to design a second focused library, which led to the most potent and selective CBX8 inhibitor reported to date (Fig. 18, **38**). In contrast to traditional DELs, which are mainly applied in hit generation, this work highlighted the potential of DELs to be used as a tool for medicinal chemistry, particularly within peptidomimetics.

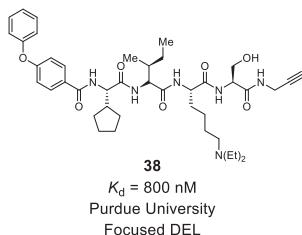


Fig. 18 Chromobox inhibitor identified from a DEL screen.

6.10 Antimicrobial

In 2017, researchers from GSK reported the results from a project targeting the identification of antibacterial leads through an automated parallel multitarget approach [16]. It was envisioned that the DELs could be screened in parallel against a panel of targets for *Staphylococcus aureus* and *Acinetobacter baumannii*, allowing a fast and cost-effective way of assessing the ligandability of antibacterial targets. In total, 39 *S. aureus* and 80 *A. baumannii* targets were considered, and out of those, 14 and 52 DEL signals were identified, respectively. Most DEL signals could be confirmed by off-DNA resynthesis, and the mode-of-action was verified for two *S. aureus* and three *A. baumannii* targets. In the same study, HTS was also used on 29 of the 38 *S. aureus* targets, and the outcome was similar for 21 of them. Moreover, the DEL approach provided hits for 5 of the targets where HTS came blank and failed to identify hits towards three targets where the HTS proved successful. From target production, selection, hit analysis, and biological testing, the DEL screening campaign took 5–7 months to run for multiple targets in parallel. In comparison, the HTS screening campaign took 9–12 months, probing a single target. A third panel of *Mycobacterium tuberculosis* proteins was provided by various collaborators and screened. The objective of this screen was to access target tractability and identify tool/lead compounds that could validate potential antituberculosis targets. In fact, a signal for 27 out of the 42 studied targets was identified and 13 of those were followed up on upon hit analysis. The antimicrobial agents (**39–41**) identified for three of the strains are depicted in Fig. 19. This study highlighted the potential of DELs as a rapid tool for target tractability and validation, and its complementarity to HTS.

Undecaprenyl pyrophosphate synthase (UppS) is an enzyme involved in the cell wall system. It has been shown to be essential for the cell viability of many bacteria and is being studied as a potential antimicrobial target [219].

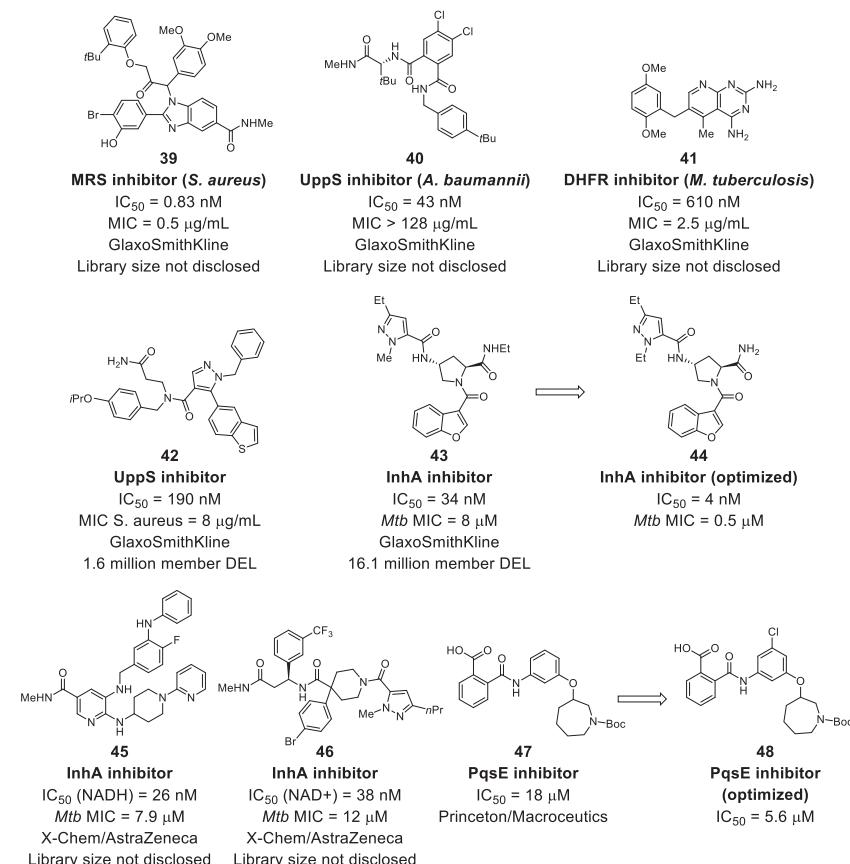


Fig. 19 Antimicrobial hits identified from DEL screens.

Researchers from GSK screened a DEL of 1.6 million members towards *S. aureus* UppS and managed to find two hits from the selection [220]. The most potent of those (**42**) showed an IC₅₀ of 190 nM in an enzymatic assay and a minimum inhibitory concentration (MIC) of 6 µg/mL. However, structural modifications were unsuccessful, and this series was not progressed further.

Enoyl-acyl carrier protein reductase (InhA) is a NADH-dependent enzyme involved in the production of mycolic acids and its inhibition leads to cell death in *Mycobacterium tuberculosis*. This is in fact the target of the compound isoniazid. Upon activation of this pro-drug by catalase-peroxidase (KatG), it forms a covalent adduct with NADH, which then works as a competitive inhibitor of NADH. The emergence of multidrug resistant strains

against isoniazid, which prevents its activation, has made the direct inhibition of InhA an attractive approach [221]. Although the discovery of potent InhA inhibitors with cellular activity has proven challenging, several groups are still pursuing this line of research. Among these, researchers from GSK reported in 2014 the identification of a potent InhA inhibitor from a 16.1 million membered DEL screen [100]. One of the greatest potentials of DEL screens, is the possibility of running several screens in parallel where different mode of actions can be interrogated at once. In this case, InhA was screened in the Apo form with NADH and NAD⁺, and a specific chemotype was identified in the presence of NADH. Subsequent off-DNA resynthesis of three putative compounds led to the identification of a hit (**43**) with an IC₅₀ of 34 nM. Further optimization of the physicochemical properties of the initial hit led to a more potent compound (**44**) with an IC₅₀ of 4 nM and a MIC₉₀ of 0.50 μM. Unfortunately, lack of *in vivo* activity in an acute tuberculosis infection model halted the development of this lead compound. In a more recent study, researchers from X-Chem and Astra Zeneca reported the identification of several series of InhA inhibitors from DEL screens [222]. A parallel screen approach was followed, and InhA was screened in the Apo form, with NADH or NAD⁺, and in the presence of a known NADH-dependent InhA inhibitor. In addition, a second tight-binding ligand was conjugated to DNA and added to the pool in order to evaluate the enrichment of the selection. After screening 11 DELs with a combined total of more than 66 billion members, 5 chemical series were identified, with two of them (**45** and **46**) showing a novel binding mode to the InhA.

Recently, a study from a collaboration between Princeton and Macroceutics reported the identification and optimization of PqsE thioesterase inhibitors [223]. This enzyme has been shown to have a critical role in *Pseudomonas aeruginosa* virulence [224]. The screen was made from a combination of 5 DELs, making a combined pool of 550 million members. The most potent hit showed an IC₅₀ of 18 μM (**47**), which was further optimized to 5.6 μM by introduction of a chlorine in the middle ring (**48**).

6.11 Antiviral

Successful DEL screens have led to the identification of antiviral compounds, and in 2015, researchers from GSK reported the identification and optimization of a NS4B inhibitor. The NS4B is a membrane-associated protein that is thought to be involved in viral replication and assembly [225].

A total of 28 DELs holding between 1 million and 8 billion members were combined and screened, and after several rounds of enrichment, target binders were identified from one of the libraries [225]. Several hits were resynthesized *off*-DNA and the most potent compound showed a K_d of 53 nM and an EC₅₀ of 172 nM (Fig. 20, **49**). This compound was optimized, (**50**) but only small improvements in potency and physicochemical properties were obtained, and the lack of selectivity over one of the genotypes halted its further development. In another study, researchers from GSK reported the identification of a potent Respiratiorial Syncytial Virus N-protein (RSV N) binder (**51**) from a 2.4×10^{12} membered macrocyclic DEL generated from six cycles of chemistry with a final macrocyclization step using click chemistry [226]. In order to rule out false positives, both the macrocycles and their linear counterpart were tested after *off*-DNA hit resynthesis. Several of the macrocycles showed a much stronger activity in comparison to their linear counterpart, indicating that the difference in binding was influenced by the more rigid structures of the macrocycles.

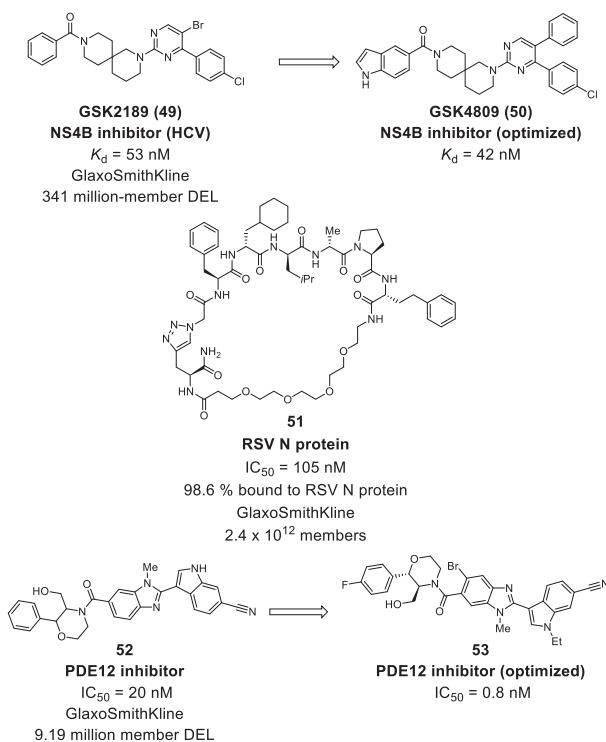


Fig. 20 Antiviral compounds identified from DEL screens.

Phosphodiesterase 12 (PDE12) is being evaluated as a potential antiviral target and researchers from GSK subjected this target to a DEL screening campaign with a benzimidazole library of 9.19 million members [227]. Strong inhibitors were identified and the most potent hit (**52**) was co-crystallized with the PDE12. Structure-guided optimization led to the identification of a competitive inhibitor with low nanomolar binding affinity (**53**).

6.12 G protein-coupled receptors (GPCRs)

GPCRs comprise one of the largest therapeutic families and account for almost 37% of the global market share (2011–2015) [228]. This important family has been outside of the scope of DELs since the DEL technology is mainly limited to purified, soluble, and tagged proteins that are screened using affinity protocols at high concentrations. However, great efforts have gone into the screening of DELs on membrane proteins, and recently several studies have reported the identification of GPCR binders from DELs. Researchers from GSK reported in 2015 the identification of several NK3 tachykinin antagonists with nanomolar binding affinity (Fig. 21, **54–57**) by screening DELs directly on cells overexpressing this receptor [114]. It was stated that the methodology was applicable to other targets than NK3, however, no detailed description was disclosed. The research group of Lefkowitz *et al.* in collaboration with Nuevolution, described the identification of allosteric modulators of β_2 -adrenergic receptors by screening DELs against purified and unliganded protein, which was maintained solubilized in detergent [99]. The identified compound with lowest micromolar binding affinity (**58**) was further characterized and proved to be an allosteric binder that enhanced the orthosteric inverse agonist binding while decreasing orthosteric agonist binding. More recently, a third approach was reported for the identification of PAR2 modulators [229]. In this case, two thermostabilized mutated PAR2 versions (StaRs) were screened in parallel against 20 DELs and in the presence of PAR2 ligands. Interestingly, two families of compounds were enriched after the screening and upon biological testing, opposing activities were revealed. One of the families was the agonist (**59**), which shared structural resemblance with the natural ligand and a synthetic agonist. The second family exhibited an antagonist activity (**60**) with an IC_{50} of 90 nM for the most active compound. This hit was further optimized to 23 nM (**61**) and co-crystallization with PAR2 StaR indicated binding to an allosteric pocket in the transmembrane region [229,230].

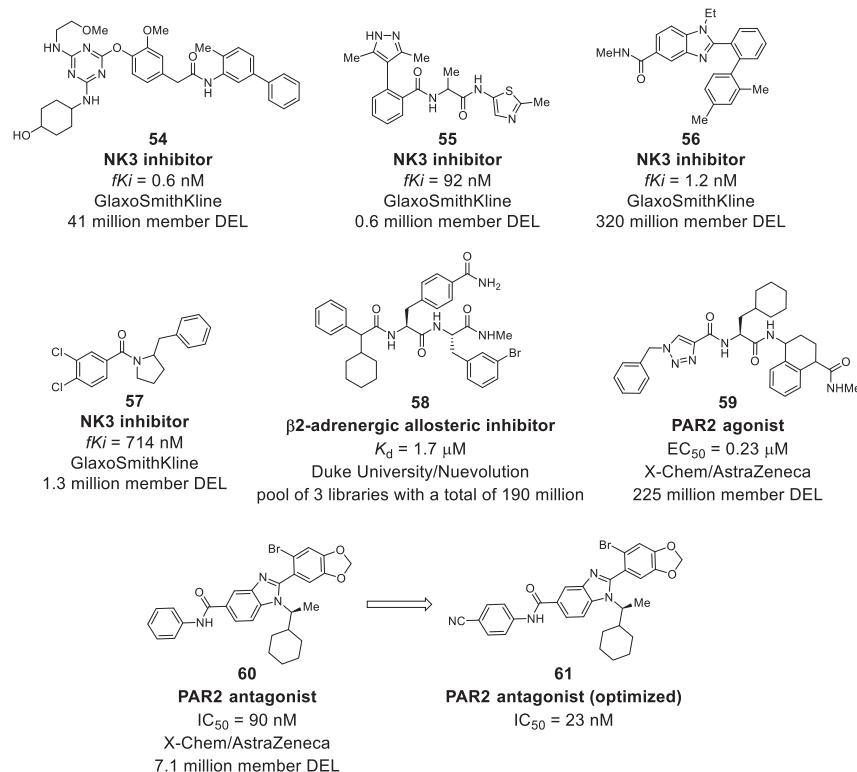


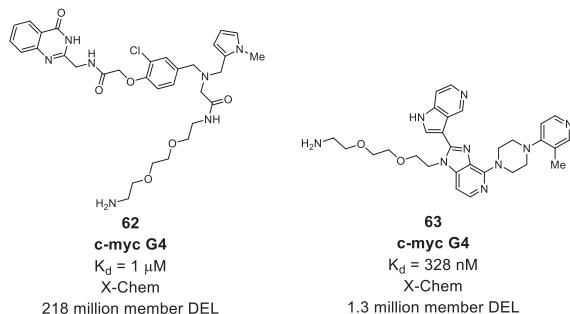
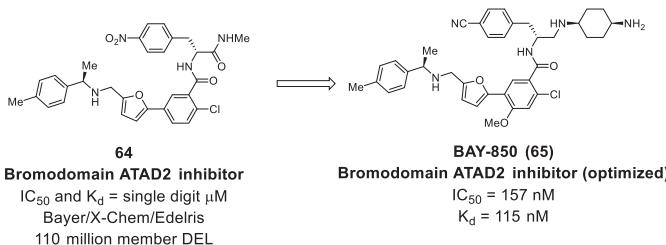
Fig. 21 GPCR modulators identified from DEL screens.

6.13 Nucleic acid binders

Researchers from X-Chem recently reported the identification of sub-micromolar binders of DNA G-quartet motifs from a DEL screening campaign using a pool of 33 libraries, with a total of 120 billion compounds [231]. These libraries were screened against a series of oligonucleotides containing G-quartets and two hits (Fig. 22, 62 and 63) from two different libraries were identified for the c-myc promoter gene, which is an oncogene associated with different tumours such as Burkitt's lymphoma. This work expanded the scope of DELs to the world of nucleic acids, which is an area that has received much attention recently, particularly for small molecules targeting RNA [232].

6.14 Transcription factors

AAA-domain containing protein 2 (ATAD2) is an epigenetic regulator and transcription factor that is highly expressed in multiple tumours, but its exact

**Fig. 22** DNA binders identified from DEL screens.**Fig. 23** ATAD2 hit identified from DEL screens.

role in cancer is yet to be discovered. ATAD2 associates with chromatin through its bromodomain, and so the ATAD2 bromodomain was interrogated against a combined pool of 11 libraries with a total of 66 billion compounds. From the screen, a single hit series was found to be highly enriched. Upon subsequent *off*-DNA resynthesis, the results from the screen were confirmed, and a hit with a binding affinity in the single digit micromolar range was identified (Fig. 23, **64**). Optimization of the hit yielded a selective inhibitor of the ATAD2 bromodomain, BAY-850 (**65**), which exerts its activity by inducing ATAD2 dimerization [233].



7. Developability of hits coming from DELs and changes in the medicinal chemistry landscape

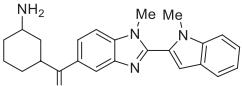
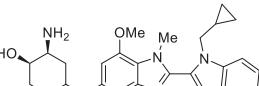
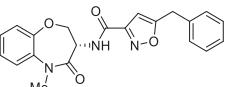
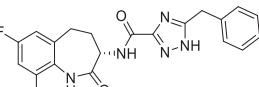
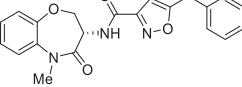
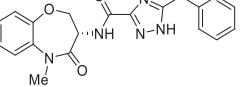
Early drug discovery has gone through significant innovation in the last 30 years due to the introduction of new tools for discovery and a gradual revision of screening cascades, in-depth profiling, selections, filtrations, and subsequent medicinal chemistry approaches. Initially, compounds were profiled, selected, and optimized primarily based on their potency and

selectivity. The failure of many compounds in *in vivo* assays and the parallel Lipinski's analysis on the importance of keeping physicochemical parameters in check from the very early phases [123], have progressively revolutionized the general process. Early *in silico* calculations and *in vitro* assays have been developed to be employed in hit-to-lead (H2L) screening cascades to profile molecules based on their 'drug-likeness'. Therefore, the choice of hits and their chemical modifications in the H2L and lead-to-candidate (L2C)-phases have started to be driven by a common objective, where the yielding of higher valuable molecules has been in focus.

In a comprehensive and extensive publication, Brown and Boström showed how candidates are originated from different approaches and highlighted the general trend of chemical modification going from the hit to the candidate structure [203]. The analysis of physicochemical properties showed a progressive increase of the molecular weight (around +85) and no significant changes on the cLogP. This reinforces the importance of keeping the molecular weight in check from the initial stages. Hence, every time a new approach joins the drug discovery toolbox, there are two main questions we need to answer. Firstly, does it access unmet medical needs, and secondly, does it provide valuable and developable chemical matter? Allowing an interrogation of a large chemical space with deep sampling [234], DELs were expected to increase the probability of discovering new chemical entities that are able to modulate the activity of challenging yet druggable targets. First successes have immediately fulfilled the initial expectations, making DEL technologies an active and attractive player in the drug discovery integrative toolbox by proving that they are able to deliver hits for demanding targets. On the other hand, drug-likeness of DEL members has been considered to be the main limitation of the approach. The number of diversity points used for building the library has a crucial impact on the molecular weight of the library members. Adding one additional diversity point can rapidly cause an increase of the molecular weight [124]. This intrinsic and inevitable limitation has predictably raised concerns around the developability of hits born from DELs. Fortunately, successful stories described in the previous section of DEL-born hits are proving that the technology is also able to provide valuable and developable molecules.

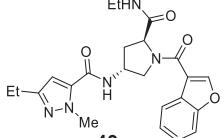
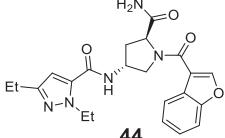
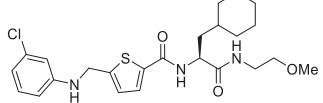
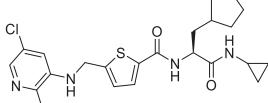
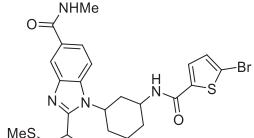
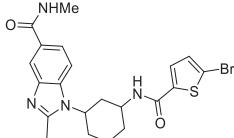
Table 3 summarizes the variation of some of the key structural parameters occurring during the chemical optimization of hits identified from DELs, and as presented, six out of the eight optimization studies led to an unvaried or decreased molecular weight (entries 3–8). Furthermore, seven out of eight hits showed high potency (single- or double-digit nanomolar

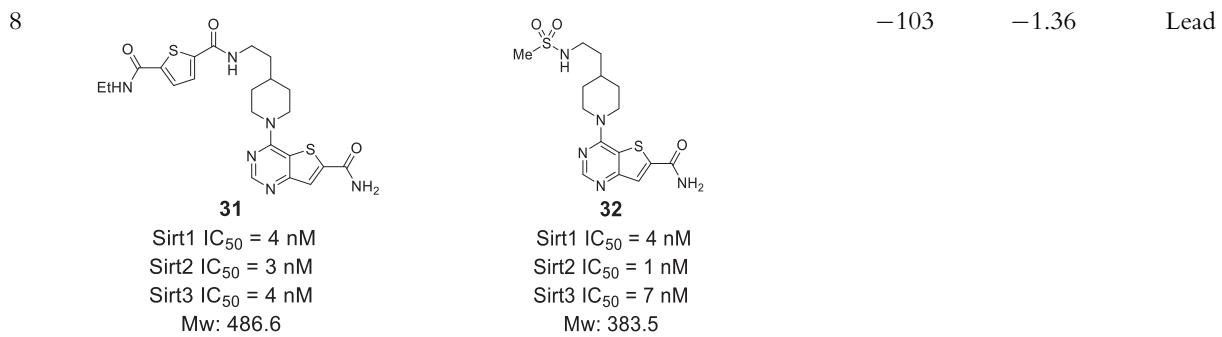
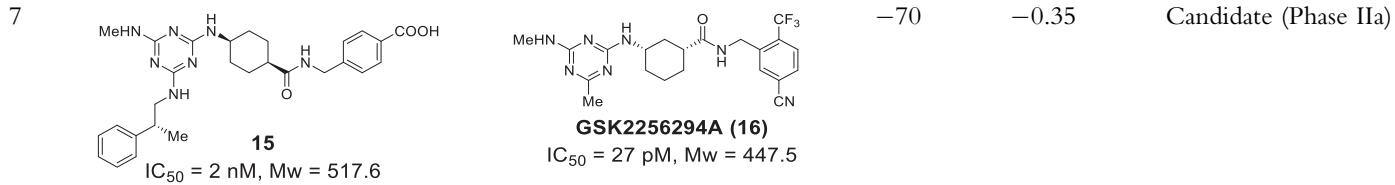
Table 3 Optimized compounds derived from DELs.

#	DEL hit structure	Optimized structure	ΔMW	ΔcLogP	Stage
1	 GSK121 (25) $\text{IC}_{50} = 3.2 \mu\text{M}$, Mw = 386.5	 GSK484 (26) $\text{IC}_{50} = 50 \text{nM}$, Mw = 472.6	+86	-0.35	Lead
2	 GSK'481 (5) $\text{IC}_{50} = 1.6 \text{nM}$, Mw = 377.4	 GSK3145095 (4) $\text{IC}_{50} = 6.3 \text{nM}$, Mw = 397.4	+20	-0.54	Candidate (Phase I)
3	 GSK'481 (5) $\text{IC}_{50} = 1.6 \text{nM}$, Mw = 377.4	 GSK2982772 (6) $\text{IC}_{50} = 1.0 \text{nM}$, Mw = 377.4	0	-0.97	Candidate (Phase IIa)

Continued

Table 3 Optimized compounds derived from DELs.—cont'd

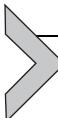
#	DEL hit structure	Optimized structure	ΔMW	$\Delta cLogP$	Stage
4	 <p>43</p> <p>$IC_{50} = 34 \text{ nM}$, Mw = 437.5</p>	 <p>44</p> <p>$IC_{50} = 4 \text{ nM}$, Mw = 423.5</p>	-14	-0.50	Lead
5	 <p>21</p> <p>$IC_{50} = 13 \text{ nM}$, Mw = 478.1</p>	 <p>GSK2830371 (22)</p> <p>$IC_{50} = 6 \text{ nM}$, Mw = 461.0</p>	-17	-0.32	Lead
6	 <p>19</p> <p>$IC_{50} = 251 \text{ nM}$, Mw = 583.6</p>	 <p>20</p> <p>$IC_{50} = 50 \text{ nM}$, Mw = 538.5</p>	-45	-1.39	Lead



range). Hence, for most of them, few structural alterations were carried out from the initial hit, focusing on improving their physicochemical properties, which involved removal of unnecessary moieties from the original hit. Interestingly, in all cases the cLogP values decreased during the optimization of the structure, even when the molecular size increased (compounds **26** and **4** entries 1 and 2, respectively). For these compounds the molecular weight of the hit was relatively low (<400) which gave a window for further optimization with an increase of the molecular weight. For compound **15** (Table 3, entry 7), minimal data of the H2L process is disclosed [17]. However, it was described that the regions bearing the methyl amine and the 2-phenylpropan-1-amine on the triazine core had little effect on the binding to the protein. Further structure optimization, including substitution of the latter part with a methyl group, led to the discovery of **16**. Hereby, the researchers from GSK were able to improve the potency and physicochemical properties of the compound, while reducing the molecular weight, and compound **16** is currently progressing in clinical studies. Hit **31** (Table 3, entry 8) was identified from screening a 1.2 million member DEL and the compound was confirmed as a pan-inhibitor of Sirt1, Sirt2, and Sirt3 with high potency [21]. It was found that the NH of the thiophene amide on **31** interacted *via* a hydrogen bond to the Val292 of Sirt3. Upon extensive SAR studies, improved potency was observed when the acidity of the NH donor to Val292 was increased. Consequently, the high potency of **31** could be maintained when the thiophene part was substituted with the sulphonamide of compound **32**, while the molecular weight was significantly reduced and the physicochemical properties were improved.

It is worth noticing how the hits undergo a H2L chemical transformation path, which varies from conventional structure optimization routes. The general trend from H2L and L2C phases defines an overall increase of the molecular weight and no significant changes in the cLogP [203], but this might not be the case when a hit originates from a DEL. Although it is premature to draw a statistical conclusion, due to the limited number of cases, the examples reported in Table 3 seem to indicate that hits coming from DEL screening undergo a different chemical approach during their structure optimization. This is not surprising, and it probably finds roots in the intrinsic chemical nature of DEL members, which are assembled with three or four diversity points. In general, this gives higher molecular weight starting points when in comparison to hits coming from other approaches (fragment-based or HTS). However, DEL hits are in many cases characterized by high potency, giving broad space of manoeuvre during subsequent

chemical modifications. For these reasons, in most of the cases, the first chemical optimization of the structure of a DEL-born hit might involve the removal of the unnecessary features with the consequent identification of a smaller active structure to be further optimized.



8. Summary and future perspectives

The field of DELs is rapidly expanding and it has become an accepted method for discovering biologically and pharmaceutically relevant chemical entities. In industry, the technology is increasingly being employed as a complement to HTS campaigns and it has proven to be especially fruitful when a predefined assay for this is unavailable [68]. DEL screenings require only standard laboratory infrastructure, with minute amounts of the library and target, offering a cost-effective approach to screening for target binders [94]. Ultimately, the DEL platform is amenable for screening several conditions in parallel, enabling a time-efficient approach to survey a large chemical space with deep sampling towards a set of protein targets, which facilitates the identification and prioritization of the most tractable targets [16,234]. Moreover, by correlating enrichment values with affinity within small focused libraries, DELs have shown to be a valuable support to classical design-make-test-analysis used during medicinal chemistry optimization [217,218].

DELs have mainly been screened on purified and soluble proteins, that are either tagged or immobilized on a solid support. This approach has some limitations since the properties of a protein target can potentially be altered upon tagging or immobilization. Therefore, the possibility of screening DELs in a more biologically relevant environment has recently been explored. Indeed, screening a target in its native environment enables the protein to undergo post-translational modifications and interact with other proteins, cofactors, or metabolites. Furthermore, such methods are especially advantageous for targets that are difficult to purify, poorly soluble, intrinsically unstable, or prone to aggregation. Advancements in this area could potentially assist the identification of small molecule binders from DELs, which would not be possible by conventional screening methods. One example is the screening of a DEL directly inside living cells by attaching cyclic cell-penetrating peptides to the library, allowing the DEL to gain access to the cell interior [110]. Another relevant example is the identification of GPCR modulators identified by screening DELs directly on cell membranes highly expressing the target of interest [114]. Technological

developments involving interaction dependant PCR techniques enable screening in cell lysates, and assist the progression of selecting target binders out of complex biological samples [105]. Although most methods require modification of the protein, selection methods with unmodified targets have been described [108]. It is worth noting that hits identified from a DEL selection do not necessarily alter the function of the target protein as the library interrogation is based on binding only. In most cases the activity cannot be assessed during post-selection analysis, since current selection methods do not provide a functional output. However, the first example of an activity-based screening has been reported on solid-supported DELs [119].

Since the initial developments of DELs, there has been a focus on increasing the chemical diversity of the libraries, while maintaining molecular and physicochemical properties that are in compliance with those of orally absorbable drugs. One approach to achieve this has been by focusing on creating diverse sets of scaffolds, where the BBs going into the library synthesis have been the major contributor to appendage diversity, and the libraries are assembled by well-established chemistries, such as acylations, substitutions, reductive aminations, or Suzuki cross-couplings. Alternatively, new DNA compatible reactions have been explored to create pharmaceutically interesting motifs, such as compounds with heterocyclic cores or novel BB assemblies. The combination of robust chemistries and quick building block access facilitate an efficient off-DNA hit resynthesis and rapid structure evaluation. In recent years, many new DNA compatible reactions have been introduced and advancements in new technologies have enabled chemistries that were believed to be damaging to the DNA [27,30,36,145]. However, despite the progress in *on*-DNA chemistry, only a subset of the new reactions have been reported for use in DELs.

The initial DELs were dominated by compounds with poor physicochemical properties, displaying large, flexible, and lipophilic compounds. With the progression of DNA compatible chemistries along with advancements in the structural complexity and diversity of available building blocks, compounds with pharmaceutically interesting structural features are increasingly being represented in DELs. Furthermore, pooling of several libraries prepared by miscellaneous chemistries and BB assemblies increase the chemical space presented to the target within a single screen. These developments significantly enhance the potential of identifying new hits/leads for potential clinical candidates. As described herein, DELs have been used to identify novel and potent binders over a broad selection of validated and non-validated

pharmaceutically relevant targets. In many cases, minimal hit-to-lead structure optimization was required, and several examples of molecular weight reductions have been reported, where unnecessary structural features were altered or removed. This demonstrates how the structure optimization of hit compounds from DELs differ from conventional paths where an increase in the molecular weight is typically observed [203].

The expansion of the DEL field opens fascinating frontiers and there continues to be high expectations and interest in the technology. Today, exploiting DELs as a source of new chemical matter is an integrated part of many pharmaceutical organizations, either by internal programs or strategic partnerships. This, in conjunction with increasing research in academia, is expected to fuel further development. In the future, it is expected that biologically active compounds with more ‘drug-like’ and diverse structural motifs will continue to be identified from DEL selections.

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