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FULL PAPER

Selective fragments for the CREBBP bromodomain identified from an Encoded Self-Assembly Chemical Library

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Abstract: DNA-encoded chemical libraries (DECLs) are collections of chemical moieties individually coupled to distinctive DNA barcodes. Compounds can be displayed either at the end of a single DNA strand (i.e., single-pharmacophore libraries) or at the extremities of two complementary DNA strands (i.e., dual-pharmacophore libraries). In this work, we describe the use of a dual-pharmacophore Encoded Self-Assembly Chemical (ESAC) library for the affinity maturation of a known 4,5-dihydrobenzodiazepinone ring (THBD) acetyl-lysine (KAc) mimic for the cyclic-AMP response element binding protein (CREB) binding protein (CREBBP or CBP) bromodomain. The new pair of fragments discovered from library selections showed a sub-micromolar affinity for the CREBBP bromodomain in fluorescence polarization and ELISA assays, and selectivity against BRD4(1).

Introduction

Since its conception by Brenner and Lerner in 1992[1], DNA-encoded chemical library (DECL) technology has steadily grown and found many practical applications in both academic and industrial laboratories.[2] The technology relies on the covalent linkage of individual chemical compounds to distinctive DNA sequences that serve as amplifiable identification barcodes.[3,4] DNA-conjugated binders enriched during affinity-based selections are therefore identified through PCR amplification and high-throughput DNA sequencing.[5,6] Using suitable synthetic schemes (e.g., "split-and-pool" synthesis, followed by the stepwise addition of DNA fragments serving as barcodes), it is possible to synthesize and screen combinatorial chemical libraries containing millions of compounds.[7] Several DNA-encoded libraries with different molecular designs have been proposed and a growing number of chemical reactions are being explored in order to enable DNA-compatible assembling of building blocks.[8–12]

Chemical moieties can be covalently attached on a single DNA strand (i.e., single-pharmacophore libraries) or at the extremities of two individual complementary DNA strands (i.e., dual-pharmacophore libraries).[13–16] Dual pharmacophore libraries can be particularly useful in the discovery of synergistic pairs of fragments that simultaneously bind to two adjacent sites on the protein surface, benefiting from the chelate effect.[17] An example of dual-pharmacophore library, also called Encoded Self-Assembly Chemical (ESAC) library, was successfully employed for the discovery and the affinity maturation of high affinity ligands against alpha-1-acid glycoprotein and carbonic anhydrase IX.[18] In another case, an ESAC library was implemented with Michael acceptor moieties and led to the discovery of a selective covalent binder of JNK-1.[19] The ESAC strategy relies on the self-assembly of mutually complementary sub-libraries, yielding to a large combinatorial diversity of building block pairs. Each library member is individually HPLC-purified, offering high level of purity even in presence of low-conversion chemical reactions.

Cyclic-AMP response element binding protein (CREB) binding protein (CREBBP) bromodomain presents two adjacent binding pockets (i.e., the KAc binding pocket and the induced-fit pocket) that can be targeted by dual-pharmacophore ligands [**Figure 1A**].[20] CREBBP is a crucial transcription factor which is involved in more than 400 protein-protein interactions (PPIs).[21] Inhibition of CREBBP-related PPIs can be particularly relevant for the treatment of severe diseases, such as prostate cancer.[22]

Structure-activity relationship (SAR) studies led to the discovery of an inhibitor of CREBBP (**1**) which enjoyed the interaction of a tetrahydroquinoline (THQ) and a dihydroquinoxalinone moieties with residue R1173 and KAc binding pocket, respectively.[20]

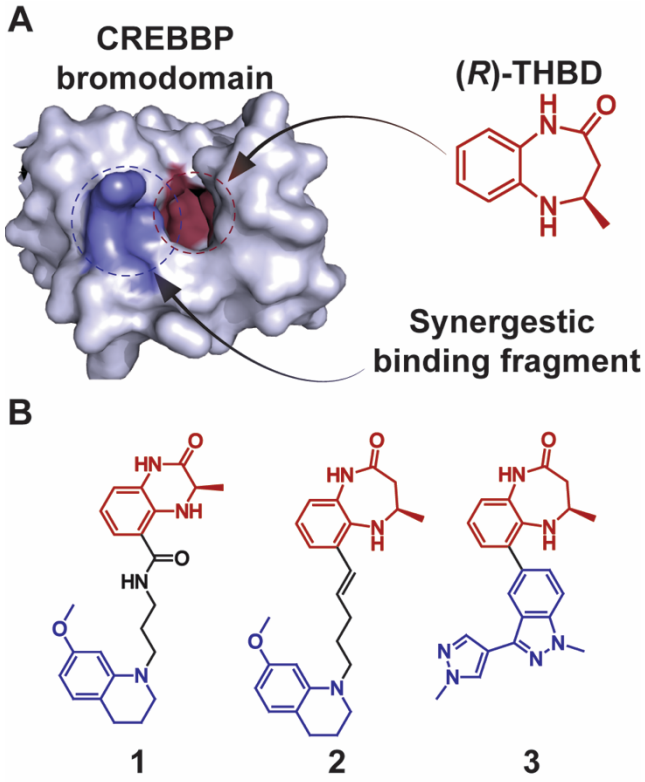

Figure 1. Dual pharmacophore approach in binding CREB-binding protein bromodomain. **A**) 4,5-Dihydrobenzodiazepinone ring (THBD)-based ligand binds to the KAc binding pocket of CREBBP bromodomain (red), granting access to a synergistic binding interaction with a second fragment in the induced-fit pocket (blue). **B**) Three known dual-pharmacophore sub-micromolar inhibitors of the CREBBP bromodomain (**1**, **2** and **3**). Protein Data Bank access number 4YK0.

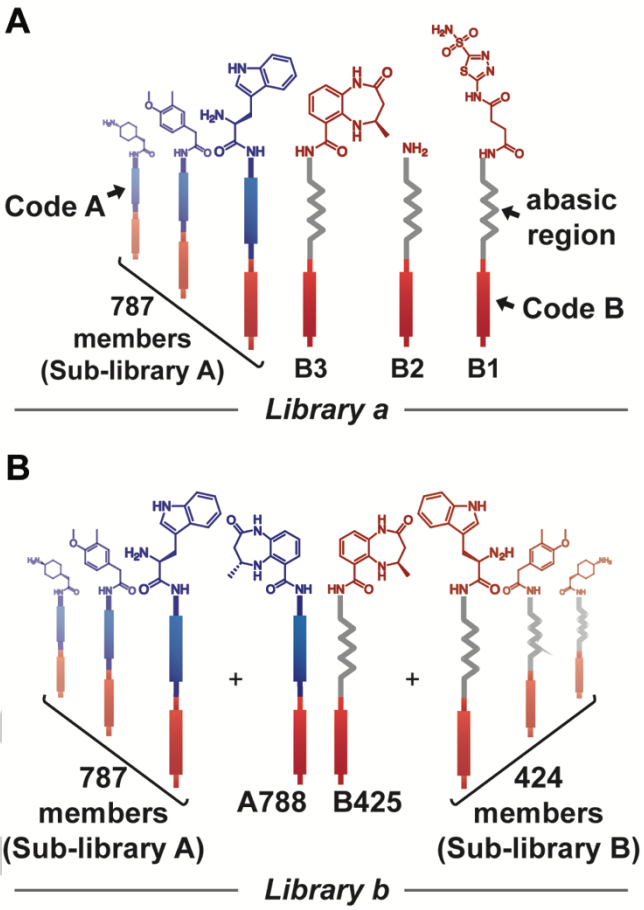

Figure 2. A set of dual-pharmacophore Encoded Self-Assembly Chemical (ESAC) libraries can be screened for the discovery of synergistic binding fragments of CREBBP bromodomain. **A**) *Library a* is constructed with three individual 3'-amino-modified DNA-fragments displaying an acetazolamide derivative (**B1**), a free-amino group (**B2**) and the (*R*)-THBD derivative moiety (**3**) hybridized with the whole 787 membered sub-library A; **B**) *Library b* is constructed with the (*R*)-THBD fragment conjugated, codified and included at the extremities of both sub-library A (code A788) and sub-library B (code B425).

Further SAR investigations of ligand **1** led to the identification of ligand **2**, which has improved affinity and selectivity profile.[23] Additionally, another 4,5-dihydrobenzodiazepinone ring (THBD)-based ligand (**3**) was shown to bind CREBBP bromodomain with >350-fold selectivity over BRD4(1).[24] For both ligands **2** and **3**, (*S*)-THBD stereoisomer displays greatly reduced affinity for the CREBBP bromodomain.[23,24]

We reasoned that the CREBBP bromodomain could represent an ideal target for ESAC selections as it contains two surface regions suitable for interaction with chemical compounds. The dual pharmacophore approach was reasoned to promote the development of selective ligands, as bromodomain KAc binding pockets have high sequence identity, while the loop regions are more sequence diverse. In this work, we have constructed a set of ESAC libraries which incorporated a carboxylate derivative of the (*R*)-THBD moiety in order to discover novel fragments that bind to the CREBBP bromodomain. One particular building block (i.e., carboxymethyl dithiobenzoic acid) was specifically enriched in presence of CREBBP and increased the binding affinity of THBD in fluorescence polarization and ELISA assays.

Results and Discussion

(*R*)-THBD binds to the KAc binding pocket of the CREBBP bromodomain, which is adjacent to the induced-fit pocket, that can be potentially occupied by a synergistic fragment [**Figure 1A**]. In order to discover synergistic binders, we screened ESAC libraries containing a carboxylic acid derivative of (*R*)-THBD. We used the ESAC assembly strategy described by Wichert *et al.*[18] to assemble three dual-pharmacophore libraries containing the (*R*)-THBD moiety at the 3'-end (i.e., *Library a*), at the 5'-end (*Library S2*) and on both extremities of the sub-libraries (i.e., *Library b*) [**Figure 2** and **Supplementary Figure S2**]. In this work, we employed two partially complementary single-stranded sub-libraries made by 5'-amino-modified DNA strands conjugated with 787 pharmacophores (sub-library A) and a 3'-amino-modified sub-library displaying 424 pharmacophores (sub-library B) [**Figure 2B**]. The assembled ESAC libraries were screened in presence of the immobilized CREBBP bromodomain, and enriched sequences were analyzed following a previously reported automated strategy.[5]

Figure 3 presents graphic plots of high-throughput sequencing data obtained from affinity-based selections in presence of CREBBP with *Library a* and *Library b*. Selections with *Library a* yielded to a selective enrichment of DNA sequences annealed to the THBD-conjugated strand (code **B3**) [**Figure 3A**]. In full

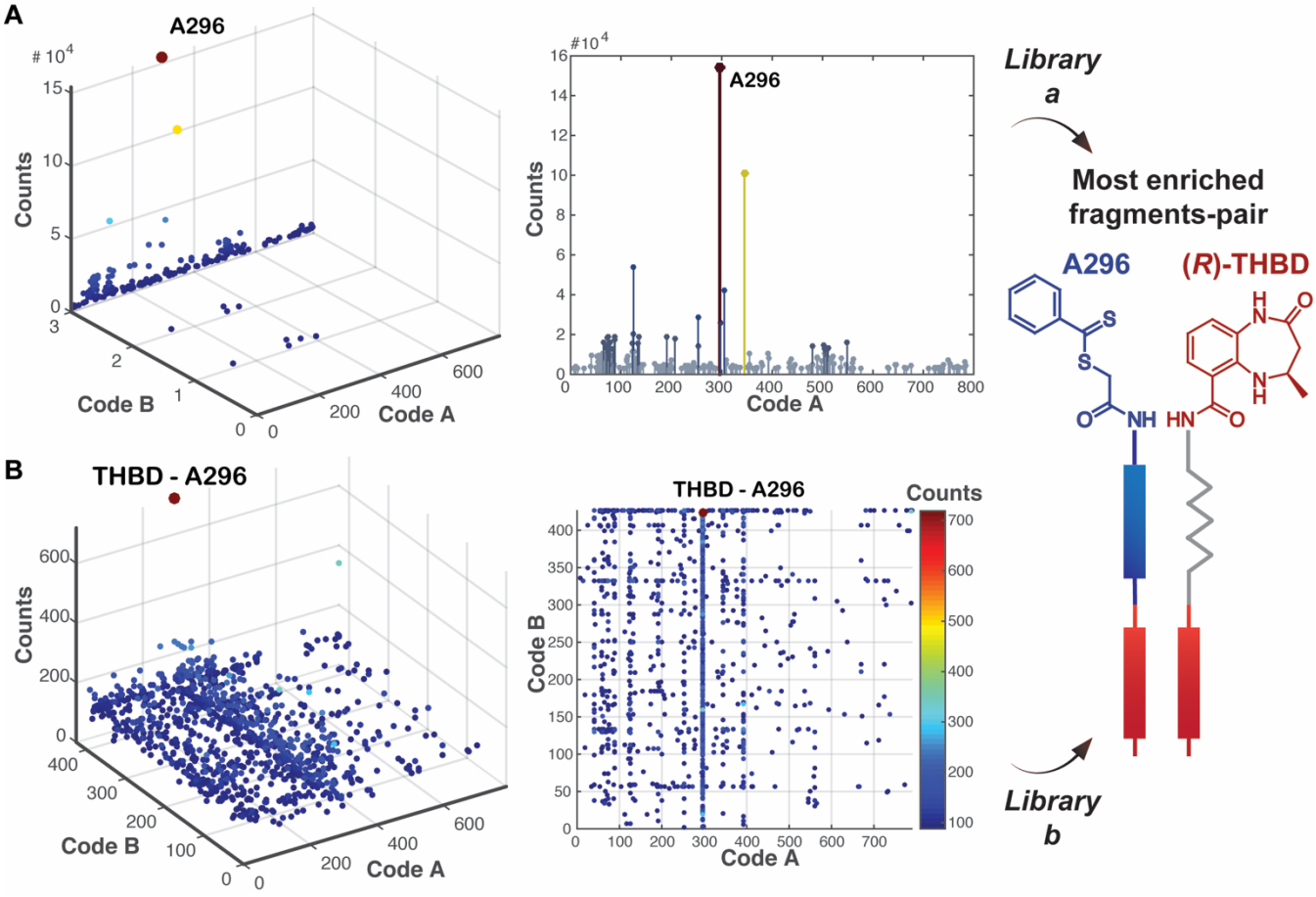

Figure 3. Three- and bi-dimensional plots of the high-throughput DNA sequencing data after selection against the CREBBP bromodomain. The most enriched fragment pair corresponds to a carboxymethyl dithiobenzoate derivative (**A296**) hybridized to the (*R*)-THBD moiety (red spots). **A**) Selection of *Library a* in presence of the CREBBP bromodomain yielded to the specific enrichment of sequences paired to the (*R*)-THBD fragment (code **B3**) over sequences paired to a free-amino group (code **B2**) and an acetazolamide derivative (code **B1**). **B**) Selection of *Library b* predominantly enriched sequence **A296** (sub-library A) and yielded to the highest enrichment factor when hybridized to the (*R*)-THBD moiety. For both sequencing data, cutoff level of counts was set to 100.

analogy, selection experiment with *Library b* selectively enriched THBD-based pair of fragments (code **B425**) [**Figure 3B**]. The sequence **A296** included within the sub-library A was specifically enriched in both libraries contributing with an increased number of counts compared to the rest of library members [**Figure 3**]. This sequence corresponded to a carboxymethyl dithiobenzoate derivative which resulted in the most enriched combination of building blocks when paired to the (*R*)-THBD fragment [**Figure 3**]. Screening of *Library a* and *Library b* against a positive-control protein target (i.e., carbonic anhydrase 9, CAIX) resulted in the specific enrichment of sequences linked to a known acetazolamide-based CAIX binder (Code **B1**), while selections against streptavidin-coated beads resulted in the selective enrichment of desthiobiotin-linked sequences [**Supplementary Figures S3**]. Surprisingly, the presentation of the (*R*)-THBD fragment at the 3'-end of sub-library A (code **A788** and **A3**) did not yield to the enrichment of specific pair of fragments [**Figure 3** and **Supplementary Figure S2**].

These selection results indicate the carboxymethyl dithiobenzoate derivative **A296** as a helping fragment that can enhance the binding affinity of the (*R*)-THBD moiety. To assess the affinity of the new pair of fragments, we synthesized hit compounds at the extremities of two complementary 12-mer locked-nucleic acid (LNA) strands [**Figure 4A**]. The 5'-amino-modified LNA strand also bears a fluorescein moiety that facilitate binding affinity measurements, in full analogy with an already described strategy.[25] In a first setup, fragments derived from known CREBBP-inhibitors (i.e., compounds **2** and **3**) were linked to LNA in order to validate the strategy for the measurement of dual-pharmacophore ligands of CREBBP through fluorescence polarization [**Supplementary Figure S6**]. Dissociation constants of ligands measured on-LNA were 5-10 fold higher than those of the parent ligands measured using isothermal titration calorimetry (ITC).[23] We have previously described how the linkage of binders on nucleic acid scaffolds might underestimate the affinity of covalently linked dual-pharmacophore ligands, presenting an appropriate connecting linker.[26] Although resulting in the measurement of weaker dissociation constants, this result validated the strategy for the rapid evaluation of binding properties of new dual-pharmacophore ligands against CREBBP.

Fluorescence polarization measurements of the combination of (*R*)-THBD fragment paired with the **A296** moiety, performed in solution, showed a 30-fold affinity enhancement compared to the (*R*)-THBD paired with an acetyl moiety [**Figure 4B**]. However, fragments paired with the enantiomer (*S*)-THBD showed much lower affinity for the CREBBP bromodomain. We further investigated the selectivity of the new pair of fragments against BRD4(1), a member of the BET family of bromodomains. Typically,

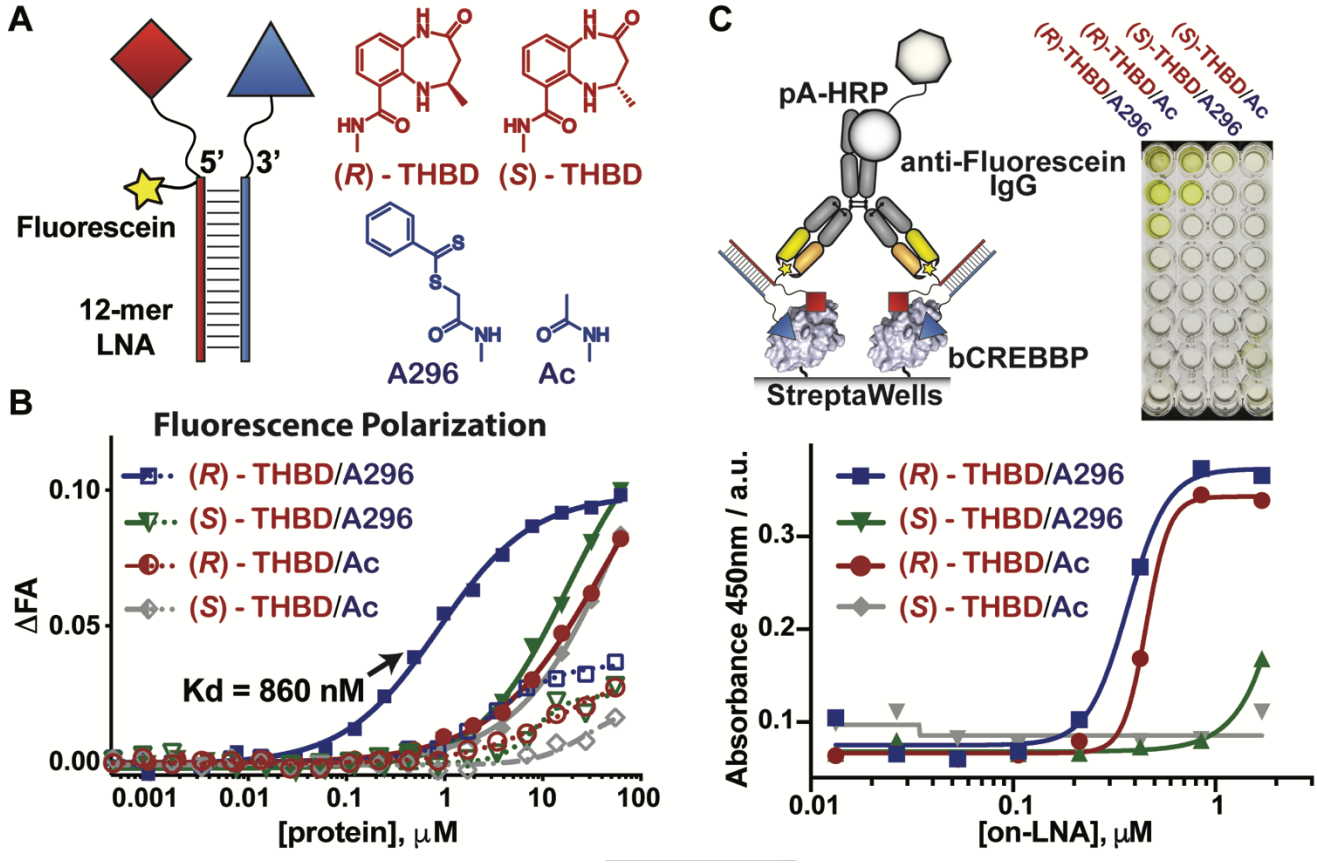

Figure 4. Hit-validation experiments of the carboxymethyl dithiobenzoate derivative **A296** in presence of THBD moieties. **A**) both (*R*) and (*S*) enantiomers of a carboxylic acid derivative of THBD were coupled to a 12-mer 5'-amino-modified LNA oligonucleotide presenting a fluorescein moiety on the last nucleic base (red strand), while the **A296** fragment and an acetylic group were coupled to the complementary 3'-amino-modified LNA-oligonucleotide (blue strand). **B**) Fluorescence polarization measurement of the four fragments pairs in presence of the CREBBP bromodomain (bold lines) and BRD4(1) bromodomain (dashed lines). **C**) Image representation and absorbance measurement of the enzyme-linked immunosorbent assay (ELISA) in presence of LNA-conjugated fragment pairs.

it is difficult to achieve some selectivity against protein of this closely related family. A weaker binding interaction was detected, confirming the selectivity of the fragment pair for the CREBBP bromodomain [**Figure 4B**].

Following a recently described procedure[27], binding ability of LNA-conjugated fragments was assessed *via* enzyme-linked immunosorbent assay (ELISA) [**Figure 4C**]. Plastic microtiter wells coated with biotinylated CREBBP were incubated with a serial dilution of fluorescein-linked LNA derivatives. Also, in this case, the pairing of (*R*)-THBD with **A296** showed a higher binding affinity compared to the (*R*)-THBD fragment alone. The (*S*)-THBD stereoisomer did not show detectable binding curves within the investigated concentration range, either alone or in presence of the **A296** fragment.

We have previously described that the substitution of nucleic acid scaffolds with short chemical linkers may lead to an increase or a decrease in binding affinity, depending on the chemistry of the linker.[26] We have therefore investigated the use of three different chemical linkers varying length and rigidity to connect the carboxymethyl dithiobenzoate **A296** fragment to the THBD moiety. The ITC affinity measurement of the covalent linked binding partners did not show an increased affinity compared to that measured on-LNA [**Supplementary Figure S4**], suggesting that a wider screening of linkers may be necessary.

Conclusion

We have validated the use of ESAC library technologies for the identification of novel fragments that bind to the CREBBP bromodomain. This has led to the identification of the carboxymethyl dithiobenzoic acid fragment **A296**, with a K_D = 860 nM, when paired with (*R*)-THBD through complementary LNA strands.

This work paves the way towards the employment of ESAC libraries in order to isolate potent small organic CREBBP binders. The same strategy could be extended for other bromodomain containing proteins and eventually yield to dual-pharmacophore binders of pharmaceutical interest.

Experimental Section

Design of the libraries. ESAC libraries were constructed as described by Wichert *et al.* [18]. The (*R*)-stereoisomer of 4,5-dihydrobenzodiazepinone ring-carboxylic acid [(*R*)THBD] was conjugated in order to be included within both sub-libraries with two distinctive code-sequences (i.e., A788 and B425, respectively for sub-library A and sub-library B).

In a first library setup (i.e., *Library a*) the full 787 members sub-library A was annealed with three single-molecules displayed on 3'-modified

strands (i.e., acetazolamide **B1**, free-amino group **B2**, (*R*)-THBD **B3**) [**Figure 2A**].
A second library (i.e., *Library S2*) was constructed in order to have the full 424 members sub-library B annealed with three single-molecule displayed on 5'-modified strands (i.e., acetazolamide **A1**, free-amino group **A2**, (*R*)-THBD **A3**) [**Supplementary Figure S2**].
In a third library setup (i.e., *Library b*), the full 787 members sub-library A containing the (*R*)-THBD fragment (code A788) was annealed with the full sub-library B also containing the (*R*)-THBD fragment (code B425) [**Figure 2B**].
The two sub-libraries were mixed in a 1:1 ratio, hybridized and code-filled by Klenow polymerase, as previously described.[18] The screening of the library was performed against the immobilized protein on streptavidin coated magnetic beads and the enriched sequences were read-out *via* high-throughput sequencing after PCR amplification, as previously described.[5] In addition to CREBBP, the libraries were screened against carbonic anhydrase IX (CAIX) and not-coated beads as positive and negative control, respectively [**Supplementary Figure S3**].

Expression and biotinylation of the CREB-binding protein. The CREBBP bromodomain (Addgene plasmid # 38977) construct including an N-terminal His-tag was transformed into *E. coli* BL21 (DE3) cells for expression, as previously described.[28] Random biotinylation of the protein was performed in presence of 3 equivalents of EZ-link™ NHS-LC-Biotin (ThermoFisher, cat. n. 21336) yielding a normal distribution of biotin-adducts with a single modification as the main component [**Supplementary Figure S4**].

Selection experiment. The screening of the three libraries was performed following a previously described automated method.[5] The magnetic streptavidin-coated beads were incubated with 2 μM biotinylated-CREBBP, with 1 μM biotinylated-CAIX and without any protein. The eluted libraries were PCR amplified and sequenced *via* high-throughput sequencing analysis.

Further experimental data are available in the **Supplementary Information** available in the online version of this article.

Contributions

M.C., M.M., S.J.C. and D.N. designed the project and the experiments. M.M. and J.C. synthesized the THBD fragments. M.M. provided the CREBBP and BRD4(1) bromodomains. M.C. and F.M. synthesized nucleic acid-conjugates. M.C., F.M. and M.B. constructed ESAC libraries and performed selections. M.C. and J.S. analysed high-throughput sequencing data. M.C. and P.B. synthesized off-DNA compounds. M.M. and K.H.A. performed ITC measurements. M.C. performed fluorescence polarization and ELISA assays. M.C. and D.N. wrote the manuscript. M.M., K.H.A., and S.J.C. edited the manuscript.

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Notes

The authors declare the following competing financial interest(s): D.N. is a cofounder and shareholder of Philochem AG and J.S. is a board member of Philochem AG.

Keywords: CREBBP Bromodomain; DNA-Encoded Chemical Library; Encoded Self-Assembly Chemical library; Inhibitors; Medicinal chemistry

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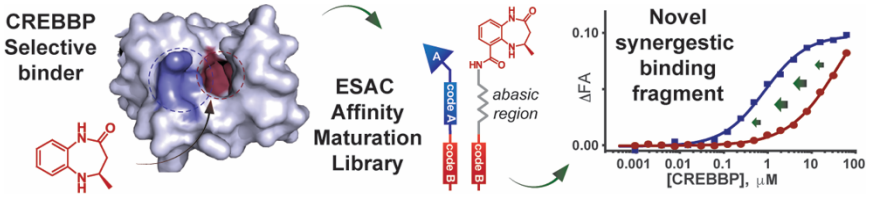

Screening against CREB-binding protein (CREBBP) bromodomain of a dual-pharmacophore Encoded Self-Assembly Chemical (ESAC) Library led to the discovery of a novel synergistic pair of binding fragments, presenting sub-micromolar affinity for CREBBP.