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Article

On-DNA Binder Confirmation: Increasing Confidence in DEL Hits

Mark A. Mantell,* Karanbir S. Pahil,* Karli R. Holman, Joshua D. Alper, Chloe M. Jones, Gang Yao, and Lisa A. Marcaurelle

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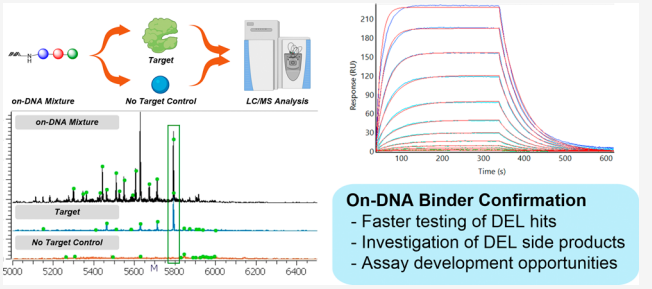
ABSTRACT: DNA-encoded libraries (DELs) are used throughout small-molecule drug discovery to identify new lead compounds for protein targets. DEL hits are traditionally evaluated via off-DNA resynthesis and subsequent biological testing. This approach can be time- and resource-intensive, limiting the number of putative hits selected for follow-up. Additionally, these hits often fail to confirm off-DNA, leading to lost time and effort. Here, we introduce GSK's on-DNA hit resynthesis and binder confirmation workflow, which increases throughput and emulates the original library synthesis, thereby enabling identification of side product binders and increasing confidence in DEL hits. We utilized thermal shift, microscale thermophoresis, activity, and compound-immobilized SPR assays to develop a robust platform for the derisking of DEL hits. This is followed by a mass-spectrometry-based assay to identify specific binders in mixtures of on-DNA compounds. We share GSK's application of on-DNA binder confirmation to both evaluate and expand hits from DEL screens.



INTRODUCTION

DNA-encoded libraries (DELs) have become a foundational tool for early-phase lead discovery across the pharmaceutical industry.[1−3] The field of DEL technology has expanded rapidly since its inception and DEL screens have been used to generate pharmaceutical lead compounds for a large number of protein targets, including RIP1K,[4,5] BMPR2,[6] and BET BD2.[7] The concept, which was originally described by Brenner and Lerner, involves "split and pool" library synthesis, where each chemical reaction is encoded with a unique DNA barcode cataloging the identity of the building block used.[8] The split and pool method can be used to rapidly generate libraries with member counts in the millions to billions of unique compounds, which can then be screened simultaneously against a host of protein targets.

The high diversity of these libraries contributes to the enormous volume of data that comes from each individual DEL screen. Each library screened against a protein target may reveal hundreds to thousands of potential binders, and the number of binders will increase as the number of libraries screened increases. Therefore, the number of chemotypes prosecuted from a DEL screen will typically be limited by the resources available for off-DNA hit resynthesis. This leads to some DEL screening hits being deprioritized in favor of alternative compounds that are considered to have an increased likelihood of success according to our traditional guiding principles for triaging DEL screening hits, described below.

With an increase in diversity also comes an increase in the potential of unintended side products in a library, which can arise from incomplete chemical reactions or unforeseen reactions between building blocks and the on-DNA material.[9] The presence of these unintended side products increases the likelihood of binding driven by non-enumerated species, which can result in wasted time and materials if only the enumerated compounds are resynthesized for further testing. The resources that must be committed to resynthesize hits from a DEL screen and the uncertainty surrounding chemical structures of interest can be some of the chief limiting factors in deriving the best possible hits from the screening platform.

Conceptualization. After nearly two decades of use in pharmaceutical research at GSK, we have largely standardized our DEL discovery workflow. Once a target has been identified and validated, we screen the target against our collection of DELs. We sequence the DNA barcodes of compounds that bind to the target and then translate the sequences into putative chemical structures. We then select binders for off-DNA synthesis and testing based on an assessment of enrichment, evidence of SAR, and physicochemical properties (Scheme 1).

Our guiding principle behind triaging screening hits for follow-up is to assess the likelihood of the hit becoming a lead compound compared to the amount of time and effort that can

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Scheme 1. DEL Discovery Workflow from Validation of Screening Conditions through off-DNA Small-Molecule Hit Identification, Triage, and Confirmation; This Figure Was Created with BioRender.com

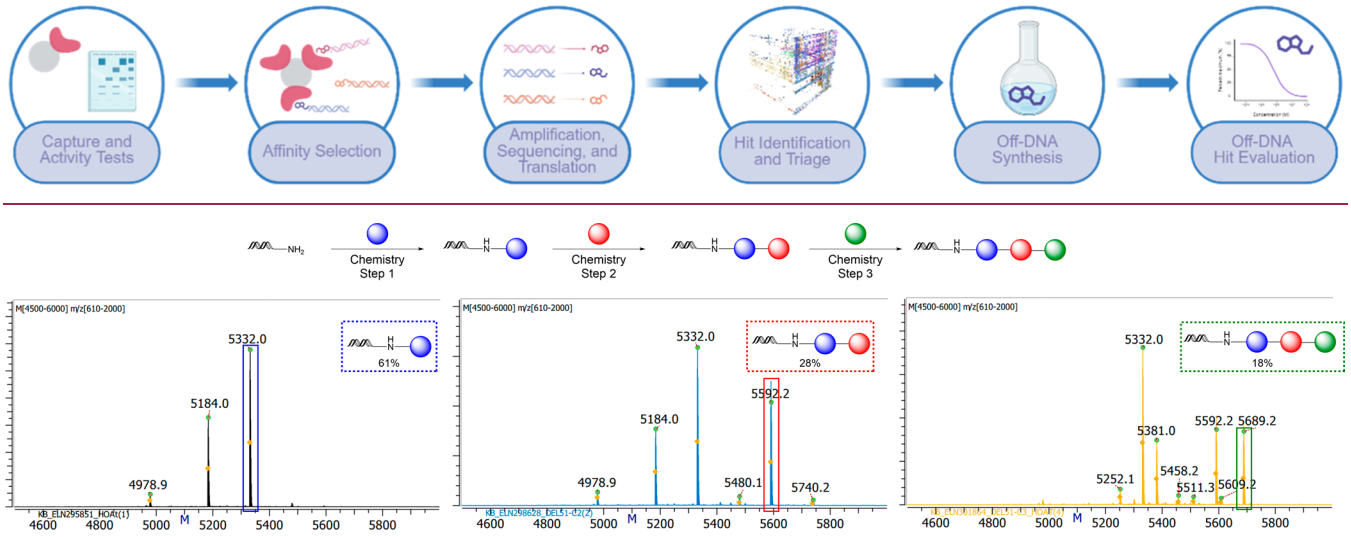

Figure 1. Tracking the synthetic purity of an on-DNA mixture using LC−MS. The desired product of each chemistry step is marked with a colored box, corresponding to the framed on-DNA compound. Purity is denoted as a percentage of the total oligo within the colored frame.

be spent on synthesizing that hit in a timely manner. Some hits that are potentially interesting are passed over due to challenges in their synthesis, weak evidence of SAR in the DEL screening data, suboptimal physicochemical properties, or overall limited resources. To expand the total number of compounds that can be investigated for a given target, we aimed to develop a robust platform for on-DNA binder confirmation (ODBC) as a supplement to our traditional DEL hit discovery workflow. This platform would serve to increase the number of "shots on goal" for each target by derisking progression of compounds that we did not select as top priorities for off-DNA synthesis, recommending only proven binders for advancement.

We find that the synthesis of compounds on-DNA is faster and less resource-intensive than traditional off-DNA synthesis due to the presence of the DNA tag, which allows for facile separation from small-molecule impurities through ethanol precipitation and size exclusion filtration, avoiding the need for aqueous workup and purification steps. The inclusion of the DNA tag also allows for replication of the same synthetic conditions used in the library's original production, generating compound mixtures that are approximations of those present in the library itself. Additionally, the DNA tag used in ODBC should not hinder the evaluation of on-DNA compounds in binding and activity assays, because the compounds of interest are identified through affinity selection with a DNA tag present. In fact, the DNA can be used as a handle for appending oligo-conjugated probes, enabling assay development. These qualities, as well as the inherent high-throughput nature of DEL synthesis, highlight the ability to rapidly generate and test on-DNA compounds.

Our group has previously published an orthogonal method to the ODBC workflow proposed here, in which individual compounds were synthesized on a DNA headpiece bearing a photocleavable or an acid-labile linker.[9] These compounds were cleaved from their DNA tags and then evaluated for binding to their target protein by affinity selection mass spectrometry (ASMS). The platform was high-throughput in nature but suffered from challenges in solubility and ionization of the cleaved small-molecule compounds, which led to the observation of false negatives.

True on-DNA binder confirmation, meaning ODBC that is conducted with all compounds affixed to a non-cleavable DNA headpiece, has also been reported, normally making use of single binding assays to add confidence for pursuing off-DNA resynthesis.[10−12] In one example, Pfizer published a platform for the evaluation of on-DNA hits using a platform they named Bead-Assisted Ligand Isolation Mass Spectrometry (BALI-MS).[13] The platform utilized magnetic bead-immobilized proteins to isolate binding compounds from a mixture of on-DNA compounds and then characterized the binders by mass spectrometry. This method was effective but protein intensive if used on a large scale. Similarly, Prati et al. have reported SPR methods to detect binding of target proteins to on-DNA molecules that are immobilized on an SPR chip,[14] and Zimmermann et al. have reported using DNA tags to enable fluorescence polarization, microscale thermophoresis, and alphaScreen assays.[15] A recent report also utilized native mass spectrometry for the hit validation of on-DNA compounds.[16]

Herein, we demonstrate our platform for on-DNA binder confirmation by discussing two case studies of its application. These studies show the adaptability of our platform, in which we use multiple orthogonal assays to confirm binding and identify the specific binder in a mixture of on-DNA compounds. Application of this method allows for both the rapid identification of compounds that bind to protein targets and the development of confirmed binders via on-DNA hit expansion.

RESULTS

Case 1: Chemotype Triage for a High Tractability Target. In the first case, we evaluated a kinase that displayed unusually high tractability for DEL screening. We identified hundreds of thousands of potential binders in the DEL screen (Figure S1), which we triaged based on structural diversity, physicochemical properties, and suspected binding mode to give a selection of several promising compounds that were

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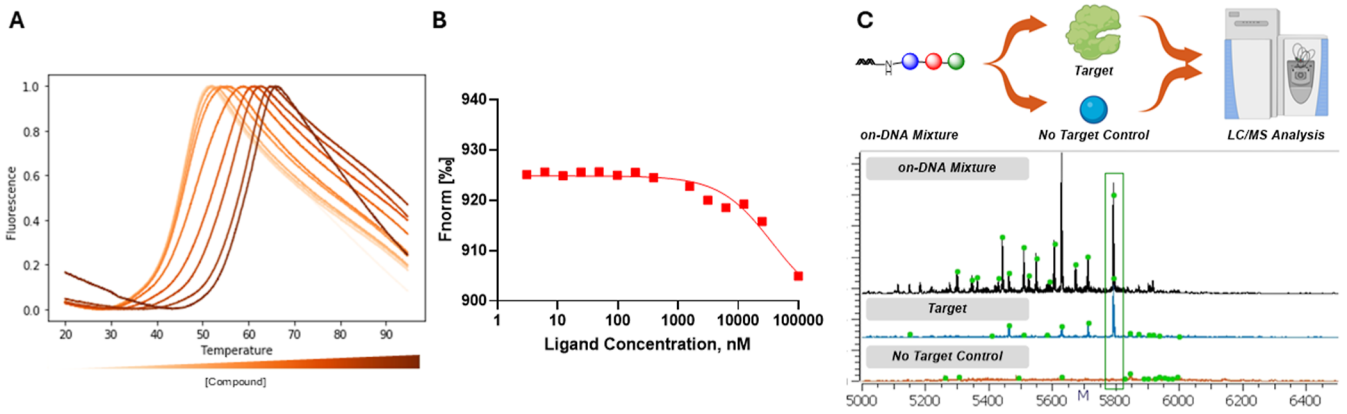

Figure 2. (A) SYPRO Orange thermal shift of mixture 7. Darkening lines show an increase in the concentration of mixture 7. (B) MST of mixture 7. (C) iASMS of mixture 7. The green box denotes the product enumerated by the library, which is present in the final mixture from the synthesis and the iASMS target condition. The peak is absent in the No Target Control.

progressed directly to off-DNA synthesis. To validate our ODBC workflow, we selected nine of those compounds, each representing a distinct chemotype of interest, to be synthesized and tested on-DNA in parallel with off-DNA hit confirmation. We completed on-DNA synthesis in a telescoped manner, following the chemical reaction conditions used in the library of origin, while making some allowances for expediency and throughput. For all compounds, we omitted DNA tag ligation steps, delivering the compounds with only the PEGylated DNA 8-base headpiece (Figure S2) as the attached DNA tag. Additionally, we restricted cleanup between chemistry steps to ethanol precipitation for the removal of the majority of excess reagent and solvent followed by size exclusion spin filtration for the removal of any remnant small-molecule impurities. Solution purity, here meaning the fraction of the oligo mixture that contained the compound enumerated by the library synthesis sequence, was assessed following each synthetic step, but we performed no further purification at any stage. Telescoped chemistry steps inevitably led to lower purity alongside increased numbers of side products after several cycles of chemistry (Figure 1). As the goal of the ODBC platform is to assess the binding ability of the mixture, as it is likely present in the library, we still evaluated final mixtures that contained low or even 0% purity of the enumerated compound in our workflow.

With the on-DNA compound mixtures in hand, we tested the ability of these compounds to bind the target in both a SYPRO Orange thermal shift assay (Figure 2A, for mixture 7, Figure S3 for other compound mixtures) and microscale thermophoresis (MST) (Figure 2B, for mixture 7, Figure S3 for other compound mixtures). Given that these compounds were not pure, it was possible that side products produced positive binding results. Therefore, we developed a method, hereafter called iASMS (immobilized affinity selection mass spectrometry), to determine the identity of the specific binder in each compound mixture. Similarly to the previously reported BALI-MS,[13] we incubated the on-DNA compound mixtures with a tagged target protein, captured the protein on affinity resin, and then washed away non-binding impurities. We also performed a no-target control, lacking the tagged protein, to account for potential on-DNA compound interactions with the resin. Heat elution of the resin-bound complexes liberated the binding components of the mixture, which we then identified via LC−MS (Figure 2C, for mixture 7, Section S5.2, for other compound mixtures). In practice, iASMS produced a qualitative measure of binding, as we determined enrichment of binders by a comparison of relative intensity of peaks in the final mixture, the target assay output, and the no-target control output (Figure S4). Further discussion of iASMS interpretation can be found in the Supporting Information.

To evaluate the predictive power of the ODBC platform, we compared the binding of all nine enumerated off-DNA compounds with their on-DNA counterparts, binning the compound mixtures based on their iASMS results (Table 1). For the on-DNA compound mixtures that failed to display binding in all three orthogonal assays, the corresponding off-DNA enumerated compound also did not bind (Table 1, mixtures 1−3). For the two on-DNA compound mixtures that produced inconclusive iASMS results, we only observed binding of the off-DNA enumerated compound in the example with positive binding on-DNA in one of the dose-dependent binding assays (Table 1, mixtures 4−5). It is notable that a thermal shift was observed for mixture 4, which contained 0% of the enumerated product; binding of the compound mixture may have been driven by a side product or by trace amounts of the enumerated product, under the limit of detection in LC−MS. For the two on-DNA compounds that displayed positive binding in thermal shift and MST, as well as positive binding of the enumerated compound in iASMS, the off-DNA compounds also confirmed as binders (Table 1, mixtures 6−7). The final two on-DNA compounds displayed positive binding in thermal shift, but iASMS revealed that binding was driven by side products. In both of these examples, the enumerated off-DNA compounds were synthesized and did not confirm as binders (Table 1, mixtures 8−9), but the one indicated side product binder that we synthesized confirmed as a binder off-DNA. Additionally, for the two compounds (Table 1, mixtures 4 and 9) that showed inconsistent thermal shift and MST results, we found that their respective iASMS-implicated binders confirmed off-DNA. Therefore, we deemed it important to run orthogonal assays to minimize the possibility of false negative results.

Together, these results led us to a general workflow for future projects that utilize the ODBC platform (Scheme 2). All compounds designated for ODBC are synthesized on-DNA and the resulting mixtures are tested in at least two orthogonal dose−response binding assays regardless of the purity of the

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Table 1. Full Assay Results of the on-DNA Compounds Synthesized for the High Tractability Target[a]

		On-DNA Results			Off-DNA Results	
on-DNA Mixture	Purity	Thermal Shift	MST	iASMS	Product Binding	Side Product Binding
1	65%	✗	✗	✗	✗	
2	13%	✗	✗	✗	✗	
3	7%	✗	✗	✗	✗	
4	0%	✓	✗	○	✓	
5	0%	✗	✗	○	✗	
6	50%	✓	✓	✓	✓	
7	17%	✓	✓	✓	✓	
8	34%	✓	✗	✓	✗	○
9	0%	✓	○	✓	✗	✓

✓ Binding ✗ No Binding ○ Indeterminate ✓ Side Product Binding

[a]iASMS designation of "Binding" implies that the enumerated compound was the binder identified by iASMS. "No Binding" implies that no binding was observable, "Indeterminate" implies that a conclusion could not be reached based on the data from the assay, and "Side Product Binding" implies that a product other than the enumerated compound drove the observed binding. This figure was created with BioRender.com.

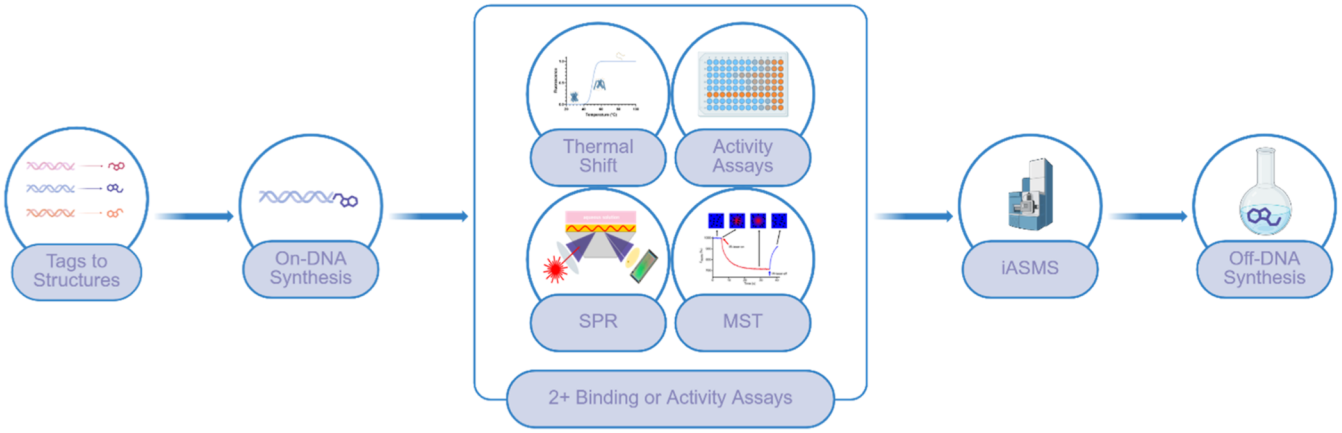
final mixture. These assays are used to determine whether the on-DNA compound mixture has a component that exhibits dose-dependent binding. Only those mixtures with positive dose-dependent binding in at least one assay are advanced to iASMS, as iASMS is both only qualitative and requires significantly more protein than any other assay. Then, the specific binders within the mixtures that are identified by iASMS are advanced to off-DNA synthesis (Scheme 2).

Case 2: Evaluation and Expansion of High-Copy Singletons. Our second case study involves a different protein target that had markedly different DEL screening results. While the Case 1 target was overwhelmingly tractable, target 2 was much less tractable: an ASMS screen of ~1 million compounds yielded no validated hits and a DEL screen yielded few hits (Figure S5A). Using the guiding principles for hit prioritization described above, the most promising compounds present in the data were singletons, which are considered higher-risk due to both the lack of corroborating evidence from the screen indicating they are true binders (e.g., evidence of SAR) and developability concerns. In an effort to derisk these compounds, we deployed ODBC to rapidly provide information on the binding and activity of the six high-copy singletons.

We synthesized these compounds on-DNA as before and evaluated each product mixture with an enzyme activity assay, a thermal shift assay, and then iASMS.[17] Only one compound displayed binding and activity against the target, and iASMS confirmed that the observed activity was driven by the enumerated compound from the library scheme. We then resynthesized the compound off-DNA and found it to be a submicromolar inhibitor of the target protein (Figure S5B,C).

Given the strong ODBC response and close alignment between the on- and off-DNA potency, we hypothesized that we could extend the ODBC platform beyond hit confirmation to explore the SAR of this confirmed hit. On-DNA synthesis is readily scalable to plate-based format, enabling higher throughput and shorter design−make−test cycle times than otherwise achievable with off-DNA synthesis alone. For the purpose of this pilot study investigating on-DNA hit expansion, we synthesized all compounds both on- and off-DNA, regardless of the ODBC outcome, to enable comparison of the results.

Scheme 2. Illustration of the ODBC Workflow. Compounds Are Tested in at Least Two of the Listed Biophysical Assays before Advancing the Binding Mixtures to iASMS. The Identified Binder is Then Advanced to off-DNA Synthesis. This Figure Was Created with BioRender.com



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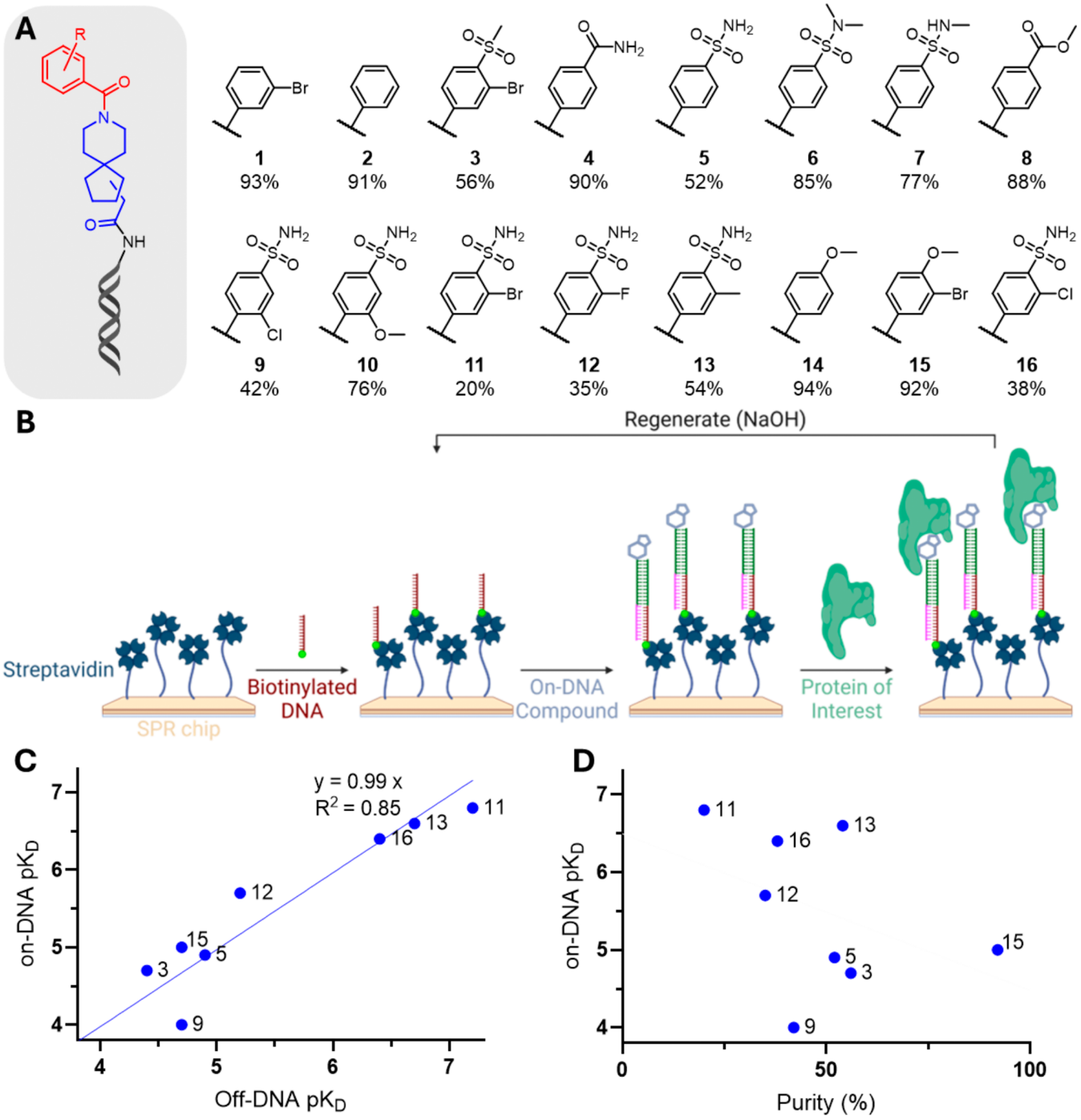

Figure 3. On-DNA SAR generation using DNA-immobilized SPR. (A) Compounds synthesized for hit expansion and their purities. (B) Illustration of the DNA-immobilized SPR method employed using the Carterra LSA^{XT}. (C) Correlation of on- and off-DNA SPR-derived pK_D values for each compound in the series. Compounds that did not bind are not pictured. All compounds that did not bind on-DNA also did not bind off-DNA. (D) Correlation of on-DNA SPR-derived pK_D values with compound purity. No trend was observed (p = 0.3). This figure was created with BioRender.com.

We synthesized 16 on-DNA compounds with varied cycle 2 building blocks, including that of the original confirmed hit, in a plate-based format. Having confirmed that the enumerated compound drove binding and activity, we determined that it was no longer important to follow the historical library procedure for these compounds; therefore, we used higher-yielding conditions to synthesize each compound. Additionally, to increase the throughput of the synthesis, we only performed ethanol precipitation for cleanup. Purities of the final compounds ranged from 20% to 94% (Figure 3A), and the purity of the hit compound synthesized using this revised protocol aligned with that of its original ODBC synthesis (Figure 3A, mixture 11).

Expanding ODBC analysis to plate-based SAR exploration also required the development of a high-throughput quantitative binding assay that is robust to varying compound purities. For this, we adapted the on-DNA SPR approach first reported by Prati et al.[14] We coated a SPR chip with a

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biotinylated single-stranded oligonucleotide and then ligated the on-DNA compounds to a complementary oligonucleotide. This allowed us to capture compounds via hybridization on the SPR chip and subsequently flow over a titration series of the target protein. We performed our experiments on a Carterra LSA^{XT}, which allows the immobilization of on-DNA compounds to up to 384 spots in parallel, enabling us to test entire plates of compounds in duplicate in a single run. Our hybridization approach allowed the chip surface to be regenerated using NaOH to dehybridize our oligos, minimizing the need to develop protein-specific regeneration conditions while maximizing chip usage (Figure 3B). Given that non-binding immobilized impurities should not show any SPR response, we hypothesized that this method should provide accurate binding affinities across a range of compound purities (Figure S6A). In fact, when tested by titrating a non-binding impurity into a known binding compound, capturing these mixtures on the chip, and then flowing over the target protein, we were able to obtain consistent binding affinities down to 1.3% compound purity (Figure S6B,D). This gave us confidence in applying on-DNA SPR to SAR exploration.

Of the 16 analogs screened, eight demonstrated dose-dependent binding both on- and off-DNA (Figures 3C, S7). The binding affinities of the ODBC compounds were within 0.7 log units of their off-DNA counterparts, with the closest correlation observed for compounds of submicromolar potency. As expected, the varying purity of the on-DNA compounds did not correlate with their respective on-DNA affinity (p = 0.3, Figure 3D). Most importantly, the decisions made regarding which compounds would be ideal for additional follow-up would have been the same with the on- and off-DNA methods of SAR evaluation, proving this as a valuable tool for rapid on-DNA SAR development. Additionally, the total synthesis and screening time for these assays was 1 week and is scalable far beyond 16 compounds at the same pace.

DISCUSSION AND CONCLUSIONS

Here, we have showcased the development of on-DNA binder confirmation as a method to rapidly derisk putative hits that are identified in a DEL screen. Implementation of this platform has allowed us to cover a larger diversity of chemical space, derisk compounds from challenging data sets, identify side product binders, and obtain high-throughput SAR of confirmed hits.

In addition to increasing the chemical space pursued for a particular target, ODBC compounds can also serve as a springboard to access challenging target space. As the drug development industry increasingly moves toward pursuing traditionally recalcitrant targets (e.g., membrane proteins and multiprotein complexes)[18−20] without known tools,[21,22] the discovery of tool molecules and the development of hit validation assays is becoming increasingly rate limiting. It has been argued that terms like "druggable" and "undruggable" are inappropriate, and in reality, protein targets should be considered either currently "assayable" or currently "un-assayable".[23] We believe that DEL screening followed by ODBC represent a means to overcome this bottleneck. Our ODBC molecules contain a two-base-pair overhang, allowing us to ligate on any desired probe upon the completion of synthesis. Here, we coupled the ODBC molecules to a biotinylated oligo to allow compound immobilization followed by SPR, but others have also ligated fluorophores onto on-DNA molecules for MST and fluorescence polarization experiments.[15] In our case, immobilization of the compound provides much higher theoretical signal than conventional protein-immobilized SPR, increasing the signal window, which is often exactly what is needed to rescue a target previously thought "un-assayable". Notably, conventional chemical probe design relies on an extensive knowledge of SAR, which cannot be achieved without a working assay. Our workflow uses knowledge from the DEL screen to overcome this challenge. Therefore, ODBC compounds not only provide a means to increase the chemical space pursued for a particular target but also can enable access to more challenging protein targets.

Overall, ODBC has seen rapid uptake by the Encoded Technologies group at GSK, and the workflow described herein is now applied to all protein targets that undergo a DEL screen. Implementation of this platform has led to more total "shots on goal" for our DEL platform, derisking of compounds from challenging data sets, and enabling the identification of several non-enumerated chemical species that may have otherwise been missed. Additionally, we have begun applying the ODBC method in the service of assay and chemical tool development, and we see its application growing as we apply it to future targets.

EXPERIMENTAL SECTION

Statement of Compound Purity. All off-DNA compounds are >95% pure by HPLC analysis. Purity of on-DNA compounds, defined here as the fraction of the oligo mixture bearing the compound enumerated by the library sequence, was assessed via LC−MS and is reported for each compound that was tested.

General Procedure for Ethanol Precipitation. To the reaction mixture was added NaCl (5 M, 10% v/v), followed by cold ethanol (2.5× total volume). The mixture was vortexed and transferred to a −80 °C freezer for at least 30 min. The mixture was centrifuged (4 °C, 30 min, 14,000 rcf), and the solvent was decanted off.

General Procedure for Size-Exclusion Spin Filtration. Solids were dissolved in water (100 μL per 100 nmol of oligo) and transferred to a 3k MW cut-off filter. The tube was washed with water (100 μL per 100 nmol of oligo) and transferred to the filter. The solution was centrifuged (20 °C, 10 min, 14,000 rcf), and 100 μL of water was added to the filter; the solution was mixed by a pipet. The solution was centrifuged (20 °C, 10 min, 14,000 rcf), and 100 μL of water was added to the filter; the solution was mixed by a pipet. The solution was centrifuged (20 °C, 10 min, 14,000 rcf), and the concentrate was transferred to a fresh tube. The solution was frozen in liquid nitrogen and dried in a lyophilizer overnight.

General Procedure for Enzymatic Ligation for on-Chip Immobilization. To a 250 μL PCR plate was added each on-DNA mixture (10 nmol, 1 mM, 10 μL, 1 equiv) in separate wells. In a separate tube was combined T4 DNA ligase (1 part), 10X Tris buffer (10 part), and water (4.15 part). This mixture (21 μL) was added to each well. In a separate tube was combined the tags: top strand (2 mM) (5′-/5Phos/CAT AGA CTC GGA TCG GAA GAG CGG TAT CTC G-3′ (MW = 9665.2)) and bottom splint (2 mM) (5′-CGA GTC TAT GGG-3′ (MW = 3701.5)) in a 1:1 mixture to make a 1 mM tag solution. Tag solution (9 μL, 0.9 equiv) was added to each well. The total volume of each well was 40 μL to make a 0.25 mM solution of oligo. The plate was sealed and vortexed and then left at room temperature overnight.

The plate was briefly centrifuged and then opened. Ligation was evaluated by LC−MS analysis. Reaction was subjected to the Ethanol Precipitation procedure above. Solids were left to dry in a fume hood overnight.

General Procedure for iASMS. Target His-tagged protein was prepared as a 1 μM solution in the same buffer that was used during the DEL screen. To the wells of a plate (incubation plate, corresponding to the number of compounds to be evaluated by

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iASMS) was added protein in a buffer (98 μL). To an equal number of wells was added buffer (98 μL). To each well was added the on-DNA compound (2 μL, 50 μM). The plate was covered with foil. The plate was incubated at room temperature for 45 min while being shaken at 700 rpm.

After 30 min of incubation, a 1:1 mixture of Dyna:His beads was prepared by vigorous resuspension and then transferred to a trough. The beads were again vigorously resuspended, and 50 μL beads were added to the wells of a fresh round-bottom plate (bead plate). The plate was placed on a magnet, and the bead storage solution was removed. Buffer (100 μL) was added to the beads, which were vigorously resuspended. The plate was placed on a magnet, and the bead storage solution was removed. Buffer (100 μL) was added to the beads, which were vigorously resuspended.

When incubation had finished, the bead plate was placed on a magnet, and storage buffer was removed to leave only beads. The contents of the incubation plate were transferred to the corresponding wells on the bead plate. The plate was covered and shaken (700 rpm) for 5 min at room temperature. The plate was placed on a magnet, and the solution was removed, with careful removal of the solution as much as possible. This solution was retained as the unbound fraction in the case of analytical interest. To the beads was added buffer (100 μL), and the beads were resuspended. Slurry was transferred to a PCR plate. The plate was placed on a magnet, and the supernatant was removed, being careful to remove as much as possible. This solution was retained as the wash fraction in case of analytical interest. To each well was added water (55 μL) and the beads were gently resuspended. The plate was covered with foil and heated to 95 °C for 10 min via thermocycler. As soon as heating was completed, the plate was placed on a magnet and the supernatant was transferred to a fresh plate. Samples were analyzed by LC−MS.

Thermal Shift Assays. Purified protein of interest was diluted to 1 μM in SYPRO orange assay buffer (25 mM HEPES pH 7.3, 12.5 mM NaCl, 1 mM $MgCl_2$, 500-fold diluted 5000× SYPRO Orange (Thermo Fisher Scientific) for the protein from case 1, 50 mM Tris pH 7.5, 125 mM NaCl, 5 mM $MgCl_2$, 500-fold diluted 5000× SYPRO Orange for the protein from case 2). 10 μL protein in SYPRO orange assay buffer was incubated with a 12-point 2-fold serial dilution series (25 μM maximum concentration of each compound for case 1, 50 μM maximum concentration of each compound for case 2) of relevant compounds for 1 h at room temperature in a 384-well plate. They were then subjected to a temperature ramp from 20 to 95 °C at 2.5 °C/min on a Roche LightCycler 480 II. Fluorescence was measured continuously with excitation at 483 nm and emission detected at 568 nm. The T_m (inflection point) of each melting curve was determined by fitting the temperature versus fluorescence intensity curve to a high order polynomial and finding the local maxima of the first derivative of said polynomial. The average and standard deviation of the protein melting temperature in the absence of the compound was calculated across a minimum of three replicates. The T_m in the presence of the compound was compared to the T_m measured in the absence of the compound (T_m, apo). A compound was considered to have evidence of binding if it caused a dose-dependent shift in the melting temperature greater than three standard deviations of the apo melting temperature for at least two tested compound doses.

Microscale Thermophoresis Assays. Purified protein of interest was diluted to 1 μM in MST labeling Buffer (25 mM HEPES pH 7.5, 1 mM $MgCl_2$, 12.5 mM NaCl, 250 nM second-generation Red-Tris NTA dye (Nanotemper)) and incubated at room temperature for 30 min. It was then centrifuged for 15,000 rcf for 10 min at 4 °C. The supernatant was diluted 2-fold into a 16-point dilution series (2-fold serial dilution series with 200 μM maximum concentration of each compound such that, after protein addition, the maximum tested compound concentration was 100 μM) of the compound in MST buffer (25 mM HEPES pH 7.5, 1 mM $MgCl_2$, 12.5 mM NaCl). Protein was incubated with the compound for 30 min at room temperature, and then MST was measured using premium MST capillaries and a Monolith NT.115 with 40% MST power. A small number of data points had abnormally behaving capillary scans and were excluded from data analysis. MST was analyzed at the 1.5 s on-time. Compounds were denoted binders if the dose−response signal-to-noise ratio was >6 as calculated using MO.Affinity Analysis v2.3.

Off-DNA Assay for Case 1. Compounds were tested off-DNA using a FRET-based displacement assay. 1 nM Eu-anti tag antibody was incubated with 1 nM tagged protein and 1 nM fluorescently labeled tracer compound for 15 min to allow formation of a tracer:protein:antibody ternary complex. This was then added to a plate containing a dilution series of the test compounds. Plates were centrifuged at 500 rpm for 1 min and incubated for 60 min, and the trFRET signal was measured. Compounds were deemed binders if they were able to displace the fluorescent tracer from the protein, as measured by a decrease in the trFRET signal.

Activity Assay for Case 2. The protein tested in case 2 had catalytic activity that could be monitored using an ADP-Glo assay (Promega). Purified protein was diluted to 400 nM in assay buffer (50 mM Tris pH 7.5, 25 mM NaCl, 10 mM $MgCl_2$, and 0.02% Tween-20). 2.5 μL diluted protein was mixed with 0.5 μL of a 12-point 2-fold serial dilution series of the compound (compound dilution series was prepared in assay buffer, prepared such that the maximum tested concentration of each compound would be 50 μM). After 30 min of incubation at room temperature, 2 μL 450 μM ATP was added to each well. The reactions were allowed to incubate at room temperature for 2 h, after which 5 μL ADP-Glo reagent 1 was added to each assay well. This was allowed to incubate for a further 40 min, after which 10 μL ADP-Glo reagent 2 was added to all wells. This was incubated for 40 min, during which 20 μL of the reaction mix was transferred into a 384-well white plate. After the incubation was complete, luminescence was read with a 1 s integration time using a Tecan Spark plate reader. Compounds were called active if they caused a significant change in protein activity.

On-DNA Surface Plasmon Resonance. Experiments were performed using a Carterra LSA^{XT} (Carterra-bio) and an SAD200M sensor chip (Carterra-bio). 50 mM Tris (pH 7.5), 125 mM NaCl, 5 mM $MgCl_2$, 1 mg/mL ovalbumin, and 0.02% Tween-20 was used as the running buffer. /5BiotinTEG/CGAGATACCGCTCTTCC-GATC was used as the capture oligonucleotide. The chip was first conditioned with a 1 min pulse of 25 mM NaOH + 1 M NaCl followed by a 1 min pulse of 10 mM glycine pH 1.5 using the single-channel flow cell. The capture oligonucleotide was then captured on the chip by flowing 2 μM oligo in buffer for 30 min followed by a 1 min wash using the single-channel flow cell. The on-DNA compounds prepared as described above were then captured on the chip in duplicate prints using the 96-channel flow cell, each involving a 20 min capture followed by 30 s buffer baseline. Depending on the number of compounds being tested at a time, between 4 and 12 concentrations of on-DNA compound were tested (typically ranging between 1000 nM compound and 1 nM compound). Acylated headpiece (meaning, on-DNA acetamide ligated to the SPR capture strand as described above was always included as a negative control condition. Compound immobilization was followed by 10 blank injections using the single-channel flow cell. Protein samples were run as single-cycle kinetics with an 8-point 1:1 dilution series of protein, with a highest concentration of 1 μM protein. Protein runs had a 5 min association followed by 10 min dissociation using the single-channel flow cell, with 0.25 mg/mL streptavidin supplemented into the association phase buffer. Affinities were calculated using kinetic fits after double referencing.

Off-DNA Affinities for Case 2. Experiments were performed using a Biacore T200 (GE Healthcare) and a NiHC100M sensor chip (Xantec Bioanalytics) and 1× HBS-P+ pH 7.5 (GE Healthcare) supplemented with 0.5 mM TCEP and 1% v/v DMSO as the running buffer. The chip was regenerated followed by protein capture, startup, and solvent correction after every 44 cycles of kinetics. Positive controls were run over the chip after every 22 cycles of kinetics. Regeneration was performed using 2× 2 min pulses of 0.35 M EDTA at a flow rate of 30 μL/min followed by 2× 2 min pulses of 0.05 M NaOH at a flow rate of 30 μL/min. Capture consisted of a 60 s flow of 10 μL/min of 0.5 mM $NiCl_2$ across the entire chip followed by 3 min contact of 10 μg/mL protein flowed at 5 μL/min. Note, spot 1 was left empty, and negative control proteins were immobilized on

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spots 2 and 4. Startup was 2× 1 min pulses of running buffer at a flow rate of 30 μL/min. Samples were run with 80 s association time followed by 6 min dissociation times, all at a flow rate of 30 μL/min. Affinities were calculated using both steady-state and kinetic fits.

Compound Purity Effects on on-DNA SPR Response. The affinity of the positive and negative control compounds for the target was determined using MST using methods similar to those described above, except with the following modifications: the assay buffer was 50 mM Tris pH 8, 100 mM NaCl, 1 mM CHAPS, and 1 mM DTT, and labeling was conducted using 100 nM protein (such that the final protein concentration in the assay would be 50 nM) and 50 nM Red-Tris NTA second-generation dye (such that the final dye concentration would be 25 nM), and the highest concentration of the compound tested was 20 μM.

The on-DNA SPR was conducted similarly as described in the "On-DNA Surface Plasmon Resonance" section except with the following modifications: the running buffer was 50 mM Tris pH 8, 100 mM NaCl, 1 mM CHAPS, 1 mM DTT, and 1 mg/mL ovalbumin, and the association phase buffer was identical to the running buffer. For the purity titration experiments, a dilution series was prepared by diluting the ligated positive-control compound 1:1 into the ligated negative-control compound, such that the overall compound concentration was constant at 4 μM but the percentage of the active compound ranged from 100% to 0.1%. We then formed an 8-point 1:1 dilution series of this such that the overall compound concentration varied between 4 μM and 31 nM, in net creating a grid of 100−0.1% pure compounds at overall concentrations of 4 μM to 31 nM. This matrix was immobilized in duplicate using the 96-channel printhead, and the rest of the experiment was conducted as described in the "On-DNA Surface Plasmon Resonance" section.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.5c01885.

Supplementary figures, general experimental, and additional discussion (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Mark A. Mantell − *Encoded Technologies, Molecular Modalities Discovery, GSK, Cambridge, Massachusetts 02140, United States;* orcid.org/0000-0003-1549-865X; Email: mark.x.mantell@gsk.com

Karanbir S. Pahil − *Encoded Technologies, Molecular Modalities Discovery, GSK, Cambridge, Massachusetts 02140, United States;* orcid.org/0000-0003-0247-5934; Email: Karanbir.s.pahil@gsk.com

Authors

Karli R. Holman − *Encoded Technologies, Molecular Modalities Discovery, GSK, Cambridge, Massachusetts 02140, United States;* orcid.org/0000-0001-6424-9479

Joshua D. Alper − *Encoded Technologies, Molecular Modalities Discovery, GSK, Cambridge, Massachusetts 02140, United States*

Chloe M. Jones − *Encoded Technologies, Molecular Modalities Discovery, GSK, Cambridge, Massachusetts 02140, United States*

Gang Yao − *Encoded Technologies, Molecular Modalities Discovery, GSK, Cambridge, Massachusetts 02140, United States*

Lisa A. Marcaurelle − *Encoded Technologies, Molecular Modalities Discovery, GSK, Cambridge, Massachusetts 02140, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jmedchem.5c01885

Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare the following competing financial interest(s): All authors are current or former employees of GSK. Gang Yao is currently employed at Septerna. Joshua Alper is currently employed at Alper Consulting.

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■ ABBREVIATIONS

ASMS, affinity selection mass spectrometry; BALI-MS, bead-assisted ligand isolation mass spectrometry; BET BD2, second bromodomain of the bromodomain and extraterminal domain proteins; BMPR2, bone morphogenetic protein receptor type 2; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DEL, DNA-encoded library; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid; iASMS, immobilized affinity selection mass spectrometry; pK_D, negative log of the dissociation constant; MST, microscale thermophoresis; NTA, nitrilotriacetic acid; ODBC, on-DNA binder confirmation; RIP1K, receptor-interacting serine/threonine kinase 1; SPR, surface plasmon resonance; TCEP, tris(2-carboxyethyl)phosphine

■ REFERENCES

(1) Dragovich, P. S.; Haap, W.; Mulvihill, M. M.; Plancher, J.-M.; Stepan, A. F. Small-Molecule Lead-Finding Trends across the Roche and Genentech Research Organizations. *J. Med. Chem.* **2022**, *65*, 3606−3615.

(2) Foley, T. L.; Burchett, W.; Chen, Q.; Flanagan, M. E.; Kapinos, B.; Li, X.; Montgomery, J. I.; Ratnayake, A. S.; Zhu, H.; Peakman, M.-C. Selecting Approaches for Hit Identification and Increasing Options by Building the Efficient Discovery of Actionable Chemical Matter from DNA-Encoded Libraries. *SLAS Discovery* **2021**, *26*, 263−280.

(3) Goodnow, R. A., Jr.; Demulin, C. E.; Keefe, A. D. DNA-encoded chemistry: enabling the deeper sampling of chemical space. *Nat. Rev. Drug Discovery* **2017**, *16*, 131−147.

(4) Harris, P. A.; King, B. W.; Bandyopadhyay, D.; Berger, S. B.; Campobasso, N.; Capriotti, C. A.; Cox, J. A.; Dare, L.; Dong, X.; Finger, J. N.; Grady, L. C.; Hoffman, S. J.; Jeong, J. U.; Kang, J.; Kasparcova, V.; Lakdawala, A. S.; Lehr, R.; McNulty, D. E.; Nagilla, R.; Ouellette, M. T.; Pao, C. S.; Rendina, A. R.; Schaeffer, M. C.; Summerfield, J. D.; Swift, B. A.; Totoritis, R. D.; Ward, P.; Zhang, A.; Zhang, D.; Marquis, R. W.; Bertin, J.; Gough, P. J. DNA-Encoded Library Screening Identifies Benzo[B][1,4]Oxazepin-4-Ones as Highly Potent and Monoselective Receptor Interacting Protein 1 Kinase Inhibitors. *J. Med. Chem.* **2016**, *59*, 2163−2178.

(5) Harris, P. A.; Berger, S. B.; Jeong, J. U.; Nagilla, R.; Bandyopadhyay, D.; Campobasso, N.; Capriotti, C. A.; Cox, J. A.; Dare, L.; Dong, X.; Eidam, P. M.; Finger, J. N.; Hoffman, S. J.; Kang, J.; Kasparcova, V.; King, B. W.; Lehr, R.; Lan, Y.; Leister, L. K.; Lich, J. D.; MacDonald, T. T.; Miller, N. A.; Ouellette, M. T.; Pao, C. S.; Rahman, A.; Reilly, M. A.; Rendina, A. R.; Rivera, E. J.; Schaeffer, M. C.; Sehon, C. A.; Singhaus, R. R.; Sun, H. H.; Swift, B. A.; Totoritis, R. D.; Vossenkämper, A.; Ward, P.; Wisnoski, D. D.; Zhang, D.; Marquis, R. W.; Gough, P. J.; Bertin, J. Discovery of a First-in-Class Receptor Interacting Protein 1 (RIP1) Kinase Specific Clinical Candidate (GSK2982772) for the Treatment of Inflammatory Diseases. *J. Med. Chem.* **2017**, *60*, 1247−1261.

(6) Modukuri, R. K.; Monsivais, D.; Li, F.; Palaniappan, M.; Bohren, K. M.; Tan, Z.; Ku, A. F.; Wang, Y.; Madasu, C.; Li, J.-Y.; Tang, S.; Miklossy, G.; Palmer, S. S.; Young, D. W.; Matzuk, M. M. Discovery of Highly Potent and BMPR2-Selective Kinase Inhibitors Using DNA-Encoded Chemical Library Screening. *J. Med. Chem.* **2023**, *66*, 2143−2160.

(7) Rianjongdee, F.; Atkinson, S. J.; Chung, C.; Grandi, P.; Gray, J. R. J.; Kaushansky, L. J.; Medeiros, P.; Messenger, C.; Phillipou, A.; Preston, A.; Prinjha, R. K.; Rioja, I.; Satz, A. L.; Taylor, S.; Wall, I. D.; Watson, R. J.; Yao, G.; Demont, E. H. Discovery of a Highly Selective BET BD2 Inhibitor from a DNA-Encoded Library Technology Screening Hit. *J. Med. Chem.* **2021**, *64*, 10806−10833.

(8) Brenner, S.; Lerner, R. A. Encoded combinatorial chemistry. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5381−5383.

(9) Xia, B.; Franklin, J.; Lu, X.; Bedard, K. L.; Grady, L. C.; Summerfield, J. D.; Shi, E. X.; King, B. W.; Lind, K. E.; Chiu, C.; Watts, E.; Bodmer, V.; Bai, X.; Marcaurelle, L. A. DNA-Encoded Library Hit Confirmation: Bridging the Gap Between On-DNA and Off-DNA Chemistry. *ACS Med. Chem. Lett.* **2021**, *12*, 1166−1172.

(10) Wichert, M.; Krall, N.; Decurtins, W.; Franzini, R. M.; Pretto, F.; Schneider, P.; Neri, D.; Scheuermann, J. Dual-Display of small molecules enables the discovery of ligand pairs and facilitates affinity maturation. *Nat. Chem.* **2015**, *7*, 241−249.

(11) Lin, W.; Reddavide, F. V.; Uzunova, V.; Gür, F. N.; Zhang, Y. Characterization of DNA-Conjugated Compounds Using a Regenerable Chip. *Anal. Chem.* **2015**, *87*, 864−868.

(12) Su, W.; Ge, R.; Ding, D.; Chen, W.; Wang, W.; Yan, H.; Wang, W.; Yuan, Y.; Liu, H.; Zhang, M.; Zhang, J.; Shu, Q.; Satz, A. L.; Kuai, L. Triaging of DNA-Encoded Library Selection Results by High-Throughput Resynthesis of DNA-Conjugate and Affinity Selection Mass Spectrometry. *Bioconjugate Chem.* **2021**, *32*, 1001−1007.

(13) Ratnayake, A. S.; Flanagan, M. E.; Foley, T. L.; Hultgren, S. L.; Bellenger, J.; Montgomery, J. I.; Lall, M. S.; Liu, B.; Ryder, T.; Kölmel, D. K.; Shavnya, A.; Feng, X.; Lefker, B.; Byrnes, L. J.; Sahasrabudhe, P. V.; Farley, K. A.; Chen, S.; Wan, J. Toward the assembly and characterization of an encoded library hit confirmation platform: Bead-Assisted Ligand Isolation Mass Spectrometry (BALI-MS). *Bioorg. Med. Chem.* **2021**, *41*, 116205.

(14) Prati, L.; Bigatti, M.; Donckele, E. J.; Neri, D.; Samain, F. On-DNA hit validation methodologies for ligands identified from DNA-encoded chemical libraries. *Biochem. Biophys. Res. Commun.* **2020**, *533*, 235−240.

(15) Zimmermann, G.; Li, Y.; Rieder, U.; Mattarella, M.; Neri, D.; Scheuermann, J. Hit-Validation Methodologies for Ligands Isolated from DNA-Encoded Chemical Libraries. *ChemBioChem* **2017**, *18*, 853−857.

(16) Bittner, P.; Gloger, A.; Keller, M.; Oehler, S.; Lessing, A.; Zhang, Q.; Ma, Z.; Su, W.; Satz, A. L.; Kuai, L.; Zenobi, R.; et al. Native Mass Spectrometry Facilitates Hit Validation in DNA-Encoded Library Technology. *Angew. Chem., Int. Ed.* **2025**, *64*, No. e202504470.

(17) The results from all compounds in this study are included in the Supporting Information.

(18) Dang, C. V.; Reddy, E. P.; Shokat, K. M.; Soucek, L. Drugging the 'undruggable' cancer targets. *Nat. Rev. Cancer* **2017**, *17*, 502−508.

(19) Xie, X.; Yu, T.; Li, X.; Zhang, N.; Foster, L. J.; Peng, C.; Huang, W.; He, G. Recent advances in targeting the "undruggable" proteins: from drug discovery to clinical trials. *Signal Transduction Targeted Ther.* **2023**, *8*, 335.

(20) Pahil, K.; Gilman, M. S. A.; Baidin, V.; Clairfeuille, T.; Mattei, P.; Bieniossek, C.; Dey, F.; Muri, D.; Baettig, R.; Lobritz, M.; Bradley, K.; Kruse, A. C.; Kahne, D. A new antibiotic traps lipopolysaccharide in its intermembrane transporter. *Nature* **2024**, *625*, 572−577.

(21) Antolin, A. A.; Tym, J. E.; Komianou, A.; Collins, I.; Workman, P.; Al-Lazikani, B. Objective, Quantitative, Data-Driven Assessment of Chemical Probes. *Cell Chem. Biol.* **2018**, *25*, 194−205.

(22) Edwards, A. M.; Isserlin, R.; Bader, G. D.; Frye, S. V.; Willson, T. M.; Yu, F. H. Too many roads not taken. *Nature* **2011**, *470*, 163−165.

(23) Zhang, X.; Cravatt, B. F. Chemical Proteomics-Guided Discovery of Covalent Ligands for Cancer Proteins. *Annu. Rev. Cancer Biol.* **2024**, *8*, 155−175.

https://doi.org/10.1021/acs.jmedchem.5c01885
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