Published in final edited form as:

Chembiochem. 2017 May 04; 18(9): 853–857. doi:10.1002/cbic.201600637.

Hit validation methodologies for ligands isolated from DNA-encoded chemical libraries

Dr. Gunther Zimmermann^a, Dr. Yizhou Li^a, Dr. Ulrike Rieder^b, Dr. Martin Mattarella^b, Prof. Dr. Dario Neri^{*,a}, and Dr. Jörg Scheuermann^{*,a}

^aDepartment of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology (ETH Zürich), Vladimir-Prelog-Weg 3, CH-8093 Zürich (Switzerland) ^bPhilochem AG, Libernstrasse, 3 CH-8112 Otelfingen (Switzerland)

Abstract

DNA-encoded chemical libraries (DECLs) are large collections of compounds linked to DNA fragments, serving as amplifiable barcodes, which can be screened on target proteins of interest. In typical DECL selections, preferential binders are identified by high-throughput DNA sequencing, comparing their frequency before and after the affinity capture step. Hits identified in this procedure need to be confirmed, by re-synthesis and by performing affinity measurements. In this article, we present novel methods, based on the hybridization of oligonucleotide conjugates with fluorescently-labeled complementary oligonucleotides, which facilitate the determination of affinity constants and kinetic dissociation constants. The experimental procedures were exemplified using acetazolamide, a binder to carbonic anhydrase IX with dissociation constant in the nanomolar range. The detection of binding events was compatible not only with fluorescence polarization methodologies, but also with Alphascreen technology and with microscale thermophoresis.

Keywords

drug discovery; DNA-encoded chemical libraries; hit validation; affinity; k_{off} ; fluorescence anisotropy; AlphascreenTM; microscale thermophoresis

Introduction

DNA-encoded chemical libraries (DECLs) are compound collections that feature a stable linkage between individual chemical moieties and cognate nucleic acid structures, serving as amplifiable identification barcodes.[1] The encoding procedure allows the construction of very large combinatorial libraries, which can be interrogated by screening on target proteins of interest. In most cases, affinity-based selection procedures are performed (e.g., using magnetic beads coated with the protein target),[2] but alternative selection procedures have also been proposed.[3] In a typical selection experiment, the relative frequency of individual

* dario.neri@pharma.ethz.ch, joerg.scheuermann@pharma.ethz.ch.

compounds before and after affinity capture is determined by counting the number of DNA tag identifications in high-throughput DNA sequencing experiments.[4]

DNA-encoded chemical libraries have been successfully utilized for identifying binders of pharmaceutically relevant proteins (for recent reviews, see[5]). Using split & pool methodologies[4a, 6] or alternative methodologies[7] very large combinatorial DECLs, containing millions of compounds, can be synthesized within weeks. One often distinguishes between single-pharmacophore chemical libraries (in which individual molecules are attached to a single DNA fragment) and dual-pharmacophore chemical libraries (in which pairs of distinct molecules are attached to the extremities of complementary DNA strands). Dual-pharmacophore chemical libraries enable the simultaneous discovery of ligand pairs, [8] in analogy to certain fragment-based ligand-pair-finding technologies from complex mixtures.[9]

Library decoding after the affinity capture step typically results in the identification of preferential binding molecules, which need to be resynthesized and tested in suitable assays, in order to confirm specificity of binding and to provide information about affinity constants. To date, hit validation is typically performed by re-synthesizing a given number of compounds in the absence of DNA and by using a functional assay (e.g., an enzymatic inhibition assay, if the target is an enzyme).[2b, 10] Alternatively, ligands can be synthesized as fluorophore conjugates, as the binding properties of the corresponding compounds can be studied in solution, using fluorescence anisotropy techniques.[1a] These procedures are time-consuming, as individual compounds need to be synthesized and purified. The linkage of a fluorophore moiety may add synthetic steps to the preparation of the hits. In some cases, compound re-synthesis on DNA may facilitate experimental procedures[1], as DNA conjugates can be easily purified by selective precipitation, followed by mass spectrometric confirmation of structural features.

In this article, we present a general and versatile methodology for the study of the binding properties of organic ligands coupled to nucleic acids. The technology relies on the hybridization of oligonucleotide conjugates with a complementary strand, labeled with a suitable fluorophore and/or additional chemical moieties. The methodology was tested on acetazolamide, a nanomolar binder to carbonic anhydrase IX (CAIX),[1b, 11] using fluorescence anisotropy detection, Alphascreen technology and with microscale thermophoresis. The proposed methodology promises to accelerate the hit validation step, after DECL selections.

Results and Discussion

We have devised a high-throughput hit-validation procedure that allows the determination of affinity constants of organic ligands, coupled to suitable nucleic acid structures. Our workflow makes use of synthesis intermediates, which are generally available as a result of library construction.

We have focused on hit validation procedures for single-pharmacophore chemical libraries, as fluorescence-based methodologies for dual-pharmacophore chemical libraries have

recently been reported by our group (Figure 1).[1b] We started our investigations using a previously described 48mer oligonucleotide, coupled to the CAIX binder acetazolamide.[1b]

This ligand was reported to bind to CAIX with a dissociation constant of 12 nM and with a dissociation constant of 35 nM as a LNA conjugate.[1b, 11d] We first investigated the possibility of labeling the acetazolamide conjugate by hybridization with a fluorescently-labeled oligonucleotide derivative. Since fluorescence anisotropy may be dependent on the size of the labeled molecule, we aimed at comparing the fluorescence anisotropy (FA) profiles obtained with DNA structures differing in oligonucleotide length. We used locked nucleic acids (LNAs), as these structures can form stable heteroduplexes also with short oligonucleotide sequences. We hybridized a fluorescently-labeled 10mer LNA strand either to a complementary 10mer DNA strand or to a partially-complementary DNA structure (48mer). In both cases, the complementary strand was coupled to acetazolamide. Both structures induced a small change in fluorescence anisotropy (0.03 units), indicating that the size of the DNA-acetazolamide conjugate did not have a strong influence on the amplitude of the FA signal (Figure 2b).

In order to investigate the influence of the distance between fluorophore and protein ligand, we used a DNA heteroduplex, consisting of a fluorescently-labeled 19-mer strand (modification at the 5' end) and of a 41-mer acetazolamide conjugate (modification at the 3' end). The resulting DNA-acetazolamide complex induced a strong increase in FA (0.12 units) upon titration with a molar excess of CAIX. Fitting of the binding curve yielded a K_D value of $32(\pm 8)$ nM, in good agreement with previous reports (Figure 2c).[1b, 11d]

For pharmaceutical applications, it is often convenient to characterize binders not only in terms of affinity constants, but also measure the kinetic dissociation constant k_{off} , as this value may predict the residence time of a ligand on the cognate target protein.[12] (Figure 3). In a complementary experimental approach, we studied the properties of a complex, generated by the binding of CAIX to a heteroduplex, featuring fluorescein and acetazolamide at the complementary ends of the two DNA strands). The competition could be performed using acetazolamide either as individual compound or as DNA conjugate. The measurement of changes in FA over time yielded a single exponential profile, which could be fitted to yield a k_{off} value of 0.0063 s^{-1} (Figure 3). The published k_{off} value for acetazolamide, determined by surface plasmon resonance, was 0.0093 s^{-1} . [1b]

The use of ligand-DNA conjugates reduces synthetic efforts, increases ligand solubility and generates products, which are compatible both with K_D and k_{off} measurements.

In addition to fluorescence anisotropy, we also tested Alphascreen technology as an avenue to confirm the binding properties of hit molecules, coupled to DNA.

Initially, we investigated the interaction between a biotinylated oligonucleotide-acetazolamide conjugate and His₆-tagged CAIX (Figure 4a), which binds to Ni-NTA-coated beads. Upon interaction of the two binding partners, a strong concentration-dependent chemoluminescent signal was generated, with an apparent half-maximum signal in the double-digit nanomolar range, consistent with FA data (see above). Due to the low-binding capacity of the streptavidin-coated donor beads, a bell-shape titration curve was observed,

indicating donor bead saturation (“hook effect”;^[13]). Repetition of the experiment in the presence of a molar excess of acetazolamide confirmed that the Alphascreen signal was dependent on the acetazolamide-CAIX interaction (Figure 4a). The Alphascreen methodology could be successfully implemented by annealing a short biotinylated LNA oligonucleotide with a double-stranded DNA structure, carrying two acetazolamide moieties (Figure 4b). Also in this case, a concentration-dependent signal could be detected, suggesting that the hybridization-based technology could potentially be applied to dual-pharmacophore DNA-encoded chemical libraries. In a further approach, we investigated the use of microscale thermophoresis (MST) for the hit validation of compounds, coupled to DNA. The MST methodology detects binding interactions in solution^[14] and is therefore particularly attractive for affinity measurements. An acetazolamide-LNA conjugate was hybridized with a complementary fluorescently-labeled LNA strand (10mer). Using MST as readout, we obtained a K_D value of 77 ($\pm$ 35) nM for the interaction with CAIX (Figure 4c).

Conclusions

The methodologies described in this article are conveniently performed using ligands coupled to oligonucleotides, exploiting the versatility of heteroduplex formation of LNA and DNA to generate molecular structures, which are suitable for affinity measurements. As several dozens of hits are commonly identified in screening campaigns using large DNA-encoded chemical libraries, it is important to rely on high-throughput and high-quality hit validation approaches. The use of DNA-based methodologies is advantageous, not only because of synthetic speed and convenience, but also because of the beneficial solubilization effect on hydrophobic organic ligands.

While methodologies based on ELISA- or time-resolved FRET-technology (e.g., DELFIA)^[15] might also be conceivable for hit-validation on DNA, we chose to investigate Alphascreen technology, microscale thermophoresis and fluorescence anisotropy, as these instruments are available in most biochemistry laboratories and provide a broader dynamic range compared to ELISA. Among these three methodologies, fluorescence anisotropy is particularly attractive, as binding events take place in solution, with fewer possible confounding factors. Additionally, fluorescence anisotropy offers the possibility to simultaneously measure several compounds, individually labeled with different fluorophores (e.g., by suitable DNA hybridization techniques). This option is particularly valuable whenever low amounts of target protein are available, thus allowing the measurement of several binding interactions in the same well. Such multiplexing approaches can therefore either be used to increase throughput or to investigate whether the apparent affinity constants are influenced by the chemical nature of the fluorophore used in the assay.

Experimental Section

Materials

Modified oligonucleotides were purchased from Microsynth or LGC Biosearch (DNA) or Exiqon (LNA). The sequences of the oligonucleotides (DNA 41mer and 48mer) and (LNA both 8mers) have previously been described.^[1b] The LNA (7mer) used for the Alphascreen technology has the following sequence /5BioTEG/G+C+T+G+CGC, where + denotes a

LNA building block. Amino-modified oligonucleotides were coupled to acetazolamide and the NT-647 dye as previously described.[1b] Alphascreen Histidine (nickel chelate) detection kit was purchased from Perkin-Elmer.

Fluorescence anisotropy measurements

Fluorescein-modified oligonucleotide was mixed with a 3.5 molar excess of acetazolamide-labeled DNA in standard PBS buffer. Complementary oligonucleotides were heated to 65°C for 3 min and allowed to passively cool down to room temperature. The resulting mixture was diluted to a final concentration of 2 nM fluorescein-modified oligonucleotide and 7 nM partially complementary acetazolamide DNA-conjugate. To the mixture was added carbonic anhydrase IX (CAIX)[1b] from a stock solution in PBS. The resulting mixture was serially diluted holding the concentration of fluorophore constant. Fluorescence anisotropy measurements were performed in 384-well plates (Greiner, non-binding, low volume, 10 µL final volume) after an incubation of 1 h with the protein at room temperature. The obtained data were fit to a quadratic equation derived from the law of mass action as previously described.[1a] Fluorescence anisotropy measurements to determine the k_{off} value were carried out analogously using 2 nM of fluorescein-labeled DNA. CAIX was used at a concentration of 1 µM. The dissociation of DNA-bound acetazolamide was induced by adding a large excess of free acetazolamide (20 µM final concentration). Data were fitted to a single exponential model.

MST measurements

NT-647 modified LNA was mixed with a ten-fold molar excess of acetazolamide labeled-LNA and paired as described above. The resulting mixture was mixed with CAIX and diluted to a final concentration of 1 nM NT-647 modified LNA, holding the fluorophore concentration constant. Measurements were carried out in capillaries using a Pico spectrometer (Nanotemper). The obtained data were fitted to a quadratic equation derived from the law of mass action as implemented in the software of the manufacturer.

Alphascreen measurements

CAIX protein was serially diluted in standard PBS buffer in the presence of one equivalent biotinylated acetazolamide conjugate in white 384-well well plates. The resulting mixture was incubated for 10 min. To the mixture were added a mixture of Donor and Acceptor beads in PBS /Tween-20 so that the final concentration is 20 µg/ml of each bead and tween-20 (0.06%). The resulting bead suspension was incubated for 15 min at room temperature and the chemoluminescent signal was detected using an Alphascreen detection cartridge (SpectraMax, Paradigm). For the hybridization-based labeling of acetazolamide-DNA conjugates with Biotin, 1 µL of biotinylated LNA (7mer, 1 µM, PBS with tween) was mixed with 3 µL complementary 48mer (540 nM) in PBS and heated to 82 °C for 2 min, then mixed with 0.5 µL 41mer DNA (2.1 µM), and again heated to 82°C. The mixture was allowed to cool to RT. The mixture was diluted to a final concentration of 15 nM (biotinylated LNA), incubated with CAIX and mixed with beads as described above.

Acknowledgements

Funding from the ETH Zürich, the Swiss National Science Foundation (310030B_163479/1 Grant and CRSII2_160699/1 Grant), from the ERC Advanced Grant “ZauberKugel”, from Leopoldina, Nationale Akademie der Wissenschaften and from Philochem AG is gratefully acknowledged.

References

- [1]. a) Franzini RM, Ekblad T, Zhong N, Wichert M, Decurtins W, Nauer A, Zimmermann M, Samain F, Scheuermann J, Brown PJ, Hall J, et al. *Angew Chem Int Ed Engl*. 2015; 54:3927–3931. [PubMed: 25650139] b) Wichert M, Krall N, Decurtins W, Franzini RM, Pretto F, Schneider P, Neri D, Scheuermann J. *Nat Chem*. 2015; 7:241–249. [PubMed: 25698334] c) Harris PA, King BW, Bandyopadhyay D, Berger SB, Campobasso N, Capriotti CA, Cox JA, Dare L, Dong X, Finger JN, Grady LC, et al. *J Med Chem*. 2016; 59:2163–2178. [PubMed: 26854747] d) Ding Y, O’Keefe H, DeLorey JL, Israel DI, Messer JA, Chiu CH, Skinner SR, Matico RE, Murray-Thompson MF, Li F, Clark MA, et al. *ACS Med Chem Lett*. 2015; 6:888–893. [PubMed: 26288689] e) Litovchick A, Dumelin CE, Habeshian S, Gikunju D, Guie MA, Centrella P, Zhang Y, Sigel EA, Cuzzo JW, Keefe AD, Clark MA. *Sci Rep*. 2015; 5:10916. [PubMed: 26061191] f) Soutter HH, Centrella P, Clark MA, Cuzzo JW, Dumelin CE, Guie MA, Habeshian S, Keefe AD, Kennedy KM, Sigel EA, Troast DM, et al. *Proc Natl Acad Sci U S A*. 2016; doi: 10.1073/pnas.1610978113
- [2]. a) Decurtins W, Wichert M, Franzini RM, Buller F, Stravs MA, Zhang Y, Neri D, Scheuermann J. *Nat Protoc*. 2016; 11:764–780. [PubMed: 26985574] b) Eidam O, Satz AL. *MedChemComm*. 2016; 7:1323–1331.
- [3]. a) Bao J, Krylova SM, Cherney LT, Hale RL, Belyanskaya SL, Chiu CH, Arico-Muendel CC, Krylov SN. *Anal Chem*. 2015; 87:2474–2479. [PubMed: 25582319] b) Zhao P, Chen Z, Li Y, Sun D, Gao Y, Huang Y, Li X. *Angew Chem Int Ed Engl*. 2014; 53:10056–10059. [PubMed: 25044298] c) Chan AI, McGregor LM, Liu DR. *Curr Opin Chem Biol*. 2015; 26:55–61. [PubMed: 25723146]
- [4]. a) Mannocci L, Zhang Y, Scheuermann J, Leimbacher M, De Bellis G, Rizzi E, Dumelin C, Melkko S, Neri D. *Proc Natl Acad Sci U S A*. 2008; 105:17670–17675. [PubMed: 19001273] b) Buller F, Steiner M, Scheuermann J, Mannocci L, Nissen I, Kohler M, Beisel C, Neri D. *Bioorg Med Chem Lett*. 2010; 20:4188–4192. [PubMed: 20538458]
- [5]. a) Franzini RM, Neri D, Scheuermann J. *Acc Chem Res*. 2014; 47:1247–1255. [PubMed: 24673190] b) Salamon H, Klika Škopi M, Jung K, Bugain O, Brunschweiler A. *ACS Chemical Biology*. 2016; 11:296–307. [PubMed: 26820267] c) Arico-Muendel CC. *MedChemComm*. 2016; 7:1898–1909. d) Zimmermann G, Neri D. *Drug Discov Today*. 2016; 21:1828–1834. [PubMed: 27477486]
- [6]. a) Brenner S, Lerner RA. *Proc Natl Acad Sci U S A*. 1992; 89:5381–5383. [PubMed: 1608946] b) Buller F, Mannocci L, Zhang Y, Dumelin CE, Scheuermann J, Neri D. *Bioorg Med Chem Lett*. 2008; 18:5926–5931. [PubMed: 18674904] c) Clark MA, Acharya RA, Arico-Muendel CC, Belyanskaya SL, Benjamin DR, Carlson NR, Centrella PA, Chiu CH, Creaser SP, Cuzzo JW, Davie CP, et al. *Nat Chem Biol*. 2009; 5:647–654. [PubMed: 19648931]
- [7]. a) Melkko S, Scheuermann J, Dumelin CE, Neri D. *Nat Biotechnol*. 2004; 22:568–574. [PubMed: 15097996] b) Melkko S, Zhang Y, Dumelin CE, Scheuermann J, Neri D. *Angew Chem, Int Ed*. 2007; 46:4671–4674. c) Daguer JP, Zambaldo C, Ciobanu M, Morieux P, Barluenga S, Winssinger N. *Chem Sci*. 2015; 6:739–744. d) Zambaldo C, Barluenga S, Winssinger N. *Curr Opin Chem Biol*. 2015; 26:8–15. [PubMed: 25621730]
- [8]. Scheuermann J, Neri D. *Curr Opin Chem Biol*. 2015; 26:99–103. [PubMed: 25779969]
- [9]. a) Chen J, Zhang Z, Stebbins JL, Zhang X, Hoffman R, Moore A, Pellicchia M. *ACS Chem Biol*. 2007; 2:329–336. [PubMed: 17465519] b) Harner MJ, Frank AO, Fesik SW. *J Biomol NMR*. 2013; 56:65–75. [PubMed: 23686385]
- [10]. Deng H, Zhou J, Sundersingh FS, Summerfield J, Somers D, Messer JA, Satz AL, Ancellin N, Arico-Muendel CC, Bedard KL, Beljean A, et al. *ACS Med Chem Lett*. 2015; 6:919–924. [PubMed: 26288694]

- [11]. a) Alterio V, Hilvo M, Di Fiore A, Supuran CT, Pan P, Parkkila S, Scaloni A, Pastorek J, Pastorekova S, Pedone C, Scozzafava A, et al. *Proc Natl Acad Sci U S A*. 2009; 106:16233–16238. [PubMed: 19805286] b) Bhatt A, Mahon BP, Cruzeiro VW, Cornelio B, Laronze-Cochard M, Ceruso M, Sapi J, Rance GA, Khlobystov AN, Fontana A, Roitberg A, et al. *Chembiochem*. 2016; doi: 10.1002/cbic.201600513 c) Mahon BP, Bhatt A, Socorro L, Driscoll JM, Okoh C, Lomelino CL, Mboge MY, Kurian JJ, Tu C, Agbandje-McKenna M, Frost SC, et al. *Biochemistry*. 2016; 55:4642–4653. [PubMed: 27439028] d) Krall N, Pretto F, Decurtins W, Bernardes GJ, Supuran CT, Neri D. *Angew Chem, Int Ed Engl*. 2014; 53:4231–4235. [PubMed: 24623670] e) Wichert M, Krall N. *Curr Opin Chem Biol*. 2015; 26C:48–54.
- [12]. Scharenberg M, Jiang X, Pang L, Navarra G, Rabbani S, Binder F, Schwardt O, Ernst B. *ChemMedChem*. 2014; 9:78–83. [PubMed: 24357503]
- [13]. Acker MG, Auld DS. *Perspectives in Science*. 2014; 1:56–73.
- [14]. Seidel SAI, Dijkman PM, Lea WA, van den Bogaart G, Jerabek-Willemsen M, Lazic A, Joseph JS, Srinivasan P, Baaske P, Simeonov A, Katritch I, et al. *Methods*. 2013; 59:301–315. [PubMed: 23270813]
- [15]. a) Newbatt Y, Hardcastle A, McAndrew PC, Strover JA, Mirza A, Morgan GJ, Burke R, Davies FE, Collins I, van Montfort RL. *J Biomol Screen*. 2013; 18:298–308. [PubMed: 23139381] b) Rega MF, Leone M, Jung D, Cotton NJ, Stebbins JL, Pellicchia M. *Bioorg Chem*. 2007; 35:344–353. [PubMed: 17512966] c) Schachterle C, Christian F, Fernandes JM, Klussmann E. *Methods Mol Biol*. 2015; 1294:151–166. [PubMed: 25783884]

Figure 1.

Fluorescence anisotropy based hit validation strategies for binders identified from DNA-encoded chemical libraries A) re-synthesis without DNA; B) re-synthesis on DNA or LNA; C) use of premade intermediate oligonucleotide conjugates derived from library construction paired with premade fluorescently-labeled oligonucleotides; D) re-synthesis of dual-display hits without DNA; E) re-synthesis on LNA using one fluorescently labeled oligonucleotide strand.

Figure 2.

Impact of fluorophore position and oligonucleotide size on fluorescence anisotropy based hit validation strategies; A) schematic representation of the DNA/LNA-acetazolamide conjugates used for the hit validation experiments; B) fluorescently-labeled LNA (3'-CCFAM-dTCGAAGAC) strand paired to either a complementary DNA strand (10mer, 5' modification acetazolamide) or to a partially-complementary DNA structure (48mer, 5' modification acetazolamide). Binding to the protein CAIX was investigated using fluorescence anisotropy as readout; C) DNA heteroduplex, consisting of a fluorescently-

labeled 19-mer strand (modification at the 5' end) and of a 41-mer acetazolamide conjugate (modification at the 3' end). Binding to the protein CAIX was investigated using fluorescence anisotropy as readout and data were fit to a single-binding site model derived from the law of mass action to obtain the K_D value.

Figure 3.

Competition experiments to determine the kinetic dissociation constant k_{off} for fluorescently labeled DNA heteroduplexes. Dissociation of the labeled DNA heteroduplex from CAIX may be induced by either adding an excess of unlabeled small molecule (acetazolamide) or by adding an excess of unlabeled small molecule conjugate. Time dependent measurements of fluorescence anisotropy and fitting to a single exponential model allow the determination of the k_{off} value.

Figure 4.

Different hit validation formats for binders identified from DNA-encoded chemical libraries.

A) Chemoluminescent Alphascreen assay to investigate the binding of a biotinylated, acetazolamide-DNA conjugate to His-tagged CAIX (black squares). Repetition of the experiment by initial addition of an excess (10 μM) of acetazolamide (blue squares). B) Alphascreen assay to investigate binding of pre-made oligonucleotides conjugates derived from dual-pharmacophore DNA-encoded libraries. C) microscale electrophoresis (MST) using a 10mer LNA-acetazolamide conjugate paired with a dye suitable for thermophoresis detection. Data were fitted to a single-site binding model derived from the law of mass action.