Rapid Elaboration of Fragments into Leads Applied to Bromodomain-3 Extra Terminal Domain

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Abstract

The development of low-affinity fragment hits into higher affinity leads is a major hurdle in fragment-based drug design. Here we demonstrate an approach for the Rapid Elaboration of Fragments into Leads (REFiL) applying an integrated workflow that provides a systematic approach to generate higher-affinity binders without the need for structural information. The workflow involves the selection of commercial analogues of fragment hits to generate preliminary structure-activity relationships. This is followed by parallel microscale chemistry using chemoinformatically designed reagent libraries to rapidly explore chemical diversity. Upon completion of a fragment screen against Bromodomain-3 extra terminal (BRD3-ET) domain we applied the REFiL workflow, which allowed us to develop a series of tetrahydrocarbazole ligands that bind to the peptide binding site of BRD3-ET. With REFiL we were able to rapidly improve binding affinity >30-fold. The REFiL workflow can be applied readily to a broad range of protein targets without the need of a structure, allowing the efficient evolution of low-affinity fragments into higher affinity leads and chemical probes.

Fragment-based drug design (FBDD) has proven to be a successful strategy, with a growing number of approved drugs originating from fragment-based projects.[1-2] The majority of successful FBDD campaigns rely heavily on structural information and in many cases a structure of the initial fragment hit bound to the target protein is used to guide the chemistry.[3-5] In the absence of structural information, key challenges are to identify developable fragments with regions for expansion (vectors) and to quickly find optimal, ligand efficient substituents for those vectors.[6] We set out to develop a systematic method for (a) assessing the development potential of fragment series and (b) efficiently identifying higher affinity compounds, with the restriction that the method should be applicable in the absence of structural information. Herein we demonstrate our integrated fragment screening and development approach for the Rapid Elaboration of Fragments into Leads (REFiL, Figure 1).

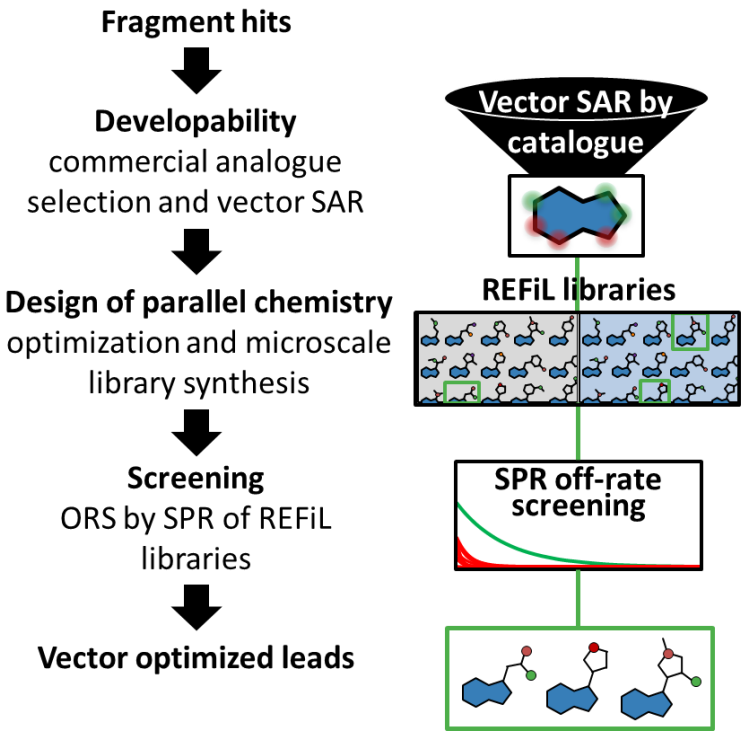


Figure 1. The Rapid Elaboration of Fragments into Leads (REFiL) workflow of fragment optimization. Initially assessing developability and prioritizing vectors for expansion followed by identification of improved affinity analogues through the use of microscale parallel chemistry and off-rate screening (ORS).

After the initial fragment screen, we aim to identify the most promising fragments and vectors for development by screening commercially available analogues with diverse substituents. These analogues are selected to generate SAR for each vector around each fragment hit and assess the ability to expand and develop the fragment series into higher affinity binders. Chemical elaboration at the most promising vectors identified by this process is achieved by using parallel microscale synthesis with robust medicinal chemistry transformations.[7] Parallel synthesis of libraries (REFiL libraries) is undertaken in 96-well plates using reagent sets designed to enable the most efficient coverage of chemical space at

each vector. Minimal purification (evaporation and filtration steps) is then conducted before the binding kinetics of the reaction products are assessed by surface plasmon resonance (SPR) using "off-rate screening" (ORS).[8-9] ORS utilizes the differences in binding kinetics observed for low affinity (predominantly fast off-rate) and high affinity (predominantly slow off-rate) compounds.[8-9] This allows higher affinity reaction products to be identified in the crude reaction sample where the slower off-rate component dominates the dissociation phase of the SPR sensorgram. Since off-rates are independent of the free ligand concentration and given the sensitivity of SPR, the chemistry can be conducted on small scale, and without the need for costly and laborious purification.[8-9] Promising compounds identified by ORS can be resynthesized on batch scale and have their target binding and other properties fully characterized. To demonstrate the utility of this approach, we have successfully developed a series of novel ligands for bromodomain-3 extra terminal domain (BRD3-ET) using REFiL.

The bromodomain and extra-terminal domain (BET) family of proteins, including bromodomain-containing (BRD) 2, 3, 4 and T, are a class of epigenetic regulators that have been implicated in a number of diseases, but most prominently cancer.[10] Despite concentrated efforts in the development of BET bromodomain inhibitors – with a considerable number currently in clinical trials,[11] the biochemical role of the extra terminal domain is not well established.[12] BRD3-ET is known to use a groove on the domain surface to bind short peptide motifs from chromatin regulatory proteins.[12] Development of small molecule probes binding to this site would allow further investigation of the mechanisms by which these domains function. Hence, we set out to develop small molecule inhibitors of the BRD3-ET domain employing fragment-based screening and the REFiL workflow.

Initially the BRD3-ET domain was screened against a library of 1148 fragments in mixtures of 3-5 fragments by saturation transfer difference (STD) NMR spectroscopy.[13] The primary screen gave 70 fragment hits that were validated by recording ^{15}N-^{1}H heteronuclear single quantum coherence (HSQC) spectra of ^{15}N-labelled BRD3-ET. Spectra of BRD3-ET (85 μM) were recorded in the absence and presence of each fragment hit (at 1 mM) to identify fragments that cause chemical shift perturbations (CSPs). Twelve fragments showed small CSPs, indicative of low-affinity binding; however, tetrahydrocarbazole fragment **1** gave significantly larger CSPs. Titration of fragment **1** revealed concentration-dependent CSPs, which could be fit to a one-site binding model with a dissociation constant (K_D) of 0.23 ± 0.04 mM (Figure 2a-c). The residues perturbed (for example Q600, E619, D608, D612, Figure 2c) are consistent with fragment **1** binding to the peptide binding site of BRD3-ET.[12]

Commercially available analogues of tetrahydrocarbazole **1** were then selected to assess the developability of the fragment and to identify the most promising vectors for expansion. A chemoinformatic workflow that enabled a customized R-group decomposition of potential analogues while allowing changes to the core and existing functional groups of tetrahydrocarbazole **1** was used. This generated a set of substituents, which were ranked by properties including size, complexity, polarity and pharmacophore diversity.[14] Analogues were selected to probe multiple different vectors, one at a time, for novel interactions with BRD3-ET, and to assess the ability to expand the fragment series at that vector. In total, a series of 16

analogues were purchased for tetrahydrocarbazole **1** (see Figure 2a for a summary and Figure S1 for a full list of analogues).

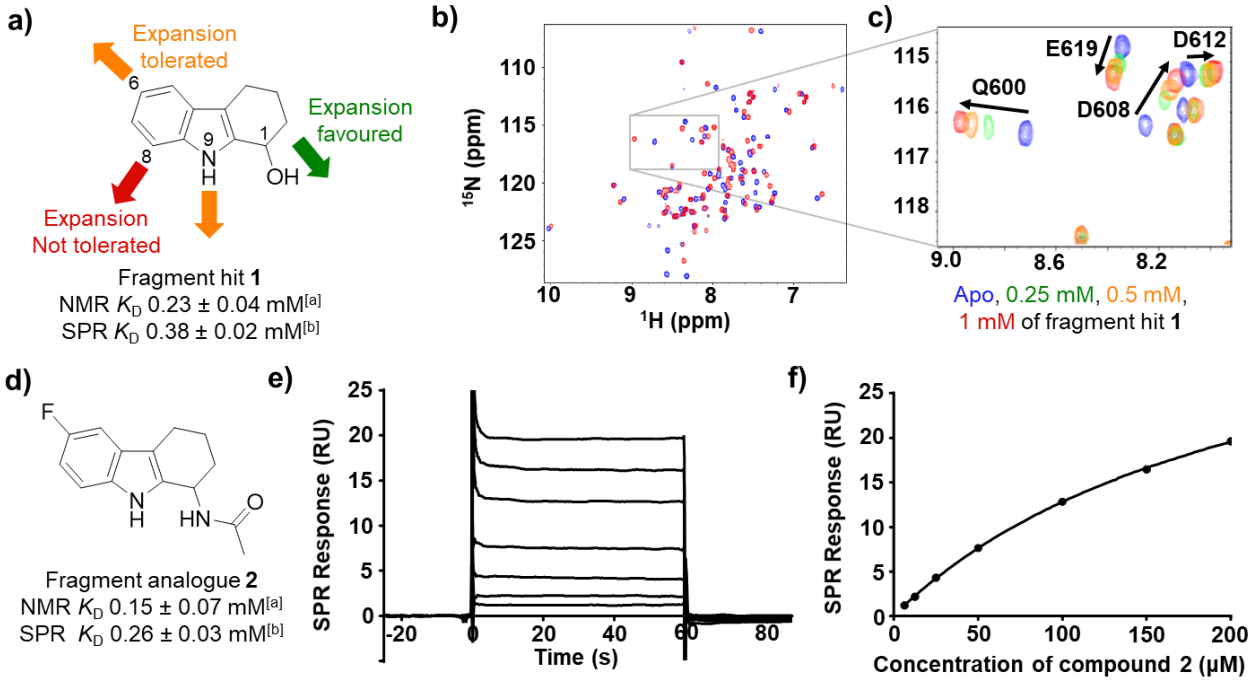
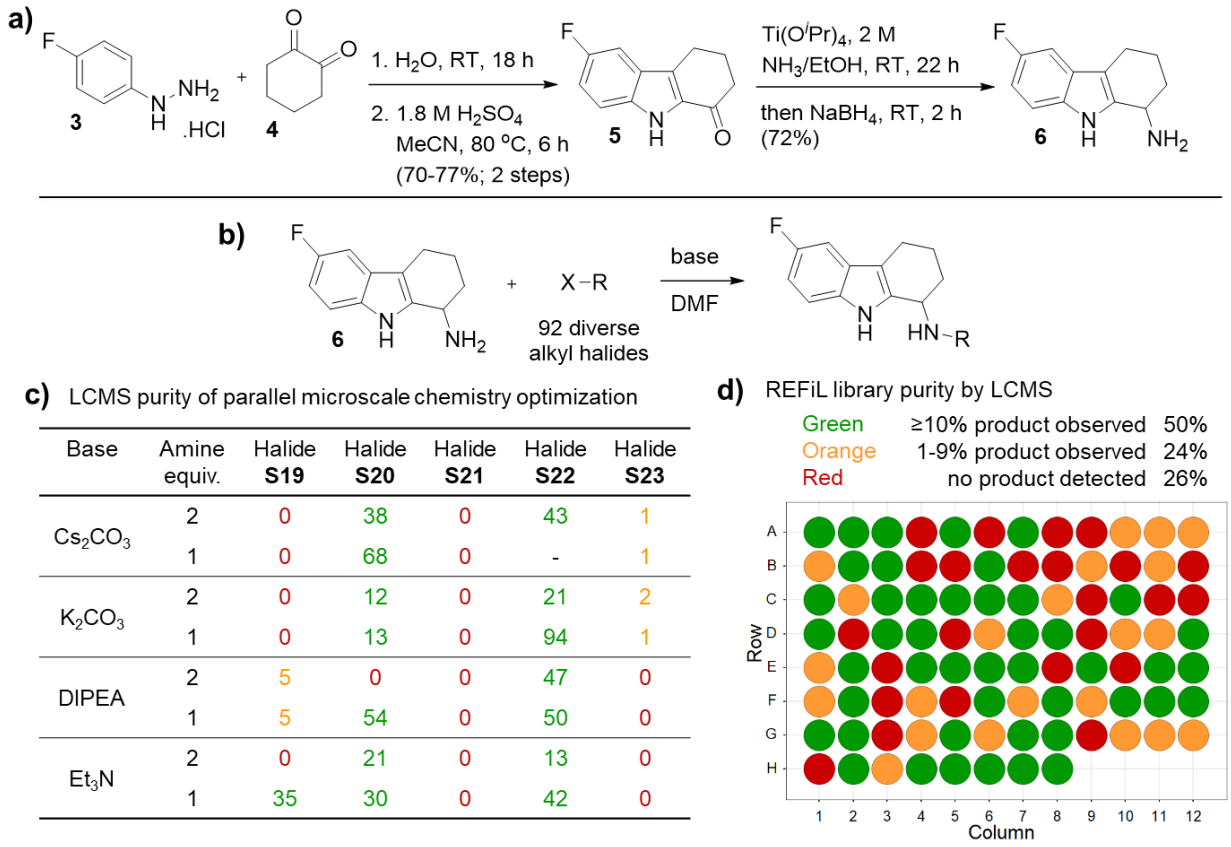


Figure 2. Fragment hit **1** and analogue **2** binding to BRD3-ET. a) Structure of fragment hit **1** with arrows indicating analogues that expanded the structure at that vector were favored (green), tolerated (orange) or not tolerated (red) for binding to BRD3-ET. b) Overlay of ^{15}N-^{1}H HSQC spectra of ^{15}N BRD3-ET (85 μM) without (blue) and with (red) tetrahydrocarbazole **1** at 1 mM. c) Expansion of ^{1}H-^{15}N HSQC spectra showing signals corresponding to residues near the peptide binding site at increasing concentrations of fragment hit **1**: blue (no ligand), green (0.25 mM), orange (0.5 mM), red (1 mM). Fitting of the CSPs yielded a K_D of 0.23 ± 0.04 mM. d) Structure and affinity of fragment analogue **2**. e) Raw SPR sensorgrams for binding of analogue **2** at 200, 150, 100, 50, 25, 12.5, 6.25 μM. f) Equilibrium binding curve derived from the data in panel e fit to a 1:1 binding model. [a] K_D is the average ± standard deviation (SD) of the K_D values calculated from fitting 4 different peak shifts in n = 1 set of titration experiments. [b] Dissociation constant from equilibrium fitting method. Errors shown are SE in the fit to the data of a single experiment.

These analogues were screened by ^{15}N-^{1}H HSQC NMR spectroscopy and the resulting structure-activity relationships (SAR) indicated that expansion was favored at position 1 and that substituents were tolerated at the 6- and 9-position of the tetrahydrocarbazole (Figures 2a and S1). Analogues showing strong CSPs were further characterized by ^{15}N-^{1}H HSQC ligand titrations, which gave K_D values in the range of 0.15–0.99 mM. The highest affinity analogue, acetamide **2** (Figure 2d), was then examined by SPR, which confirmed binding. The equilibrium response recorded in the SPR sensorgrams for **2** could be fit to a one-site binding model with a K_D = 0.26 ± 0.03 mM (Figure 2e-f). The 1-position of the tetrahydrocarbaxozole **1** was therefore chosen for the design of two REFiL libraries. The first REFiL library was based on alkylation of amine **6** (Figure 3) (described below) and the second was based on amidation

of a tetrahydrocarbazole carboxylic acid at the same position (described in supporting information (SI)).

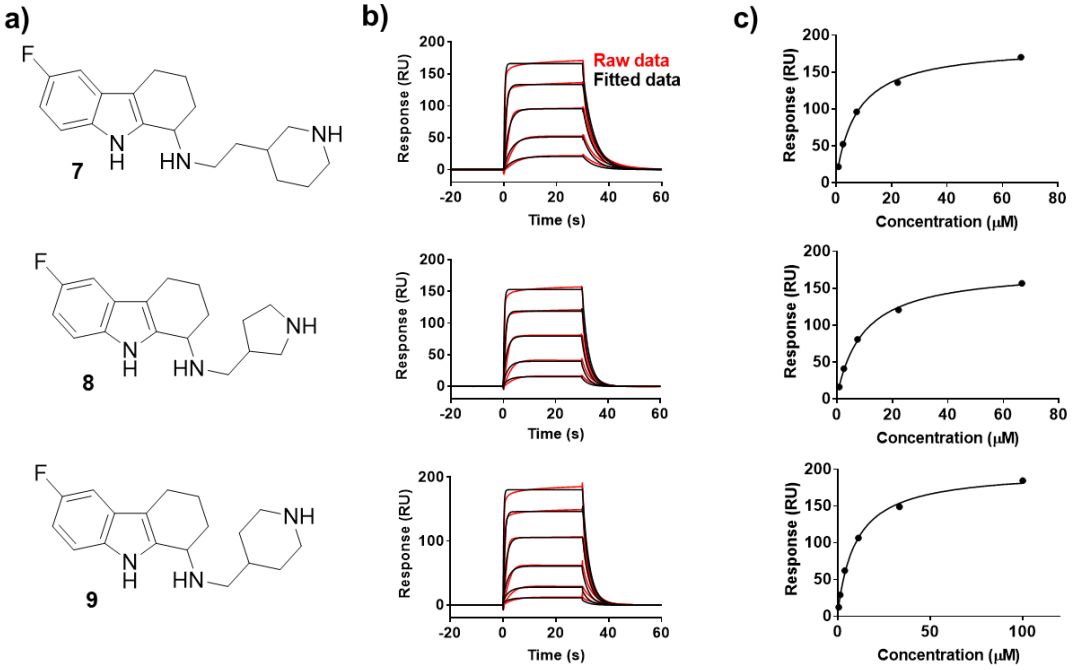


Base	Amine equiv.	Halide **S19**	Halide **S20**	Halide **S21**	Halide **S22**	Halide **S23**
Cs_2CO_3	2	0	38	0	43	1
	1	0	68	0	-	1
K_2CO_3	2	0	12	0	21	2
	1	0	13	0	94	1
DIPEA	2	5	0	0	47	0
	1	5	54	0	50	0
Et_3N	2	0	21	0	13	0
	1	35	30	0	42	0

Figure 3. Synthesis of precursors and parallel microscale REFiL library synthesis. a) Synthetic route to amine **6**. b) Alkylation of amine **6** used in the synthesis of the alkylation REFiL library. c) Reaction optimization matrix with purity values derived from LCMS % area of peaks in the 254 nm UV/Vis trace that contain the parent ion of the expected product. Reactions performed at 100 mM concentration and 5 µmol scale with respect to amine **6**. d) LCMS analysis of the alkylation REFiL library. Reactions were carried out using 1 molar equiv. of amine **6** and triethylamine as base. Plate wells are colored according to the measured LCMS purity of the desired product, ≥10% (green), 1-9% (orange) and no product detected (red).

Amine **6** was prepared *via* Fischer indole synthesis with hydrazine **3** and diketone **4** to give ketocarbazole **5**, followed by reductive amination (Figure 3a). Microscale reaction condition optimization was then undertaken with amine **6** in a matrix of 40 microscale reactions. These were designed to optimize the base used and the ratio of amine to halide against a set of halides with different reactivities (Figures 3c and S3). The most robust conditions were selected based on the highest and most consistent yields across the halide series. These conditions were then employed to synthesize a REFiL alkylation library (Figure 3b-d) using amine **6** and an internal set of 92 diverse alkyl halide reagents (Table S1). The 92 diverse halides were designed to have a common reactive handle, either alkyl chloride or bromide but with highly diverse structures that were chemoinformatically selected to maximize coverage of chemical space. 22 of the halides in the reagent set deliberately contained

protecting groups and these were reacted under the same conditions before undergoing either acid or base mediated deprotection (See SI for full details). The reactions were then evaporated and taken up in DMSO. The DMSO stocks were prepared at a notional concentration of 100 mM assuming 100% reaction conversion. Evaluation of the library by LCMS indicated 50% of reactions yielded product at ≥10% purity and a further 24% had evidence of product at <10% purity. Faux reactions containing the amine **6**, with and without base, in the absence of halide were also run simultaneously to act as controls during ORS by assessing interference in the SPR experiments caused by the reagents or background reactions. The REFiL library DMSO stocks were then further diluted to a notional concentration of 20 mM in DMSO to account for any compound insolubility and then diluted 100-fold into buffer (*i.e.*, to a notional concentration of 200 µM) and screened by SPR. A concentration of 200 µM was chosen to maximize potential slow off-rate signal while avoiding potential artefacts in SPR.[15] The dissociation phase of each sensorgram was fit to a single exponential 1:1 ligand dissociation model and hits were selected for further investigation based on strict criteria for the quality of the fit and measured off-rate (full details in the SI). For the alkylation REFiL library three reactions were identified as ORS hits and the alkylation products were resynthesized and purified on milligram scale (Figure 4 and Schemes S1-S2).



Compound	SPR			
	ORS k_{off} (s^{-1})[a]	Resynthesized k_{off} (s^{-1})[b]	Resynthesized K_D (µM)[b,c]	LE[d]
7	0.28 ± 0.002	0.28 ± 0.04	7.8 ± 0.8	0.30
8	0.22 ± 0.02	0.33 ± 0.03	11 ± 2	0.32
9	0.30 ± 0.002	0.49 ± 0.04	17 ± 6	0.30

Figure 4. Structure and SPR analysis of selected ligands with measured dissociation rate constants. a) Ligand structures. b) Raw and fitted SPR sensorgrams of ligand binding to BRD3-ET and fits to kinetics model and c) Dose response SPR equilibrium response plot with 1:1 binding model fit. [a] Errors shown are standard error of fit of a single experiment. [b] Errors shown are SD of n = 3 (compound **7**), n = 6 (compound **8**) and n = 7 (compound **9**) independent experiments. [c] Dissociation constant from equilibrium fitting method. [d] LE units are kcal.mol^{-1}.heavy atom^{-1}.

These resynthesized samples were characterized by SPR for binding to BRD3-ET and good correlation between the fitted off-rate (k_{off}) values in the minimally purified REFiL library samples and the purified resynthesized samples was observed (Figures 4 and S4). All ORS hits identified had improved binding affinity for BRD3-ET compared to acetamide **2** (Figures 2d, 4 and S4) with alkylation REFiL library hits **7-9** having K_D values of 8-17 μM by SPR. The improvement in affinity for analogues **7-9** corresponds to a 15 to 33-fold improvement in binding over acetamide **2** (260 μM by SPR), whilst maintaining a ligand efficiency (LE) of between 0.30 and 0.32 kcal.mol^{-1}.heavy atom^{-1}. The REFiL strategy allowed this significant improvement in affinity (33-fold) while maintaining ligand efficiency to be achieved from the batch synthesis of only four analogues that followed the ORS screening of two REFiL libraries.

Medicinal chemistry can be a major bottleneck for fragment-based drug discovery projects requiring design, synthesis and purification of a significant number of compounds for screening. The process described here enabled the rapid evaluation of two chemically diverse libraries, totaling 185 microscale reactions, without the need for lengthy purification. This screening led to the synthesis and purification of four compounds on milligram scale, three of which had significantly improved affinity for the target. The starting point for this implementation of the REFiL workflow was a moderate affinity fragment hit ($K_D \approx 230$ μM) and a SPR binding assay setup. The fragment hit was then quickly elaborated using REFiL to improve its affinity whilst maintaining ligand efficiency. The REFiL process is potentially applicable to a wide range of targets and provides a systematic and efficient workflow to progress fragment hits to higher affinity leads. The analogues described here may prove useful as chemical probes to further elucidate the function of the BRD3-ET domain. They may also provide a starting point for the development of molecules that complement the BET bromodomain inhibitors that are currently under development in several therapeutic contexts.

Experimental Section

Protein production, screening methods, list of analogues purchased and reagent libraries used, synthetic methods, amide library synthesis, along with ligand characterized binding to BRD3-ET are given in the supporting information.

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Conflict of Interest

The authors declare no competing financial interest.

References

(1) Ferenczy, G. G.; Keseru, G. M. How are fragments optimized? A retrospective analysis of 145 fragment optimizations. *J. Med. Chem.* **2013**, *56* (6), 2478-2486.

(2) Erlanson, D. A.; Fesik, S. W.; Hubbard, R. E.; Jahnke, W.; Jhoti, H. Twenty years on: the impact of fragments on drug discovery. *Nat. Rev. Drug Discovery* **2016**, *15* (9), 605-619.

(3) Mortenson, P. N.; Erlanson, D. A.; de Esch, I. J. P.; Jahnke, W.; Johnson, C. N. Fragment-to-Lead Medicinal Chemistry Publications in 2017. *J. Med. Chem.* **2019**, *62* (8), 3857-3872.

(4) Johnson, C. N.; Erlanson, D. A.; Jahnke, W.; Mortenson, P. N.; Rees, D. C. Fragment-to-Lead Medicinal Chemistry Publications in 2016. *J. Med. Chem.* **2018**, *61* (5), 1774-1784.

(5) Adams, L. A.; Sharma, P.; Mohanty, B.; Ilyichova, O. V.; Mulcair, M. D.; Williams, M. L.; Gleeson, E. C.; Totsika, M.; Doak, B. C.; Caria, S.; Rimmer, K.; Horne, J.; Shouldice, S. R.; Vazirani, M.; Headey, S. J.; Plumb, B. R.; Martin, J. L.; Heras, B.; Simpson, J. S.; Scanlon, M. J. Application of fragment-based screening to the design of inhibitors of Escherichia coli DsbA. *Angew. Chem. Int. Ed.* **2015**, *54* (7), 2179-2184.

(6) Murray, C. W.; Rees, D. C. Opportunity Knocks: Organic Chemistry for Fragment-Based Drug Discovery (FBDD). *Angew. Chem. Int. Ed.* **2016**, *55*, 488-492.

(7) Roughley, S. D.; Jordan, A. M. The medicinal chemist's toolbox: an analysis of reactions used in the pursuit of drug candidates. *J. Med. Chem.* **2011**, *54* (10), 3451-3479.

(8) Murray, J. B.; Roughley, S. D.; Matassova, N.; Brough, P. A. Off-rate screening (ORS) by surface plasmon resonance. An efficient method to kinetically sample hit to lead chemical space from unpurified reaction products. *J. Med. Chem.* **2014**, *57* (7), 2845-2850.

(9) Brough, P. A.; Baker, L.; Bedford, S.; Brown, K.; Chavda, S.; Chell, V.; D'Alessandro, J.; Davies, N. G.; Davis, B.; Le Strat, L.; Macias, A. T.; Maddox, D.; Mahon, P. C.; Massey, A. J.; Matassova, N.; McKenna, S.; Meissner, J. W.; Moore, J. D.; Murray, J. B.; Northfield, C. J.; Parry, C.; Parsons, R.; Roughley, S. D.; Shaw, T.; Simmonite, H.; Stokes, S.; Surgenor, A.; Stefaniak, E.; Robertson, A.; Wang, Y.; Webb, P.; Whitehead, N.; Wood, M. Application of Off-Rate Screening in the Identification of Novel Pan-Isoform Inhibitors of Pyruvate Dehydrogenase Kinase. *J. Med. Chem.* **2017**, *60* (6), 2271-2286.

(10) Xu, Y.; Vakoc, C. R. Targeting Cancer Cells with BET Bromodomain Inhibitors. *Cold Spring Harb. Perspect. Med.* **2017**, *7* (7), a026674.

(11) Perez-Salvia, M.; Esteller, M. Bromodomain inhibitors and cancer therapy: From structures to applications. *Epigenetics* **2017**, *12* (5), 323-339.

(12) Wai, D. C.; Szyszka, T. N.; Campbell, A. E.; Kwong, C.; Wilkinson-White, L. E.; Silva, A. P. G.; Low, J. K. K.; Kwan, A. H.; Gamsjaeger, R.; Chalmers, J. D.; Patrick, W. M.; Lu, B.; Vakoc, C. R.; Blobel, G. A.; Mackay, J. P. The BRD3 ET domain recognizes a short peptide motif through a mechanism that is conserved across chromatin remodelers and transcriptional regulators. *J. Biol. Chem.* **2018**, *293* (19), 7160-7175.

(13) Mayer, M.; Meyer, B. Characterization of Ligand Binding by Saturation Transfer Difference NMR Spectroscopy. *Angew. Chem. Int. Ed.* **1999**, *38* (12), 1784-1788.

(14) Allu, T. K.; Oprea, T. I. Rapid evaluation of synthetic and molecular complexity for in silico chemistry. *J. Chem. Inf. Model.* **2005**, *45* (5), 1237-1243.

(15) Giannetti, A. M. From experimental design to validated hits a comprehensive walk-through of fragment lead identification using surface plasmon resonance. *Methods in enzymology* **2011**, *493*, 169-218.