Protein-protein interaction inhibition (2P2I)-oriented chemical library accelerates hit discovery

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ABSTRACT

Protein–Protein Interactions (PPIs) represent an enormous source of opportunity for therapeutic intervention. We (and others) have recently pinpointed key rules that will help identifying the next generation of innovative drugs to tackle this challenging class of targets within the next decade. We used these rules to design an oriented chemical library corresponding to a set of diverse 'PPI-like' modulators with cores identified as privileged structures in therapeutics. In this work, the resulting 1664 structurally diverse compounds were purchased, stored in 384-well plates and evaluated on a series of representative protein-protein interfaces with distinct "druggability" potential using the Homogeneous Time-Resolved Fluorescence technology (HTRF®). For certain PPI classes, analysis of the hit rates revealed enrichment factors of up to 100 when compared to non-oriented chemical libraries. This observation correlates with the predicted "druggability" of the targets. A specific focus on selectivity profiles, 3-Dimensional molecular mode of action resolved by X-ray crystallography, and biological activities of identified hits targeting the well-defined "druggable" bromodomains of the BET family is presented as a proof-of-concept. Overall, our present study illustrates the potency of vector machine-oriented chemical library to accelerate the identification of hits targeting PPIs. A generalization of this method to larger set of compounds will allow a genuine acceleration of the discovery of original and potent probes for this challenging class of target.

INTRODUCTION

The modulation of protein-protein complexes using small molecule compounds is getting more and more popular for the development of probes that can lead to new therapeutic drugs or original tools to decipher biological processes, particularly because of the multiple and complex implication of protein-protein interactions (PPIs) in cellular signaling networks [1–5]. Over the last 15 years, the number of success stories has grown considerably. There are currently thousands of small molecule compounds targeting more than fifty protein-protein complexes (please refer to http://www.ippidb.cdithem.fr/ [6,7], https://www.ebi.ac.uk/chembl/ [8], or http://mordred.bioc.cam.ac.uk/timbal [9,10] for more details). As a consequence, a wealth of information is available to define, on the one hand, what makes a protein-protein interaction a good target for drug discovery (referred as the 'druggability' problem), and on the other hand to characterize a potential profile of PPI modulators. Despite being a popular strategy both in academic labs and in pharmaceutical companies, experimental screening of medium or large diverse collections of compounds has delivered lower hit rates than expected when targeting protein-protein complexes [11–14]. One of the reasons for these low success rates is

thought to result from the inadequacy of the libraries used to find the 'hidden gem'. It is therefore crucial to improve the quality and adequacy of the chemical libraries that are used to search for active PPI compounds to improve the success rates and to lower the costs of experimental screening campaigns. To this end, we have recently developed $2P2I_{DB}$, a hand-curated structural database dedicated to PPI with known orthosteric inhibitors ([15–17]; http://2p2idb.cnrs-mrs.fr). Analysis of the small molecule inhibitors present in $2P2I_{DB}$ led us to propose the 'rule-of-four' as a guideline to characterize PPI inhibitors [18]. Using dedicated support vector machine approaches, we have developed $2P2I_{HUNTER}$, a tool that allows potential orthosteric PPI modulators to be filtered from large collections of compounds [19]. This innovative tool has been applied to a set of 8.3 million compounds from the "big vendors" to design several *in silico* PPI-oriented chemical libraries [20]. Compounds corresponding to medicinally important privileged structures identified as core structures in therapeutics (*2,3*) were prioritized in a medicinally oriented version of the library, $2P2I_{PRIV}$. The library was subsequently filtered for diversity and with carbon bond saturation index (Fsp3) to escape from flatland, which resulted in a structurally diverse and medicinally oriented chemical library, $2P2I_{3D}$, made of 1664 compounds [20]. Interestingly, these compounds retain reasonable drug-like properties including Lipinski 'rule of five', Veber's rules and other oral bioavailability rules (please refer to [5] for more details). In the present study, the ability of this PPI-oriented library to enhance hit rates in screening campaigns was experimentally evaluated using the Homogeneous Time-Resolved Fluorescence technology (HTRF®), an established screening technology. Each molecule of the chemical library was evaluated against a representative set of protein-protein interfaces. This set includes: i) a protein-protein large-sized globular interface (Dengue virus NS3/NS5 PPI complex) - representing the globular, type I protein-protein interface, as defined by [15], ii) a protein-domain medium-sized interface (the HIV virus Nef protein in complex with the SH3 domain from the Hck kinase) type I protein/domain interface (with several screens already referenced in Pubchem and Pubmed [23–25], iii) a protein-peptide small-sized interface (a bromodomain in complex with its acetylated peptide) - representing a 'druggable' protein/peptide interface as exemplified by several compounds in preclinical and clinical development [26,27] and iv) two protein-peptide small-sized interfaces (2 PDZ domain-containing proteins in complex with their cognate peptides) - representing in this study 'poorly druggable' or challenging protein-peptide interfaces [28,29]. The physicochemical properties of the validated hits are presented and discussed as key factors to accelerate the identification of hits targeting PPIs. As an example, selectivity profiles, 3-Dimensional molecular mode of action and biological activities of original validated hits identified from the primary screening targeting the bromodomains of BET family members are revealed. The present work using our $2P2I_{3D}$ 'PPI-oriented' chemical library consolidates the proof-of-concept that the design of oriented chemical library

through vector machine-based approaches will allow a significant acceleration of probes identification targeting PPIs.

RESULTS and DISCUSSION

In vitro (HTRF) evaluation of the '2P2I$_{3D}$' library

The autofluorescence of each compound of the library were evaluated before screening on a representative set of protein-protein interfaces. When used at 20 µM, we observed that 3.2% and 2.3% of the compounds were significantly autofluorescent at 665 and 620 nm, the HTRF detection wavelengths, respectively (Fig. S1). This rate may be higher than the one obtained with classic 'non-oriented' chemical libraries but remains low, and thus compatible with HTRF experiments.

In addition to the ability of HTRF to limit compounds interferences in HTS [30], we also took advantage of its flexibility in the design of screening assays. Since this technology can accommodate the use of a large diversity of tag/anti-tag reagents, the design of robust assays to screen the 2P2I$_{3D}$ library was noticeably simplified and we could establish robust assays on the four representative protein-protein interfaces (Fig. S1*a* and Table 1). Of note, each complex has been primarily validated with a competition, when available, in presence of the untagged protein partner (refer to Line 3, Table 1 for the validated IC$_{50}$'s) or in presence of a positive control (*i.e* JQ1, a well known probe for the BRD4/Acetylated peptide complex highlighted a similar IC$_{50}$ for BRD4(BD1), 100nM, as compared to value identified in the literature, 76.9nM – (http://www.thesgc.org/chemical-probes/JQ1).

The primary screening of our 2P2I$_{3D}$ library was then performed at 20 µM against each of the above-described PPIs. Compounds exceeding 40% of the HTRF signal inhibition were then tested in a counter screen assay set-up with fusion protein composed only of the tags used to link the proteins of interest with the HTRF probes (for example GST-biotin or GST-His, Fig. S1*a*). This counter screen was used to eliminate compounds exhibiting a non-specific inhibition profile for the two proteins of interest. Compounds showing less than 15% inhibition in the counter-screen assay were then confirmed as potential inhibitors in a secondary screening assay. Finally, the IC$_{50}$ of the confirmed compounds was precisely determined using fresh powder reordered to the corresponding provider and tested in presence of diverse buffers, including BSA and DTT or tween to prevent false positives selection (please refer to Fig. S2 for more details regarding the screening cascade).

The Z'-factors of the primary screens measured for these 5 screenings ranged from 0.7 to 0.9 underlining the quality of the screening procedure [31] (Fig. S1*b* and Fig S3). Hit rates, defined as the percentage of compounds reaching pre-established activity criteria after primary and secondary screenings - *i.e.* inhibition higher than 40%, less than 15% inhibition

in counter-screen and dose-response profile in the secondary screening -, after exclusion of the autofluorescent compounds and IC_{50} measured in triplicate, ranged from 0.2% for the challenging PDZ complexes up to 1.6% for the protein-protein complex involving NS3 and NS5 proteins from the Dengue virus (Table 1).

The experimental hit rates measured in the screening campaign presented important enrichment factors (at least 10 to 100x) as compared to non-oriented libraries evaluated against PPI's (4). Interestingly, these primary hit rates also reflect the "druggability" of the targets. We indeed observed a high validated hit rate, 17 hits among which 15 are unique in this evaluation study (*i.e* 1% hit rate, at least 10 to 50x compared to non-oriented libraries) when screening the chemical library against the BRD4(1)/Acetylated peptide complex, a very well known 'druggable' protein-peptide complex. In contrast, the hit rate value drops down to 0.2% for the PDZ/C-terminus peptide complexes evaluated here. This PPI interface has been categorized as a challenging PPI interface; several research articles reported on failures to identify validated hits in large HTS campaign involving more than one hundred thousand compounds [28] or to identify very few 'mild' PDZ binders for another HTS program [29]. Our protocol applied on a larger scale, *i.e.* 10,000 to 100,000 compounds would seriously increase chances to find a true probe amenable to medicinal chemistry programs. Finally, the Nef/SH3-Hck complex, a protein/domain complex, has been presented as a potential target in several seminal papers. Among them, Emert-Sedlak *et al.* [24] presented a hit rate of 0.01% (3 different libraries from ChemDiv, Inc., were screened at 10 µM) while Breuer *et al.* observed similar ratio screening the LOPAC library [25]. We observed here a hit rate of 0.1% for the same interface, using our $2P2I_{3D}$ library as template and a screening at 20 µM. While it is difficult to calculate the enrichment factor for this complex since these different experiments were not performed in parallel, a 5 to 10 fold enrichment ratio can be predicted.

Besides hit rates, and as already mentioned, most validated hits were specific of the complex of interest and not identified as hit when screened against other PPI complexes. This result demonstrates the quality, diversity and selectivity of our small size chemical library (Table 1, # of unique molecules identified for each complex). It is however reasonable to imagine that these numbers would drop when increasing the number of complexes to be screened. To go further in this analysis, we decided to perform cross-validation experiments of these unique molecules by HTRF (*i.e* on another, PPI complex defined as the 'control' experiment, here the 'BRD4/H4' PPI complex) and by differential scanning fluorimetry (DSF) on the protein supposedly targeted. We reported two different cases according to the studied complexes (Fig 1). First case scenario, for the compounds identified for the PDZ-Grasp55/JamB peptide and for the NS3/NS5 complex: the identified hits did not inhibit the PPI 'control' (Fig 1*a* and *c*), they influenced the DSF curve of the protein target, Grasp55 or NS3 (Fig 1*b* and *d*), including during the experiments recorded in presence of DTT or tween, demonstrating that

these compounds were not acting by promiscuous, aggregation-based inhibition ([32,33]) (Fig 1*b*). Second case scenario, for the two compounds identified for the Nef/SH3-Hck complex, and the one identified for the Syntenin/Syndecan, similar IC_{50} were obtained on the PPI 'control' used for the cross validation, therefore pinpointing a warning on these compounds for biological evaluation, which were not studied in the present work. We thus decided to further investigate some of these unique hits to evaluate more deeply their selectivity profiles and biological activity (please refer to Table 2 for more details regarding chemical structure, IC_{50}'s or provider's information for all unique validated compounds). More particularly, we undertook supplementary orthogonal validation using Isothermal Titration Calorimetry (ITC), X-ray crystallography and cellular evaluation for one target of interest, the bromodomain BRD4/H4 complex.

Focus on one target of interest - Bromodomains:

Inhibitors identified during the screening of the Bromodomain/Acetylated Lysine containing peptide target complex were evaluated more deeply for three main reasons: i) the high hit rate obtained for this complex (1%), ii) its unexpected high specificity (15 out of the 17 compounds were not identified in any of the other PPI complexes evaluated in this program), and iii) Bromodomains recently received a lot of attention as they represent a new class of targets with broad interest in epigenetics and cancer, and large amount of data are thus available in the literature to compare our identified hits.

Bromodomains (BRDs) are protein-protein interaction modules that are selectively recruited by N-acetylated lysine-containing sequences, notably histone tails. For this reason, BRD-containing protein function as 'readers' of histone lysine acetylation, a component of the proposed 'histone code', thereby acting as scaffolds for the recruitment of transcription factors and chromatin organizers required in transcription initiation and elongation. BRD-containing proteins have been involved in the development of a large variety of diseases and their highly 'druggable' acetyl-lysine binding pocket architecture makes them attractive targets for the development of potent and specific inhibitors (please refer to [26] or [27] for more details about current specific inhibitors developed in the clinic). In particular, BRD2, BRD3, BRD4, and the testis-specific BRDT proteins, which form the bromo and extraterminal (BET) sub-family, recently received a lot of attention after the discovery of highly potent and cell-active pan-BET inhibitors. These inhibitors display strong phenotypic effects in a number of cell lines and affecting a range of cancers *in vivo* were developed (for more details please visit the SGC consortium website: http://www.thesgc.org/). The recent developments with this class of targets have raised large interest in the epigenetic community to discover new and potent compounds specific for each of the 61 known modules present in 46 human proteins

(25). The current challenge still lies in the identification of sub-family specific or bromodomain specific modulators. To assess the quality of our established library and screening results, we thus further evaluated the 15 hits targeting the BRD4(1)/acetylated peptide complex in an orthogonal validation assay using ITC and determined their BRD selectivity profiles.

Orthogonal validation and selectivity profile

ITC was chosen for the orthogonal validation since this assay is direct and in solution. Out of the 15 unique HTRF validated hits, we confirmed interaction of 7 molecules with the BRD4 bromodomain with K_D values ranging from 1.8 to 4.7 μM (Table 3). The large overlap observed between the number of hits for the Bromodomain/Acetylated Lysine containing peptide complex and the number of compounds confirmed by the orthogonal assay (ITC) demonstrates the robustness of our HTRF screening procedure (in particular, the use of counter screens assays to reject compounds interfering with the detection system has proven mandatory to limit false positives).

The most potent two compounds (Fig. S4a), a quinolone derivative compound #**1** (CID #20862085), and a triazolopyridazine derivative, compound #**2** (CID #46326697), exhibited ITC thermograms fitted to 1.8 and 2.0 μM K_D respectively (Fig. S4 and Table 2). DSF experiments revealed a ΔT_m of +1.9 °C and +1.5 °C respectively (at 1 : 25 protein : compound excess) (Fig. S4c). Both experiments validated a direct binding of the compounds on the BRD4(1) domain. A selectivity profile of the best two compounds on the BET family members displayed a 'pan-inhibitor' profile with IC_{50}'s ranging from 3.7 to 25 μM for compound #**1** (Fig S5*a* and *c*) and 3.6 to 20 μM for compound #**2** (Fig. S5*b* and *c*). The selectivity window was more than ten times higher when compared to ATAD2, a non-BET family member, thus representing excellent starting points for the development of more potent BET bromodomain leads.

Binding modes determined by X-Ray crystallography

To further demonstrate that our hits recognize their target specifically, we set-up a co-crystallization assay and elucidated the 3D structure for both compounds #**1** (PDB code 5DLZ) and #**2** (PDB code 5DLX) in complex with BRD4(1). BRD4(1) readily crystallized in the presence of the inhibitors, crystallographic data-collection and refinement statistics are described in Table S1. The structures of these complexes with compound #**1** and compound #**2** were solved at 1.7 Å and 1.9 Å resolution, respectively (Fig. 2).

The 2Fo-Fc map at 1σ around compound #**2** is very well defined for the entire compound (Fig. S6). The triazolopyridazinyl moiety of this compound penetrated deeply into the N-acetylated lysine pocket and occupies the same position as the acetyl head group of the

acetylated lysine present in the histone tail peptides and initiates a direct hydrogen bond with the conserved asparagine (Asn140) (Fig. 2*a*). Four water molecules were located at the bottom of the BRD4(1) cavity as seen in the N-acetylated lysine peptide complex structure (PDB ID: 3UVW) as well as other BRD4(1) inhibitor complexes. The water molecule hydrogen bond network contributes to stabilizing the ligand through an additional hydrogen bond (Fig. 2*b*). The N-substituted carbamoylpiperidinyl side chain occupied the same localization as the second acetylated lysine, as observed in the peptide complex (PDB ID: 3UVW), engaging the shelf between the BC loop and the WPF motif composed of the amino acids Trp81, Pro82 and Phe83 (Fig. 2*a* and Fig. S7). The carbonyl group establishes a water bridged hydrogen bound interaction with the NH amide of the gatekeeper residue Ile146, stabilizing the complex. The 2Fo-Fc map at 1σ around the oxoquinolinyl moiety of compound #**1** was very well defined (Fig. S6), while a weaker electron density was observed for the rest of the molecule outside of the N-acetylated binding pocket. Similar to compound #**2**, the oxoquinolinyl ring of the molecule penetrates deeply into the N-acetylated lysine pocket. The four water molecules, which contribute to stabilizing the ligand through an additional hydrogen bond, are also conserved (Fig. 2*c*, 2*d* and Fig. S7).

Cellular evaluation

BETs are transcriptional regulators that control expression of genes that play key regulatory roles in cellular proliferation, cell cycle progression, and apoptosis. BRD4 is known to be a key regulator of cell cycle control and transcriptional elongation of growth-promoting genes, including c-myc and its target genes [35–37]. *C-myc* down-regulation being a unifying, yet not exclusive, mechanistic theme reported for the various developed BET inhibitors.

We thus evaluated the anti-proliferative capacity of compounds #**1** and #**2** and their effect on c-Myc expression level in tumor cell lines. Both compounds #**1** and #**2** reduced Jurkat T cells viability in a dose-dependent manner (Fig. 3*a*), with EC_{50} values of 31 ± 1.9 µM and 21 ± 0.5 µM, respectively. Both compounds also induced dose-dependent down-regulation of c-Myc protein levels at 1, 10 and 50 µM, as seen by immunoblotting (Fig. 3*b*), and at a similar effective dose that has been found in cells viability studies. To assess if compounds #**1** and #**2** engage with their target protein BRD4 in live cells and are able to displace BRD4 from chromatin binding, fluorescence recovery after photobleaching (FRAP) experiments were performed in U2OS cells overexpressing GFP-tagged BRD4. After photobleaching, diffusion of unbleached protein back into the bleached region is retarded by protein binding to chromatin and chromatin-associated complexes and is therefore slower compared to a freely diffusible molecule. Both compounds are able to significantly accelerate half-recovery times in FRAP experiments at 10 µM (Fig. 3*c*) suggesting that the inhibitors effectively displace

BRD4(1) from chromatin. These data are thus in accordance with their *in vitro* potency and observed antiproliferative effect.

The present work demonstrates the potential to exploit PPI-oriented chemical libraries using HTRF in a general screening cascade in order to accelerate hit identification targeting PPI's. As an example, the screening of our $2P2I_{3D}$ over the bromodomain target identified 15 hits in the low µM. We set-up ITC as orthogonal assay and could validate a direct binding for 7 out of the 17 compounds tested (Table 3) with Ligand Efficiency (LE) values ranging from 0.20 up to 0.23. The best compounds (Kd of 1.8 and 2 µM), a quinolone derivative (compound **#1**) and a triazolopyridazine derivative (compound #**2**), both compatible with the 'rule of five' filter [38], demonstrated pan-inhibition profiles, with selective inhibition of the BET family proteins *vs*. non-BET members. Finally, the demonstration that the identified compounds are indeed orthosteric inhibitors of the targeted interface requires a structural biology approach where the target is co-crystallized in the presence of the identified hits. However, 3D structures obtained from primary screening are relatively rare (3D structures generally require a first hit to lead optimization, in order to obtain sufficient affinity for the target). Both hits exhibit typical acetyl-lysine mimetic head groups and our co-crystal structures indeed reveal the canonical acetyl-lysine mimetic binding mode forming a hydrogen bond with the conserved asparagine (Asn140). Furthermore, direct visualization of an on-target effect in the nucleus of live cells emphasized the quality of these hits identified from a primary screening. Quinolone derivatives have recently been described as ATAD2 inhibitors [39] and have been developed into potent probes targeting BRD9 [40]. The identified hits may serve therefore also as chemical starting points for the development of inhibitors targeting non-BET bromodomains, an area of chemical biology that has not been extensively explored.

Overall, we here functionally validated the $2P2I_{3D}$ chemical library, developed by a SVM approach based on current identified orthosteric inhibitors found in our $2P2I_{DB}$ database, and demonstrate that it is enriched in biologically active PPI modulators that present sufficient basic properties to be proposed for medicinal chemistry programs. Our report thus provides a 'proof of concept' for the development of such approaches applied to larger and more complex libraries that will provide innovations and more particularly will accelerates hit findings targeting this challenging class of target.

FIGURES

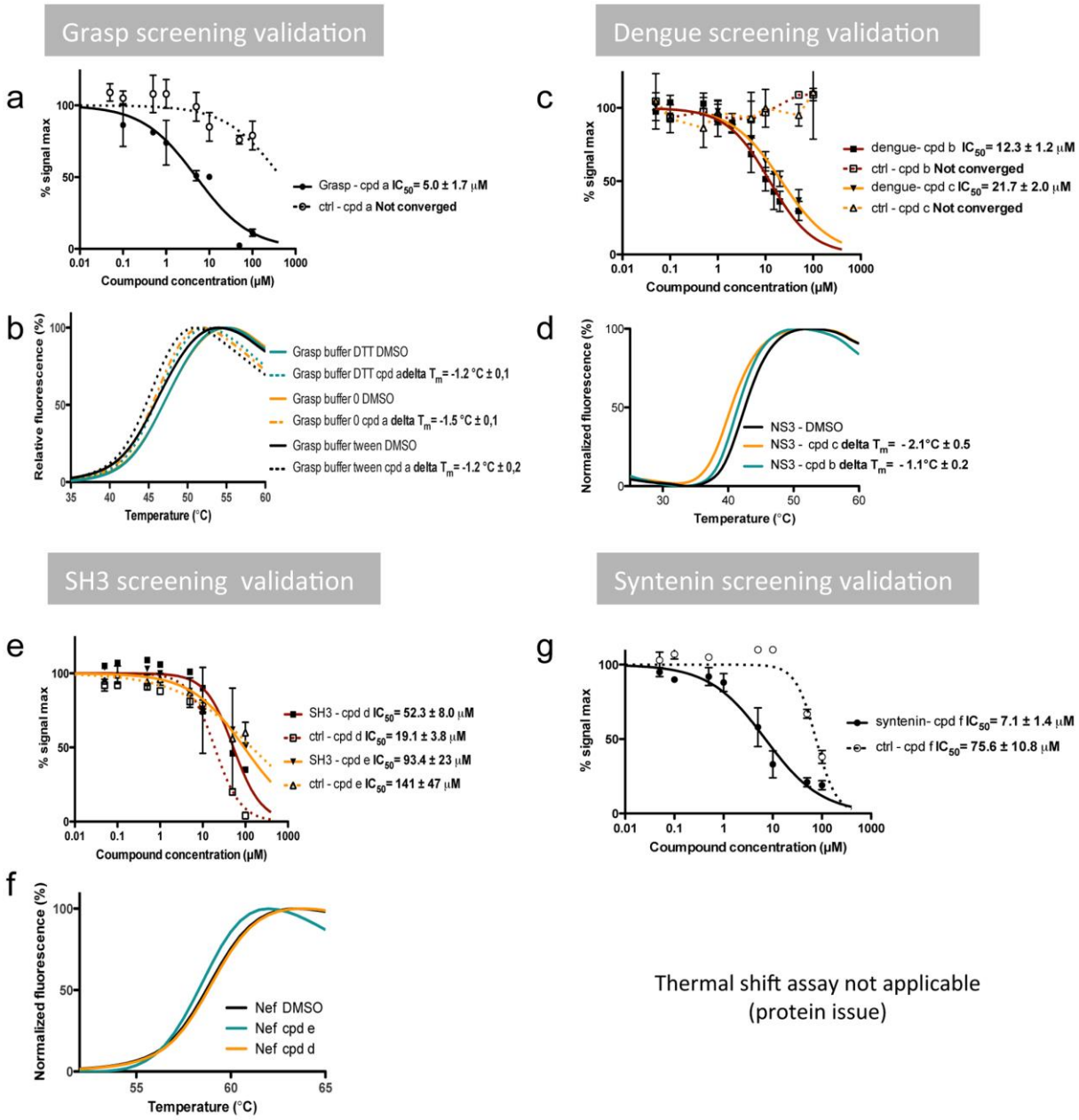


Figure 1 : Cross-validation for each identified hits

IC_{50} cross-validation experiments were performed by HTRF on a control, 'irrelevant' assay, to assess the specificity of each identified hit. In a second experiment, each potential hit were evaluated by differentiel scanning fluorimetry (DSF) experiment in order to validate their direct binding with an orthogonal method. (a) HTRF IC_{50} of compound a performed on Grasp55/JamB compared to the 'irrelevant/control' assay (BRD4/H4 complex, dashed curve). (b)Thermal shift experiments performed for compound a on Grasp55. The bold curves represents Grasp55 alone in presence of 2.5% DMSO while dashed curve represent the same experiment with a 25 fold excess of compound a. Experiments were performed in « 0 » buffer (hepes pH, 7.5, 150mM NaCl), DTT buffer (buffer 0 + 2mM DTT) and tween buffer (buffer 0 + 0.012% Tween 20). (c) HTRF IC_{50} of compound b and c performed on NS3/NS5 compared to the 'irrelevant/control' assay (BRD4/H4 complex, dashed curve). (d)Thermal shift experiments performed for compounds a and b on NS3. The black curve represents NS3 alone in presence of 2.5% DMSO while green and orange curves represent the same experiment with a 25 fold excess of compound b and c. (e) HTRF IC_{50} of compound d and e performed on Nef/SH3 compared to the 'irrelevant/control' assay (BRD4/H4 complex, dashed curves). (f)Thermal shift experiments performed for compounds d and e on Nef. The bold curve represents Nef alone in presence of 2.5%

DMSO while green and orange curves represent the same experiment with a 25 fold excess of compound d and e. (g) HTRF IC_{50} of compound f performed on syntenin/syndecan compared to the 'irrelevant/control' assay (BRD4/H4 complex, dashed curves). The thermal shift experiment could not be achieved using syntenin as target.

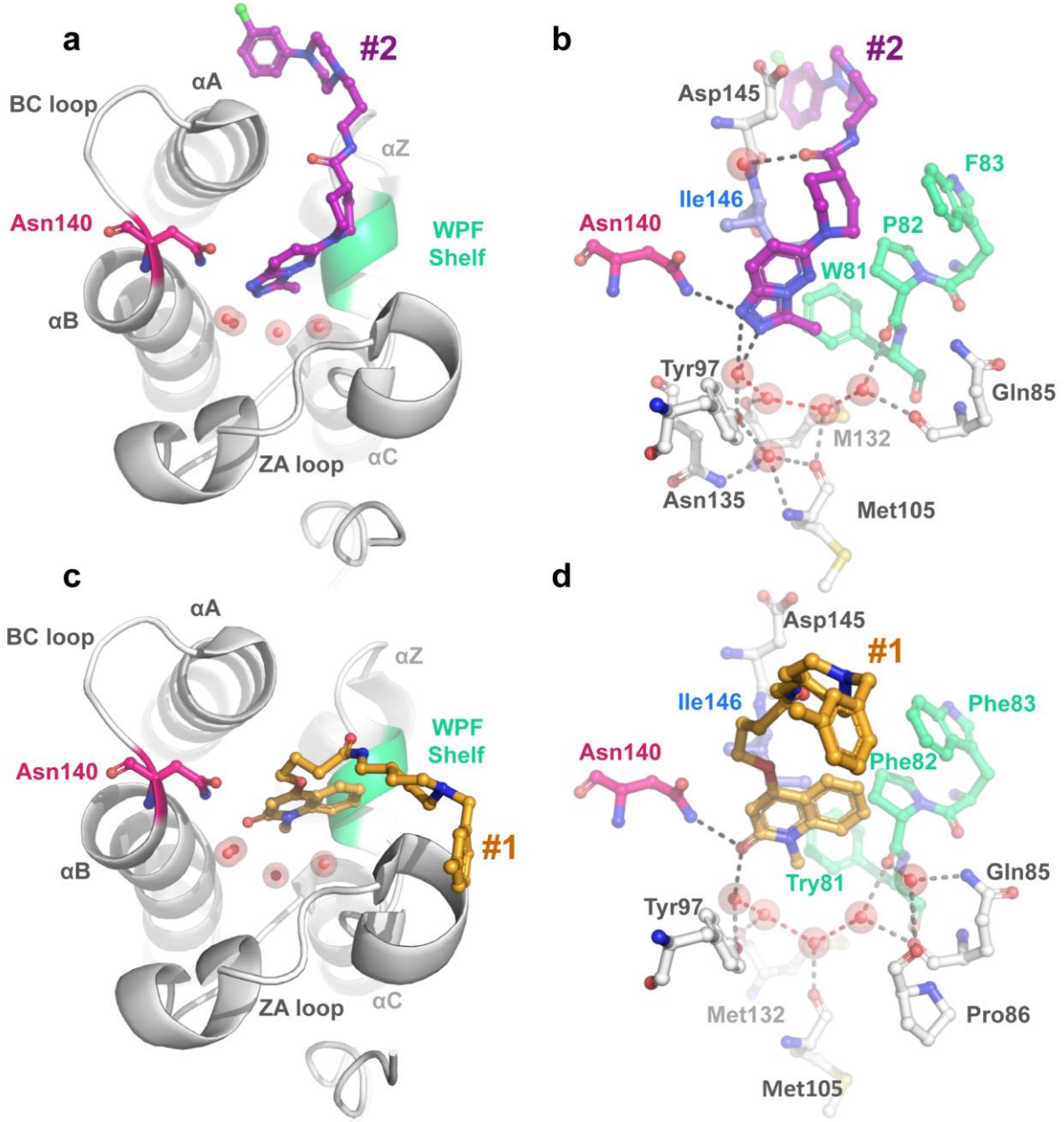


Figure 2: Structures of the BRD4(1) complexes with compounds #1 and #2. *(a) Overall fold of BRD4(1) (grey, cartoon representation) in complex with compound #2 (purple, ball and stick representation) (PDB code 5DLX). BRD4 (1) is folded into four α helices (α A, αB, αC, αZ) and two loops (BC and ZA). Compound #2 (purple) binds in the N-acetylated-lysine binding pocket through interactions with the conserved asparagine Asn140 residue (pink) and four conserved water molecules deep in the cavity (red spheres). (b) The triazolopyridazine moiety of compound #2 occupies the N-acetylated-lysine pocket and initiates a direct hydrogen bond interaction with the asparagine Asn140 (pink). Four water molecules are located at the bottom of the BRD4(1) cavity (red spheres) as seen in the N-acetylated lysine peptide complex structure (PDB ID: 3UVW). The water molecule hydrogen bond network (red dash lines) contributes to stabilize the ligand through an additional hydrogen bond. The rest of the molecule is close to the BRD4(1) WPF shelf residues (green) and the carbonyl moiety establishes a water bridge hydrogen bound interaction with the NH amide of the gatekeeper residue Ile146 (blue). (c) Overall fold of BRD4(1) in complex with compound #1 (orange, ball and stick representation)(PDB code 5DLZ). (d) As for compound #2, the oxoquinoline moiety occupies the N-acetylated-lysine pocket by contacts with the Asn140 and the conserved water molecules.*

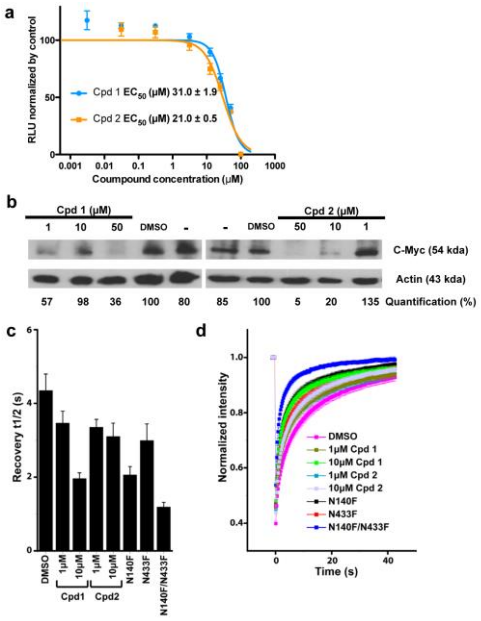


Figure 3: Cell viability and FRAP assays. (a) Viability curves and EC_{50} values (± SEM, N=3) of Jurkat cell lines treated by compound #1 (blue curve) or compound #2 (orange curve). (b) Western blot of c-Myc expression in Jurkat cells treated by compounds #1, #2 or DMSO as control. Quantifications were performed using actin expression level as reference and DMSO condition corresponding to the 100%. (c) Fluorescence Recovery After Photobleaching experiment in U2OS cells transfected with GFP-tagged BRD4 and treated with compounds #1, # 2 or DMSO as control. Shown are half times of recovery of at least 15 cells (left panel) and time dependence of fluorescence recovery in the bleached area of cells expressing wt or mutant GFP-BRD4 with the corresponding treatment. (d) Curves represent the means at each time point of at least 15 cells in each group.

TABLES

Screening results

Protein Partners	NS3/NS5 (Dengue Virus)	Nef /SH3-Hck (HIV Virus)	Bromodomain/ H4 ($K_{ac\ 5/8/12/16}$)	Grasp55/ JamB (PDZ Domain)	Syntenin/ Syndecan (PDZ Domain)
Interfaces Type	Protein/ Protein	Protein/ Domain	Protein/ Peptide	Protein/ Peptide	Protein/ Peptide
K_D (µM)	1.0 (1)	1.5 (2)	2.8 (3)	1 (4)	1.8 (5)
HTRF IC_{50} value from competition with untagged protein (µM)	0.4	N/A	5.9	1.7	9.2
Z'	0.70	0.88	0.78	0.81	0.92
# Hits Non-Autofluorescent (Inhibition > 40%)	39	16	44	44	19
# Validated Molecules (Secondary Screening)	26 (1.6%)	2 (0.1%)	17 (1%)	2 (0.1%)	3 (0.2%)
# IC_{50} Measured	26	2	17	2	3
# Unique Molecules	22	2	15	2	1
# Thermal shift sensitive	10	0	10	1	N/A

(1) Zou G, et al. (2011) Functional analysis of two cavities in flavivirus NS5 polymerase. *J Biol Chem* 286(16):14362–14372.

(2) Arold S, et al. (1998) RT loop flexibility enhances the specificity of Src family SH3 domains for HIV-1 Nef. *Biochemistry* 37(42):14683–14691.

(3) Filippakopoulos P, et al. (2012) Histone recognition and large-scale structural analysis of the human bromodomain family. *Cell* 149(1):214–231.

(4) Internal data

(5) Baietti MF, et al. (2012) Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat Cell Biol* 14(7):677–685.

N/A: not applicable

Table 1: High Throughput Screening results. Summary of screening results obtained during screening of the $2P2I_{3D}$ chemical library on various PPI targets. The fifth line corresponds to the number of compounds validated after the counter screen and the secondary screening. The sixth line indicates the number of compounds validated during IC_{50} measurement. Number of unique compounds, i.e. never validated for another screening, and Z' are indicated in the last line.

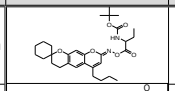
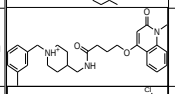
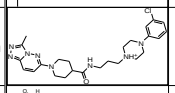
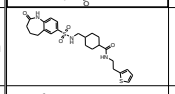
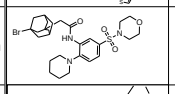
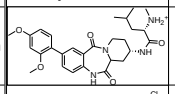
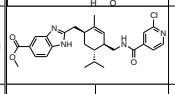
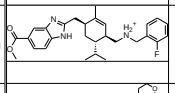
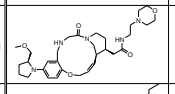
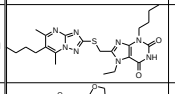
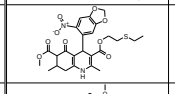
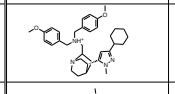
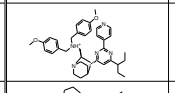
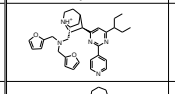
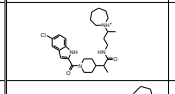
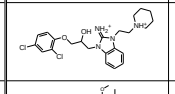
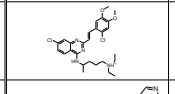
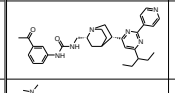
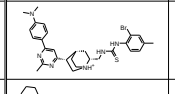
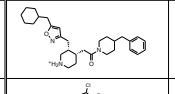
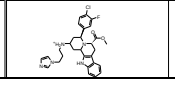
Target	Structure	IC_{50} (µM)	PAINS prediction	Potential aggregator	Molecular weight ($g.mol^{-1}$)	PubChem Compound Identifier (CID)
Bromodomain		25.2	0	no	526.7	16416191
Bromodomain		8.4	0	no	461.6	20862085
Bromodomain		7.8	0	yes	497.0	46326697
Bromodomain		8.9	0	yes	489.7	20937233
Bromodomain		37.9	0	yes	580.6	4658136
Bromodomain		12.9	0	no	509.6	7134939
Bromodomain		0.2	0	no	495.0	71693175
Bromodomain		22.8	0	no	463.6	nat (provider: Analyticon, ref: NAT28-553840)
Bromodomain		13.6	1	no	569.7	71694343
Bromodomain		18.0	0	no	484.6	60498769
Grasp		10.3	0	no	532.6	3324458
NS3 NS5		12.3	0	no	542.8	44715900
NS3 NS5		7.1	0	no	605.8	45360407
NS3 NS5		20.6	0	no	525.7	45360408
NS3 NS5		25.5	0	no	487.1	50816819
NS3 NS5		20.6	0	no	463.4	1306476 44654877 1383160 44440575
NS3 NS5		18.8	0	no	517.5	3322776 5793057
NS3 NS5		21.8	0	no	526.7	40777317
NS3 NS5		12.4	1	no	579.6	40776845
NS3 NS5		21.1	0	no	477.7	25313771
NS3 NS5		13.1	1	no	536.0	44716462

Table 2: Thermal shift sensitive compounds chemical structure and chemical features
Summary of chemical structure, molecular weight and IC_{50} values obtained during screening process with "thermal shift sensitive" inhibitors. Pan-assay interference compounds ("PAINS") and aggregators predictions are indicated based on FAF*Drugs*[3] and Aggregator advisor, respectively.

ITC thermodynamics parameters, IC_{50} and ligand efficiency values

#	ITC			MW (Da)	Ligand Efficiency
	K_D ITC (µM)	**ΔH** (cal/mol)	**ΔG** (Kcal /mol)		
1	1.8	-8664 ± 129	-7.56 ± 0.04	462.7	0.22
2	2.0	-7996 ± 84.75	-7.48 ± 0.07	498.1	0.21
3	2.0	-4547 ± 414.3	-7.49 ± 0.14	509.7	0.20
4	2.2	-9174 ± 84.75	-7.44 ± 0.02	489.7	0.23
5	3.6	-1.276^{e}4 ± 2358	-7.16 ± 0.12	464.66	0.21
6	4.5	-3915 ± 868.6	-7.04 ± 0.29	484.7	0.21
7	4.7	-1.288^{e}4 ± 3172	-6.97 ± 0.05	495.1	0.20

Table 3: ITC thermodynamics parameters, IC_{50} and ligand efficiency values. Binding affinities and thermodynamics parameters measured by ITC during the orthogonal validation process for the hits identified by HTRF on BRD4(1). Ligand Efficiency values are indicated based on the ΔG values obtained from the ITC experiments.

METHODS

An extended methods section describing molecular biology, protein expression, preparation, crystallization, structure determination, cell biology (culture conditions, reagents, cytotoxicity assays and western blot studies) and biochemical (such as HTRF) assays can be found in supporting information Methods.

SUPPORTING INFORMATION

This material is available free of charge via the Internet.

Supporting methods, supporting Figure S1 – S7, table S1 and S2

ACCESSION CODES

Coordinates and structure factors of refined BRD4(1) complexed with compound 1 and 2 have been deposited in the Protein Data Bank with accession code 5DLZ and 5DLX.

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AUTHOR CONTRIBUTIONS

JCG and XM designed experiments. S.M., B.R., S.B, C.D., A.R., E.R., A.L., M.B., R.K., JC.L., C.E., C.R. performed experiments. S.M., B.R., S.B., C.D., A.R., E.R., A.L., R.K., JC.L., ME.G., S.C., P.Z., M.AL., T.R., S.M., S.K., E.T., Y.C., JC.G., X.M. analyzed and interpreted the data. V.H., P.R. performed molecular modeling and cheminformatics. The manuscript was written by XM with input from all authors.

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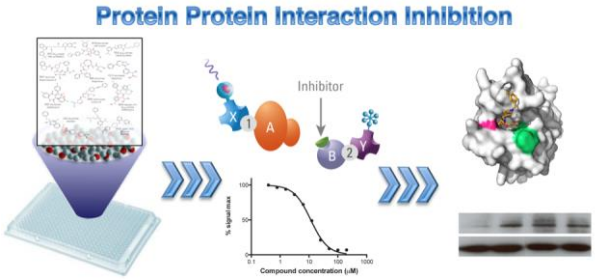
FUNDING SOURCES

The authors declare no competing financial interest

Table of contents graphic



REFERENCES

(1) Morelli, X., and Hupp, T. (2012) Searching for the Holy Grail; protein-protein interaction analysis and modulation. *EMBO Rep. 13*, 877–879.

(2) Arkin, M. R., Tang, Y., and Wells, J. A. (2014) Small-molecule inhibitors of protein-protein interactions: progressing toward the reality. *Chem. Biol. 21*, 1102–1114.

(3) Kuenemann, M. A., Sperandio, O., Labbé, C. M., Lagorce, D., Miteva, M. A., and Villoutreix, B. O. (2015) In silico design of low molecular weight protein-protein interaction inhibitors: Overall concept and recent advances. *Prog. Biophys. Mol. Biol.*

(4) Villoutreix, B. O., Kuenemann, M. A., Poyet, J.-L., Bruzzoni-Giovanelli, H., Labbé, C., Lagorce, D., Sperandio, O., and Miteva, M. A. (2014) Drug-Like Protein-Protein Interaction Modulators: Challenges and Opportunities for Drug Discovery and Chemical Biology. *Mol. Inform. 33*, 414–437.

(5) Zhang, X., Betzi, S., Morelli, X., and Roche, P. (2014) Focused chemical libraries--design and enrichment: an example of protein-protein interaction chemical space. *Future Med. Chem. 6*, 1291–1307.

(6) Labbé, C. M., Laconde, G., Kuenemann, M. A., Villoutreix, B. O., and Sperandio, O. (2013) iPPI-DB: a manually curated and interactive database of small non-peptide inhibitors of protein-protein interactions. *Drug Discov. Today 18*, 958–968.

(7) Labbé, C. M., Kuenemann, M. A., Zarzycka, B., Vriend, G., Nicolaes, G. A. F., Lagorce, D., Miteva, M. A., Villoutreix, B. O., and Sperandio, O. (2016) iPPI-DB: an online database of modulators of protein-protein interactions. *Nucleic Acids Res. 44*, D542–547.

(8) Gaulton, A., Bellis, L. J., Bento, A. P., Chambers, J., Davies, M., Hersey, A., Light, Y., McGlinchey, S., Michalovich, D., Al-Lazikani, B., and Overington, J. P. (2012) ChEMBL: a large-scale bioactivity database for drug discovery. *Nucleic Acids Res. 40*, D1100–1107.

(9) Higueruelo, A. P., Schreyer, A., Bickerton, G. R. J., Pitt, W. R., Groom, C. R., and Blundell, T. L. (2009) Atomic interactions and profile of small molecules disrupting protein-protein interfaces: the TIMBAL database. *Chem. Biol. Drug Des. 74*, 457–467.

(10) Higueruelo, A. P., Jubb, H., and Blundell, T. L. (2013) TIMBAL v2: update of a database holding small molecules modulating protein-protein interactions. *Database*

J. Biol. Databases Curation 2013, bat039.
(11) Gupta. (2011) New drug development. *Drug Discovery and Clinical Research. JayPee Brothers Medical Publishers Ltd* 1–135.
(12) Arkin, M. R., and Wells, J. A. (2004) Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. *Nat. Rev. Drug Discov. 3*, 301–317.
(13) Macarron, R., Banks, M. N., Bojanic, D., Burns, D. J., Cirovic, D. A., Garyantes, T., Green, D. V. S., Hertzberg, R. P., Janzen, W. P., Paslay, J. W., Schopfer, U., and Sittampalam, G. S. (2011) Impact of high-throughput screening in biomedical research. *Nat. Rev. Drug Discov. 10*, 188–195.
(14) Davies, J. W., Glick, M., and Jenkins, J. L. (2006) Streamlining lead discovery by aligning in silico and high-throughput screening. *Curr. Opin. Chem. Biol. 10*, 343–351.
(15) Bourgeas, R., Basse, M.-J., Morelli, X., and Roche, P. (2010) Atomic analysis of protein-protein interfaces with known inhibitors: the 2P2I database. *PloS One 5*, e9598.
(16) Basse, M. J., Betzi, S., Bourgeas, R., Bouzidi, S., Chetrit, B., Hamon, V., Morelli, X., and Roche, P. (2013) 2P2Idb: a structural database dedicated to orthosteric modulation of protein-protein interactions. *Nucleic Acids Res. 41*, D824–827.
(17) Basse, M.-J., Betzi, S., Morelli, X., and Roche, P. (2016) 2P2Idb v2: update of a structural database dedicated to orthosteric modulation of protein-protein interactions. *Database J. Biol. Databases Curation 2016*.
(18) Morelli, X., Bourgeas, R., and Roche, P. (2011) Chemical and structural lessons from recent successes in protein-protein interaction inhibition (2P2I). *Curr. Opin. Chem. Biol. 15*, 475–481.
(19) Hamon, V., Bourgeas, R., Ducrot, P., Theret, I., Xuereb, L., Basse, M. J., Brunel, J. M., Combes, S., Morelli, X., and Roche, P. (2014) 2P2I HUNTER: a tool for filtering orthosteric protein-protein interaction modulators via a dedicated support vector machine. *J. R. Soc. Interface R. Soc. 11*, 20130860.
(20) Hamon, V., Brunel, J. M., Combes, S., Basse, M. J., Roche, P., and Morelli, X. (2013) 2P2Ichem: focused chemical libraries dedicated to orthosteric modulation of protein-protein interactions. *Med Chem Commun* 797–809.
(21) Welsch, M. E., Snyder, S. A., and Stockwell, B. R. (2010) Privileged scaffolds for library design and drug discovery. *Curr. Opin. Chem. Biol. 14*, 347–361.
(22) Horton, D. A., Bourne, G. T., and Smythe, M. L. (2003) The combinatorial

synthesis of bicyclic privileged structures or privileged substructures. *Chem. Rev. 103*, 893–930.

(23) Betzi, S., Restouin, A., Opi, S., Arold, S. T., Parrot, I., Guerlesquin, F., Morelli, X., and Collette, Y. (2007) Protein protein interaction inhibition (2P2I) combining high throughput and virtual screening: Application to the HIV-1 Nef protein. *Proc. Natl. Acad. Sci. U. S. A. 104*, 19256–19261.

(24) Emert-Sedlak, L., Kodama, T., Lerner, E. C., Dai, W., Foster, C., Day, B. W., Lazo, J. S., and Smithgall, T. E. (2009) Chemical library screens targeting an HIV-1 accessory factor/host cell kinase complex identify novel antiretroviral compounds. *ACS Chem. Biol. 4*, 939–947.

(25) Breuer, S., Espinola, S., Morelli, X., Torbett, B. E., Arold, S. T., and Engels, I. H. (2013) A Biochemical/Biophysical Assay Dyad for HTS-Compatible Triaging of Inhibitors of the HIV-1 Nef/Hck SH3 Interaction. *Curr. Chem. Genomics Transl. Med. 7*, 16–20.

(26) Filippakopoulos, P., and Knapp, S. (2014) Targeting bromodomains: epigenetic readers of lysine acetylation. *Nat. Rev. Drug Discov. 13*, 337–356.

(27) Brand, M., Measures, A. M., Wilson, B. G., Cortopassi, W. A., Alexander, R., Höss, M., Hewings, D. S., Rooney, T. P. C., Paton, R. S., and Conway, S. J. (2015) Small molecule inhibitors of bromodomain-acetyl-lysine interactions. *ACS Chem. Biol. 10*, 22–39.

(28) Chen, X., Longgood, J. C., Michnoff, C., Wei, S., Frantz, D. E., and Bezprozvanny, L. (2007) High-throughput screen for small molecule inhibitors of Mint1-PDZ domains. *Assay Drug Dev. Technol. 5*, 769–783.

(29) Thorsen, T. S., Madsen, K. L., Dyhring, T., Bach, A., Peters, D., Strømgaard, K., Rønn, L. C. B., and Gether, U. (2011) A fluorescence polarization based screening assay for identification of small molecule inhibitors of the PICK1 PDZ domain. *Comb. Chem. High Throughput Screen. 14*, 590–600.

(30) Degorce, F., Card, A., Soh, S., Trinquet, E., Knapik, G. P., and Xie, B. (2009) HTRF: A technology tailored for drug discovery - a review of theoretical aspects and recent applications. *Curr. Chem. Genomics 3*, 22–32.

(31) Zhang, J.-H., Chung, T. D. Y., and Oldenburg, K. R. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen. 4*, 67–73.

(32) Ryan, A. J., Gray, N. M., Lowe, P. N., and Chung, C. (2003) Effect of detergent

on “promiscuous” inhibitors. *J. Med. Chem. 46*, 3448–3451.
(33) Feng, B. Y., and Shoichet, B. K. (2006) A detergent-based assay for the detection of promiscuous inhibitors. *Nat. Protoc. 1*, 550–553.
(34) Filippakopoulos, P., Picaud, S., Mangos, M., Keates, T., Lambert, J.-P., Barsyte-Lovejoy, D., Felletar, I., Volkmer, R., Müller, S., Pawson, T., Gingras, A.-C., Arrowsmith, C. H., and Knapp, S. (2012) Histone recognition and large-scale structural analysis of the human bromodomain family. *Cell 149*, 214–231.
(35) Rahl, P. B., Lin, C. Y., Seila, A. C., Flynn, R. A., McCuine, S., Burge, C. B., Sharp, P. A., and Young, R. A. (2010) c-Myc regulates transcriptional pause release. *Cell 141*, 432–445.
(36) Delmore, J. E., Issa, G. C., Lemieux, M. E., Rahl, P. B., Shi, J., Jacobs, H. M., Kastritis, E., Gilpatrick, T., Paranal, R. M., Qi, J., Chesi, M., Schinzel, A. C., McKeown, M. R., Heffernan, T. P., Vakoc, C. R., Bergsagel, P. L., Ghobrial, I. M., Richardson, P. G., Young, R. A., Hahn, W. C., Anderson, K. C., Kung, A. L., Bradner, J. E., and Mitsiades, C. S. (2011) BET bromodomain inhibition as a therapeutic strategy to target c-Myc. *Cell 146*, 904–917.
(37) Zuber, J., Shi, J., Wang, E., Rappaport, A. R., Herrmann, H., Sison, E. A., Magoon, D., Qi, J., Blatt, K., Wunderlich, M., Taylor, M. J., Johns, C., Chicas, A., Mulloy, J. C., Kogan, S. C., Brown, P., Valent, P., Bradner, J. E., Lowe, S. W., and Vakoc, C. R. (2011) RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature 478*, 524–528.
(38) Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev. 46*, 3–26.
(39) Chaikuad, A., Petros, A. M., Fedorov, O., Xu, J., and Knapp, S. (2014) Structure-based approaches towards identification of fragments for the low-druggability ATAD2 bromodomain. *Med. Chem. Commun.* 1843–1848.
(40) Clark, P. G. K., Vieira, L. C. C., Tallant, C., Fedorov, O., Singleton, D. C., Rogers, C. M., Monteiro, O. P., Bennett, J. M., Baronio, R., Müller, S., Daniels, D. L., Méndez, J., Knapp, S., Brennan, P. E., and Dixon, D. J. (2015) LP99: Discovery and Synthesis of the First Selective BRD7/9 Bromodomain Inhibitor. *Angew. Chem. Int. Ed Engl. 54*, 6217–6221.