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Selection of DNA-encoded Dynamic Chemical Libraries for Direct Inhibitor Discovery

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Abstract: Dynamic combinatorial library (DCL) is a powerful tool for ligand discovery in biomedical research; however, the development of DCL has been hampered by its low diversity. Recently, the concept of DNA encoding has been employed in DCL to create DNA-encoded dynamic libraries (DEDLs); however, all current DEDLs are limited to fragment identification, and a challenging process of fragment linking is required after selection. We report an anchor-directed DEDL approach that can identify full ligand structures from large-scale DEDLs. This method is also able to convert unbiased libraries to focused ones targeting specific protein classes. We demonstrated this method by selecting DEDLs against five proteins, and novel inhibitors have been identified for all targets. Notably, several selective BD1/BD2 inhibitors were identified from the selections against BRD4 (bromodomain 4), an important anti-cancer drug target. This work may provide a broadly applicable method for inhibitor discovery.

Introduction

Screening large-scale chemical libraries against biological targets to identify novel ligands is a central strategy in drug discovery and many other research fields. In traditional high throughput screening, compounds are spatially encoded and screened against the target individually. In contrast, dynamic combinatorial library (DCL) employs a mixture of building blocks (BBs) that undergo dynamic exchange through reversible chemical reactions, allowing the synthesis and screening of all library compounds in one pot.[1] With DCL, the target acts as the template to promote the formation of high-affinity ligands at the expense of non-binders, and "hit" compounds can be identified by comparing the equilibria with and without the target. In the past decade, DCL has shown wide utilities in numerous applications.[1d-h, 2] However, with a few exceptions,[3] most DCLs only contain a few hundred compounds or less, mainly due to the lack of techniques to resolve large number of compounds in a single mixture.[1a, 1h, 4] Indeed, low library diversity has been considered as the major limitation of DCL.[1a, 1h, 4-5]

DNA-encoded chemical library (DEL), originally proposed by Brenner and Lerner,[6] has recently become an important technology in drug discovery.[7] Similar to DCL, DEL also employs mixed compounds in library processing; however, DELs can contain many billions to even trillions of compounds since each compound is encoded with a unique DNA tag, and the library selection can be efficiently decoded with PCR amplification and DNA sequencing.[7a] Therefore, introducing DNA encoding to DCL should be a viable strategy to enable the preparation and selection of large DCLs.[7k] Previously, the Neri group developed an elegant dual-pharmacophore DEL approach named Encoded Self-Assembling Chemical (ESAC) library,[7c, 8] and we incorporated dynamic DNA hybridization with dual-pharmacophore DELs and developed a DNA-encoded dynamic library (DEDL) method that can process large dynamic libraries.[9] Zhang and co-workers also reported two dynamic DEL methods.[10] However, all current DEDL methods are limited to fragments or fragment pairs, and a post-selection tethering process is still required to elaborate the fragments into full ligands. As seen in many studies, finding a suitable linker connecting the fragments to achieve cooperative binding is highly challenging, which is empirical, tedious, and involves a lot of trial and error.[8b, 11] In fact, fragment linking often takes considerably more efforts than fragment identification itself. It is highly desirable to be able to directly obtain full ligand structures from library selections.

Here, we report a DEDL method without the need for post-selection fragment linking. This method is based on anchor-directed dynamic exchange and an *in situ* hit isolation strategy. Notably, existing DELs could be used in this method to create large dynamic libraries. Moreover, DEL is generally used as a one-for-all discovery platform without target bias. Recently, several studies have exploited the "focused" DELs to target specific protein classes;[12] however, they all require the preparation of a new library for each target. Here, we show that this method has a "*plug-and-play*" feature that can convert the general-purpose libraries to focused ones targeting specific proteins by switching the anchor. To demonstrate this method, we selected DEDLs against five proteins, and a series of potent and selective inhibitors were identified and validated for these targets.

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Results and Discussion

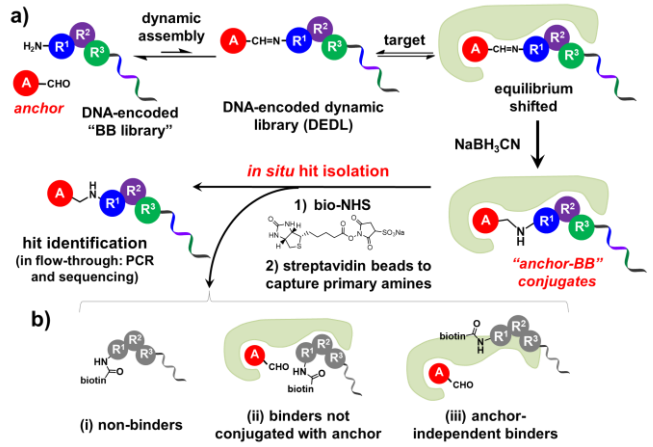

Fig. 1. a) The proposed library selection scheme. An anchor and a DNA-encoded "BB library" form the DEDL. After target addition and imine reduction, **bio-NHS** and streptavidin beads are used to capture the primary amines. Hit compounds in the flow-through are isolated and decoded by PCR and DNA sequencing. b) Species captured by **biotin-NHS**.

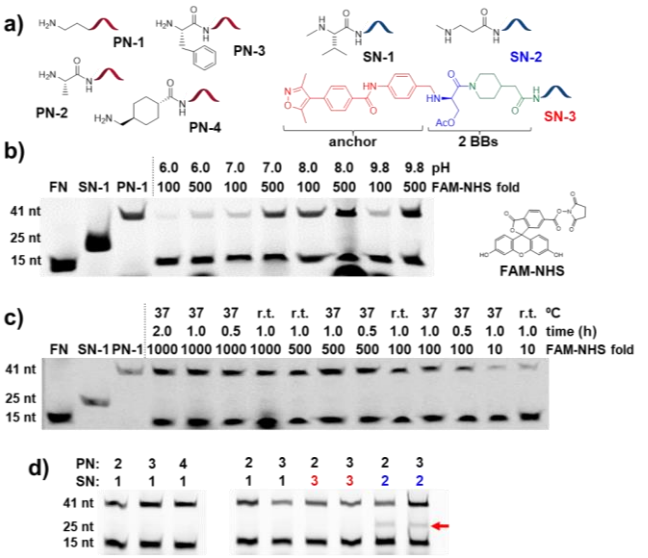

Fig. 2. a) Structure of DNA-conjugated amines. b)-c) In-gel fluorescence analysis of the **PN-1/SN-1** reactions with **FAM-NHS**. d)-e) In-gel fluorescence analysis of the reactions of various **PN/SN**s with **FAM-NHS**. **PN/SN**: 3 μM; buffer: MES (pH 6.0), MOPS (pH 7.0), HEPES (pH 9.8). In b): r.t., 1 h; in c): pH = 8.0; in d): r.t., 500-fold **FAM-NHS**, 1 h, pH 8.0. Gel: denaturing PAGE. **FN**: a 15-nt fluorescent DNA as the internal standard. Other conditions are as marked. See Fig. S1 for ethidium bromide stained gels.

Our strategy is shown in Fig. 1. An "anchor" with an aldehyde group is incubated with a DNA-encoded "building block (BB) library", which could be a regular DEL with a primary amine. The DNA-encoded BBs compete for the anchor through reversible imine formation, thereby forming a DEDL. Adding the target shifts the equilibrium and promotes the formation of high-affinity binders, and the dynamic exchange can be stopped by $NaBH_3CN$-mediated imine reduction. To decode the "anchor-BB conjugates" that bind the target, we devised an *in situ* hit isolation strategy: after the reduction, a biotin sulfo-*N*-hydroxy succinimidyl ester (**bio-NHS**; Fig. 1a) is added to capture the primary amine on the BBs, while the "anchor-BB conjugates" have a less reactive secondary amine due to the steric hindrance and are less efficiently captured.[13] As a result, the selected ligands could be separated from the biotinylated species (non-binders, binders not conjugated with the anchor, and anchor-independent binders; Fig. 1b) with streptavidin beads and then subjected to hit deconvolution. Importantly, no-anchor and non-target control selections will be performed to control for anchor-independent binding and other variables in the selection process.[9a] This design requires a known binder as the anchor, but it does not need to be a high-affinity ligand; instead, the anchor could simply be a small fragment with weak affinity or a structural moiety derived from the protein's natural substrate. Therefore, this method may be particularly suitable for the "affinity maturation" for the proteins with known but unoptimized binders.[8b, 14] Moreover, DEL is mostly a binding assay; here, using an anchor may facilitate inhibitor discovery, since the anchor could guide the library compounds to be sampled at the site with functional relevance (e.g. the catalytic pocket of enzymes). Finally, this method does not need protein immobilization and is compatible with unmodified, in-solution proteins.

A key to this method is the selective capture of the primary amines, which has two potential issues: a) the structural diversity of BBs may result in variations in capture efficiency; the less efficiently captured BBs would be "enriched" in the flow-through and become false positives; b) albeit less reactive, the secondary amines may also be captured. We reason that using a strong capture condition to overcome the structural variation of BBs may be beneficial. Although secondary amines would be captured, it is in a lesser degree and should retain sufficient materials for hit decoding. To test this, we prepared two DNAs with a simple primary and secondary amine, respectively (**PN-1**/**SN-1**, 41-nt/25-nt; Fig. 2a); they were mixed at equal ratio and reacted with excess of an activated fluorescein ester (**FAM-NHS**). Strikingly, little capture of the secondary amine was observed (Fig. 2b), while **PN-1** could be efficiently labeled at pH >7. We decided to choose pH 8.0, as it was reported to be optimal for reductive amination under physiological conditions.[15] The reaction time and temperature were varied and again little capture of **SN-1** was observed (Fig. 2c). Next, several different primary and secondary amines were tested and the selective capture was also observed (**PN-2**, **3**, **4**/**SN-1, 2**, **3**; Fig. 2d). Since the pKa's of primary and secondary amines are above 10, the selectivity was presumably due to the steric bulk of the secondary amines, rather than the difference in protonation. In fact, **SN-2**, which has relatively low steric hindrance, gave more capture products (red arrow; Fig. 2d). **SN-3** represented the compound in the actual DEDL; it has a crowded secondary amine structure and also did not show any capture (Fig. 2d). Although BB diversity is expected to affect the capture efficiency, these results indicated there is a large selectivity window. Importantly, a non-target selection will always be conducted to control for this variation.

Next, we tested the selection with a model protein CA-II (carbonic anhydrase II). Since arylsulfonamides are well-known CA-II binders, a 4-formylbenzenesulfonamide was used as the anchor (**A-1;** Fig. 3a). First, two DNA-conjugated primary amines (**PN-1** and **PN-5**; Fig. S2) were prepared. **PN-5** has a phenylglycine motif known to enhance the binding affinity of sulfonamide.[16] The two DNAs were mixed with **A-1** to form the imines and incubated with CA-II; a 10:1:1 library/anchor/target ratio was used to create a competitive environment for **PN-1**/**PN-5**. After the imine reduction, **bio-NHS** capture, and streptavidin

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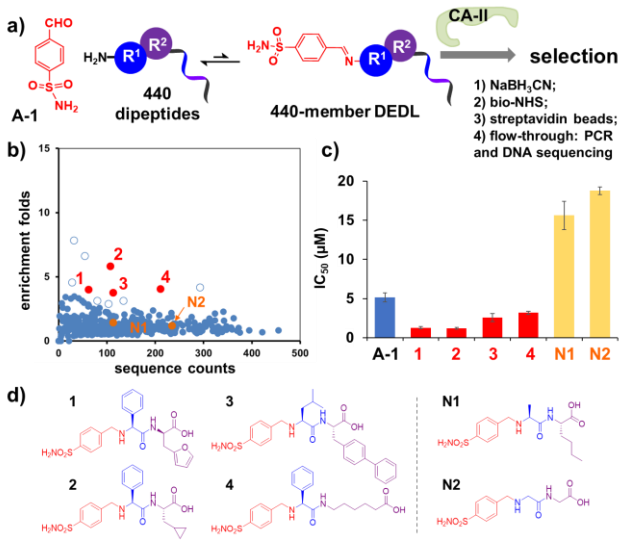

Fig. 3. a) Selection of a model DEDL against CA-II. Library, 1 μM; CA-II, 0.1 μM; **A-1**, 0.1 μM; incubation: 4 ºC, 2 h in 50 mM MOPS (pH 8.0); $NaBH_3CN$ reduction: 38.5 mM, 1 h; **bio-NHS**: 100 eq., 1 h, 37 ºC. b) Scatter plot of the selection results. Red: hits; orange: negatives; open circle: compounds also enriched in control selections (Fig. S3). *x*-axis: post-selection sequencing counts; *y*-axis: enrichment fold = (post-selection%)/(pre-selection%). c) CA-II inhibition assay. Error bar represent 3 repeats. d) Compound structures. See the SI for experimental details.

pulldown, the surviving **PN-1**/**PN-5** in the flow-through were quantified using qPCR. The results showed a ~40-fold increase of **PN-5**, indicating that the "**A-1** + **PN-5**" conjugate was enriched by CA-II (Fig. S2). Next, we prepared a 440-member dipeptide library (Fig. 3a), which formed a DEDL with **A-1** and selected against CA-II. The DNA tags of the selected compounds in the flow-through were amplified and decoded with the next-generation sequencing (NGS). The sequencing data were processed to calculate the post-selection sequence count (SC) and the enrichment fold (EF) for each compound as previously described.[9] The selection results were plotted in the form of scatter plots (Fig. 3b) and the compounds with high EF and SC were considered as potential hits. No-anchor and non-target (with bovine serum albumin; BSA) control selections were also conducted (Fig. S3). By comparing the scatter plots, the compounds also enriched in control selections were identified and excluded (open circles; Fig. 3b). Four specifically enriched compounds (**1**-**4**) were chosen as "hits", resynthesized off-DNA, and assayed for their inhibition activity against CA-II. In general, all hits exhibited enhanced activity compared with **A-1** (Fig. 3c). Interestingly, 3 hits have the same phenylglycine motif as **PN-5**, and the other one has a valine residue, also known to increase the CA-II-binding affinity.[16] To verify the selection specificity, two "negatives" with low EF were tested and both exhibited very low activity (**N1** and **N2**; Fig. 3d). Moreover, to verify that CA-II in fact shifted the equilibrium to form the imine, using **2** and **4** as the example, we incubated CA-II with **A-1** and the BBs of **2** and **4**, respectively.[17] After the equilibrium was locked by reduction, the product formation was analyzed with LC-MS. As shown in Fig. S4, CA-II significantly promoted the product formation, while nearly no product was observed without CA-II. Furthermore, assuming the quantity of the amine products reflected the imine formed in the equilibrium, the binding affinity of the imines was estimated based on the LC peak integration, which were further compared with the affinity (K_d) of **2** and **4**, determined with fluorescence polarization

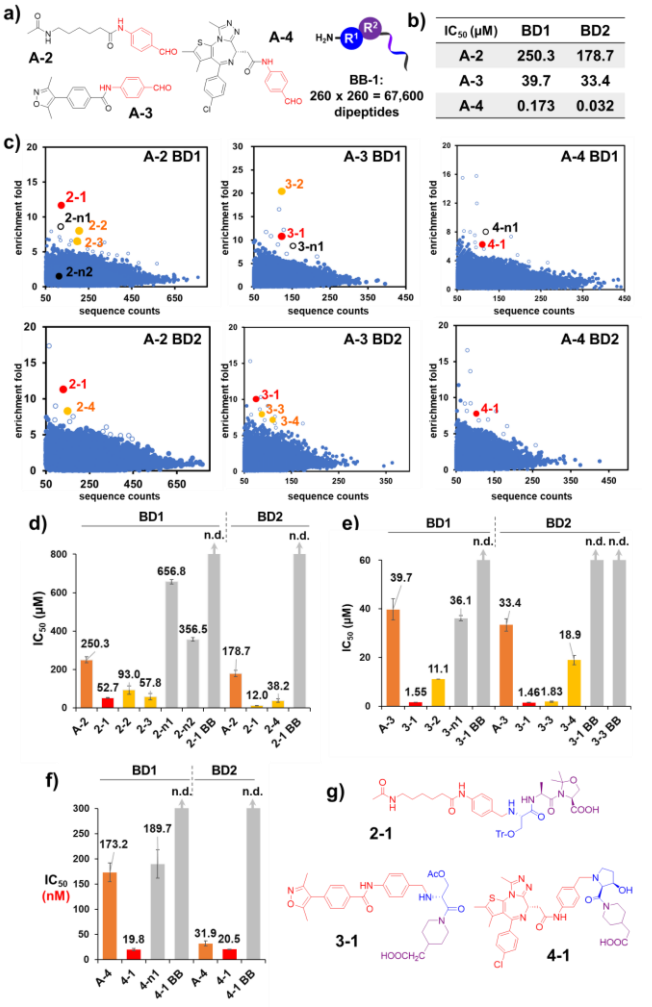

Fig. 4. a) The structure of the anchors and **BB-1** library. b) The anchors' activity against BD1/BD2. c) Scatter plots of the selection results. Red: hits enriched by both BD1 and BD2; orange: hits enriched by BD1 or BD2; open circles: compounds also enriched in control selections (Fig. S7). d)-f) BD1/BD2 assay results of the hits, anchors, non-specifics, and the BB-only controls. n.d.: not detectable. IC_{50} fitting curves are shown in Fig. S8. g) Structures of representative hit compounds. See the SI for experimental details and the full list of compound structures (Fig. S9).

(Fig. S5). The results showed that the two species had similar CA-II-binding affinity.

Next, we moved on to large libraries. A 2-BB DEL (**BB-1;** 67,600 dipeptides) was prepared using the split-and-mix DEL encoding method (Fig. S6).[18] First, **BB-1** was selected against the BD1/BD2 domains of BRD4 (bromodomain 4), an important epigenetic regulator that functions through binding to the acetylated lysine on histones.[19] Despite the high similarity, BD1 and BD2 have distinct cellular functions due to their interactions with different lysine-acetylated histones or transcriptional proteins;[20] thus, it is highly desirable to identify selective BD1/BD2 inhibitors. We prepared three BD1/BD2 anchors (**A-2**, **A-3**, **A-4**; Fig. 4a). **A-2** is derived from acetylated lysine, the natural BRD4-binding structure; **A-3** has an isoxazole ring, which is a "privileged" BRD4-binding structure;[21] **A-4** is based on the well-known, highly potent BRD4 inhibitor (+)-JQ-1.[22] The IC_{50}'s of the anchors were determined using a TR-FRET assay, and they range from high μM to nM (Fig. 4b). Thus, these anchors

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should be able to assess the method across a wide range of binding affinities.

The anchors were mixed with **BB-1** to form 3 dynamic libraries and selected against BD1 and BD2, respectively. Again, non-target and no-anchor controls were performed for all selections. The post-selection sequencing data were processed as same as in Fig. 3 and scatter plots are shown in Fig. 4c. In general, the selections exhibited relatively high number of surviving DNA, probably because the compounds were enriched in flow-through, where the background DNAs might not be completely removed by streptavidin beads and were also PCR-amplified. Nevertheless, the specifically enriched compounds could still be identified by comparing with the control selections (Fig. S7). Interestingly, for each anchor, one compound was enriched by both BD1 and BD2 (**2-1**, **3-1**, and **4-1**; Fig. 4c), strongly suggesting that they were specific binders. Next, the selected hits were re-synthesized and assayed against BD1/BD2. As shown in Fig. 4d-4f, most compounds showed enhanced activities compared with the anchor. For the weakest anchor **A-2**, the greatest increase was observed with **2-1** for BD2 (from 178.7 μM to 12.0 μM). For **A-3**, more significant increase was observed with **3-1**; the IC_{50} was reduced from mid-μM's to 1.55 μM (BD1) and 1.46 μM (BD2). For anchor **A-6**, which was already a nM inhibitor, a ~9-fold increase was still obtained (**4-1** for BD1; from 173.2 nM to 19.8 nM). To verify the selection specificity, we tested several compounds also enriched in control selections (**2-n1, 3-n1**, **4-n1**) and a negative compound with low EF (**2-n2**); the results showed that they had no activity or lower activity than the anchor (Fig. 4d-4f). In addition, all the "BB-only" compounds without the anchor motif were completely inactive. The selection data actually reflected the combined effect of three factors: target binding, imine formation/reduction, and biotin-based hit separation. Considering the structural diversity of BBs, all three are expected to affect the selection results. The non-target (with BSA) and no-anchor selections (Fig. S7) were used to control for non-specific binding and the variations in biotin capping/hit separation. Thus, to control for the effect from the differing imine reactivities, we conducted a no-target selection with the **BB-1** library and the three anchors, respectively (Fig. S10a); a simple biotin capping control was also performed to delineate the primary amine reactivity (Fig. S10b). The results showed that the identified BD-1 ligands in Fig. 4c were not significantly enriched in these controls, and they all had similar EF values and sequence counts, indicating that their enrichments in the actual **BB-1** selection were not significantly altered by the difference in imine reactivities (Fig. S11).

Collectively, these selections have demonstrated the capability of this method in ligand optimization across a wide range of binding affinity. For example, the potency of the weak anchor **A-2** was improved from high μM to low μM and the IC_{50} of **A-3** was reduced to single-digit μM, making them much more suitable for further optimization.

Next, we prepared a 3-BB tripeptide DEL (**BB-2**; 17.576-million tripeptides), considering that it may identify more potent binders. We also prepared two more anchors (**A-5**, **A-6**; Fig. 5a**)**, which were similar to **A-3** but with different heterocycles.[21] Five DEDLs were formed using **BB-2** with the anchors and selected against BD1 and BD2, respectively. The results are shown in Fig. 5c and the specific binders were identified by comparing with the control selections (Fig. S12). However, albeit with greater diversity, the inhibitors identified from **BB-2** were not distinctively more potent

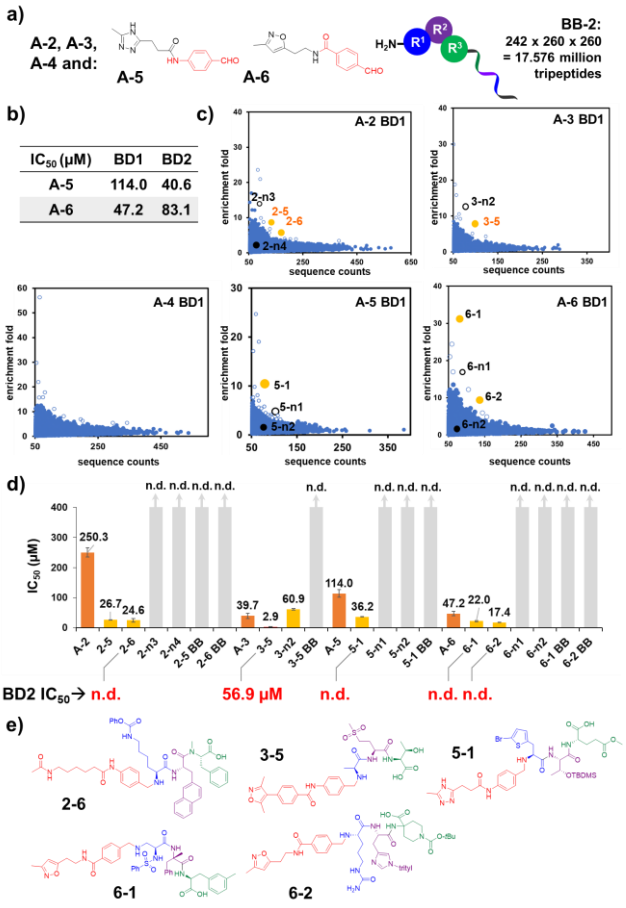

Fig. 5. a) Five anchors (**A-2** to **A-6**) were used to form 5 dynamic libraries with **BB-2**. b) BD1/BD2 inhibition assay of **A-5** and **A-6**. c) Scatter plots of the BD1 selection results. Orange: hits; open circles: non-specific compounds also enriched in control selections (Fig. S12). c) BD1 assay results of the hits, anchors, non-specifics, and BB-only controls. n.d.: not detectable. Several compounds were assayed against BD2 and the IC_{50}'s are shown below the column graph. Fitting curves are shown in Fig. S13. d) Structures of the representative compounds. See Fig. S14 for the full list.

than the ones from **BB-1**. With BD1, the potency of the hit compounds was similar to the ones from **BB-1** (Fig. 5d). Surprisingly, the selection with the high-affinity **A-4** did not identify any specific binders. With BD2, only one specific binder was identified (**A-3**; Fig. S15). We reasoned this might be due to the larger size of the **BB-2** compound; the tripeptide may dominate target-binding and have attenuated the anchor's directing effect to the acetyl-lysine-binding site on BD1/BD2. Nevertheless, although the IC_{50}'s of the BD1 inhibitors were in μM range, they exhibited good selectivity against BD2. Compounds **3-5** gave ~20-fold selectivity for BD1 and the others were inactive against BD2 (Fig. 5d), while the anchors alone showed very low selectivity. In addition, the compounds also enriched in the control selections or with low EF showed little activity. These results indicated that the method may be used to improve the target selectivity of the ligands, but large compound size appeared to be detrimental for the anchor-directed target-binding.

Usually, DELs are designed as general-purpose libraries with little consideration of the ligand space of the specific target. With this method, the same BB library could be directed to bias different proteins by changing the anchor. To test this, we conducted selections against two more proteins: acetyl-cholinesterase (AChE) and X-linked inhibitor of apoptosis protein

(XIAP). AChE is a key hydrolyzing enzyme for the neurotransmitter acetylcholine, and **A-7** (Fig. S16a) was prepared based on tacrine, a ligand that binds to the catalytic pocket of AChE.[23] The selection using **BB-2**/**A-7** against AChE identified two compounds, and they exhibited a 3- and 18-fold of improvement of activity over **A-7** (**7-1** and **7-2**; Fig. S16). XIAP is an inhibitor of cell apoptosis and has been pursued as an anti-cancer drug target. The tetra-peptides with a sequence of *N*-methyl-Ala-*t*-Leu-Pro-Xaa are potent XIAP antagonists.[24] Based on this sequence, we prepared an XIAP anchor by truncating the key proline and the 4th amino acid and replacing them with a benzaldehyde (**A-8**; Fig. S17a); as expected, **A-8** showed very weak affinity (>600 μM; Fig. S17).[25] The selection of **BB-2**/**A-8** against XIAP identified two compounds with significantly improved affinity (**8-1** and **8-2**; 32.9 μM and 53.2 μM; Fig. S17). In addition, their corresponding BBs without **A-8** and a non-specific compound **8-n1**, showed no or very weak affinity, which verified the selection specificity and that **A-8** was required for binding (Fig. S17d). Although the identified compounds in these selections were weak, they all exhibited considerably improved potency towards the target, indicating that the same BB library could be used for different proteins by changing the anchor.

Conclusion

In summary, we have developed a method to create and select large DNA-encoded dynamic libraries for ligand optimization. Compared with other approaches, this method avoids the post-selection fragment linking and full ligands could be directly obtained from the selection. Although a primary amine is required, it could be feasibly incorporated in library synthesis using regular amino acids, which are the most commonly used BBs in DELs. In addition, this method does not require special library design; in principle, existing DELs prepared with different methods or encoded with double-stranded DNA tags could also be used, as long as the library contains a primary amine.[8a, 8b, 18a, 26] One caveat of this method is that the actual binders in the selection are imines, while the hit compounds are isolated and characterized as amines; thus, although the imine formation/reduction chemistry has been widely used in numerous DCL studies,[27] an assumption is taken that the amines largely represent the properties of their imine counterparts. In addition, in principle, some of the other well-developed reversible reactions in DCL may be implemented to diversify the ligand structure.[1h]

The data have shown that this method could be used for ligand optimization in a wide range of affinities; however, the results from the **BB-2** library indicated that the large compound size may offset the anchor's directing effect. Thus, using BB libraries with more compact structures[28] may be more favorable; although this would limit the library size, a recent study showed that focused DELs with 2 sets of BBs were sufficient to identify high-quality inhibitors.[12a] Moreover, in previous reports on focused DELs,[12] the target-biasing elements were permanently built-in, so that the library was less suitable for other types of targets. Here, this method converts the unbiased DELs to focused ones by simply changing the anchor in the selection.

On another aspect, this method does not require protein immobilization, and all the selections were performed in solution. We envision this method may be suitable for selections in more complex biological environment. However, this in-solution selection reflected the combination of three implicating factors: target binding, imine formation/reduction, and biotin-based hit separation. Considering the vast BB diversity in a DEL, BB-dependent variations are expected for all three factors. Our data suggested that non-target (using BSA or other unrelated proteins) and no-anchor control selections were essential to exclude the potential false positives arising from non-specific binding and the variation in biotin capture, and the no-target selection was important to control for the differing imine reactivities. Finally, this method is limited to the optimization of known ligands. Future studies will focus on the method development to realize completely *de novo* ligand discovery.

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Keywords: DNA-encoded chemical library • dynamic combinatorial library • high-throughput screening • drug discovery • DNA

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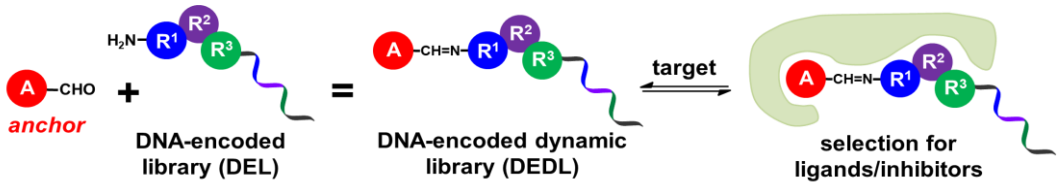

An amine-bearing DNA-encoded chemical library (DEL) could be converted to DNA-encoded dynamic library (DEDL) and subjected to the selection against biological targets for inhibitor discovery. Full ligand structures could be obtained without the need for fragment-linking.