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Discovery of BRD4 bromodomain inhibitors by fragment-based high-throughput docking

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ABSTRACT

Bromodomains (BRDs) recognize acetyl-lysine modified histone tails mediating epigenetic processes. BRD4, a protein containing two bromodomains, has emerged as an attractive therapeutic target for several types of cancer as well as inflammatory diseases. Using a fragment-based in silico screening approach, we identified two small molecules that bind to the first bromodomain of BRD4 with low-micromolar affinity and favorable ligand efficiency (0.37 kcal/mol per non-hydrogen atom), selectively over other families of bromodomains. Notably, the hit rate of the fragment-based in silico approach is about 10% as only 24 putative inhibitors, from an initial library of about 9 million molecules, were tested in vitro.

Bromodomains (BRDs) are protein interaction modules that are exclusively recruited to ε-N-lysine acetylation motifs, a key event in the reading process of epigenetic marks.[1] Among the 61 BRDs of eight families present in human transcriptional co-regulators and chromatin modifying enzymes, BRD4 of the bromo and extra-terminal (BET) family has been characterized as a key determinant in acute myeloid leukemia, multiple myeloma, Burkitt's lymphoma, NUT midline carcinoma, colon cancer, and inflammatory diseases.[2–5] BRD4, a bromodomain containing protein consisting of two bromodomains called BRD4(1) and BRD4(2), has emerged as an exciting new therapeutic candidate for the development of inhibitors targeting gene transcription.

Recent fragment-based biophysical screening approach and QSAR-based hit optimization has led to the discovery of potent BRD4 inhibitors.[6–9] Fragment-based drug discovery (FBDD) has progressed over the years as a viable alternative to more traditional methods of hit identification, such as high throughput screening (HTS).[10] A small set of fragments can cover a larger chemical space than a typical HTS collection. Despite their weak affinity, fragments often exhibit high ligand efficiency (LE), defined as the ratio of the free energy of binding to the number of non-hydrogen atoms.[11]

Previously, we have developed a fragment-based in silico procedure called ALTA (Anchor-based Library Tailoring) to select subsets of large libraries of compounds for high-throughput docking.[12,13] As illustrated in Figure 1, the molecules in a multi-million library are first decomposed into (mainly rigid) fragments, which are then screened in silico to identify those with the most favorable predicted binding free energy (called anchor fragments). Secondly, only compounds containing at least one of the anchor fragments are retained for flexible-ligand docking. Given the small fragment space, this procedure is computationally very efficient in inquiring the entire library. Moreover, hits identified by this approach bear a relatively large anchor fragment and often have high LE, a favorable feature for lead elaboration by medicinal chemistry. In previous high-throughput docking campaigns, we have shown that low-micromolar hits identified by the ALTA procedure can be efficiently optimized into low-nanomolar tyrosine kinases inhibitors.[13–15] Here, we report the discovery of novel BRD4(1) inhibitors by the ALTA fragment-based approach.

Briefly, the nearly 9 million compounds in the ZINC all-now library (version of 2012) were firstly filtered to 4.6 millions by molecular weight ranging from 200 to 400 Dalton, more than one hydrogen-bond acceptor, number of rotatable bonds lower than 8, and number of rings ranging from 2 to 6. This focused library was automatically decomposed into 375,897 fragments with rich chemical features by a previously reported algorithm.[13] The generated fragments were further filtered to 238,408 fragments by molecular weight from 60 to 300 Dalton, at least one hydrogen-bond acceptor, and number of rotatable bonds lower than 4. The focused library of fragments was docked into BRD4(1) (PDB code 3MXF) by an in-house developed docking

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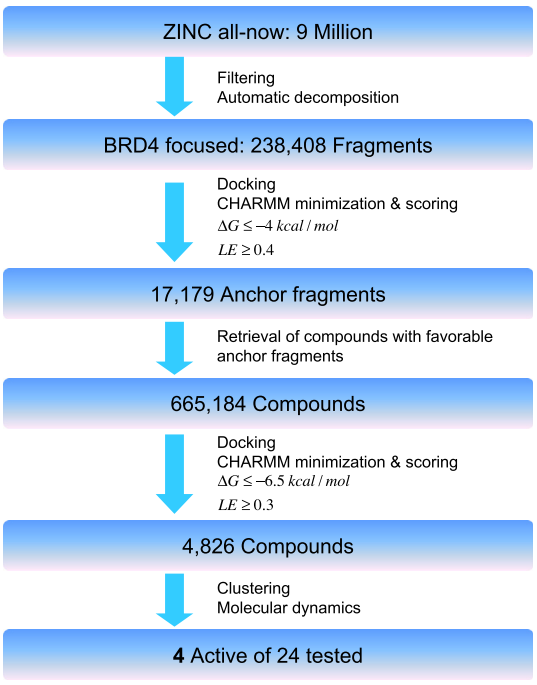

Figure 1. Illustration of the ALTA procedure[12,13] for fragment-based in silico screening of BRD4(1) inhibitors. Fragment docking and flexible ligand docking were carried out with an in-house developed program[17,18] using the X-ray structure of BRD4(1) (PDB code 3MXF). Units of LE are kcal/mol per non-hydrogen atom.

tool,[16] which in previous applications has led to the discovery of novel inhibitors of the ZAP70 and Syk tyrosine kinases.[17,18] The 511,417 docking poses of the fragments were further minimized in the rigid protein by CHARMM[19,20] with the CHARMm22 force field.[21] The poses were then filtered by hydrogen bonding to the conserved asparagine that is involved in a hydrogen bond with the natural ligand (Asn140 in BRD4(1)), and hydrogen bonding penalty[22] smaller than 1. With these two filters, unrealistic poses of the fragments were removed efficiently before rescoring by an energy function[22] which takes into account solvation and whose calculation requires about 90 s per pose. This energy function is transferable among kinases and has been used to identify novel classes of kinase inhibitors with activity ranging from low micromolar to nanomolar.[13,17,18,22,23] Docking of the 238,408 fragments and CHARMM minimization of 511,417 poses together with scoring required about 0.7 and 2.7 days, respectively, of a cluster of 100 cores of Xeon 2.6 GHz processors. The 17,179 fragments with predicted binding affinity more favorable than −4 kcal/mol (corresponding to 1 mM) and predicted LE more favorable (i.e., larger) than 0.4 kcal/mol per heavy atom were retained as anchor fragments. Next, the 665,184 molecules containing at least one anchor fragment were retrieved and docked using the same docking tool as for the fragments, which required nearly 5 days on 100 cores. Finally, 4826 compounds (for a total of about 1000 anchor fragments) remained after filtering by predicted binding affinity more favorable than −6.5 kcal/mol (corresponding to 20 μM) and predicted LE more favorable than 0.3 kcal/mol per heavy atom. By using ECFP4 fingerprints and a maximum intracluster distance of 0.7,[24] the 4826 compounds belong to 616 clusters and their anchor fragments belong to 286 clusters. Selection of compounds for molecular dynamics (MD) was guided by predicted LE as well as chemical diversity, rigidity, novelty and actual commercial availability. Multiple MD simulations of 55 compounds with different anchor fragments were carried out starting from their docked pose into BRD4(1) to investigate the main binding interactions, for example, the stability of the hydrogen bond with Asn140 (Fig. S1). The MD simulations revealed that the binding mode of some of the selected chemotypes was not stable. These MD results were used to further reduce the number of compounds. Finally, only 24 compounds (Fig. S2) were selected for in vitro validation.

The 24 compounds were tested by differential scanning fluorimetry (DSF, also called thermal shift assay) at a protein concentration of 2 μM. Four compounds (**1**–**4**, Fig. 2) increased the thermal stability of BRD4(1) by ΔT_m values larger than 1 °C at a ligand concentration of 50 μM (Table 1). With increase in the ligand concentration, larger thermal shifts were observed, particularly for compounds **2** and **3** with ΔT_m values around 4 °C at a ligand concentration of 200 μM. Increase in ligand concentration at constant protein concentration gives rise to higher ratio of bound protein, leading to larger thermal shift. This dose-dependency provides additional evidence of the binding of the four compounds to BRD4(1). The four compounds were further tested by the Alpha-screen assay which monitors the competitive displacement of a histone peptide from BRD4(1) at Reaction Biology Corp. (Malvern, PA). Compounds **1** and **4** show a moderate inhibition of about 40% at 50 μM, while compounds **2** and **3** exhibit an IC_{50} value of about 7 μM (Fig. S3 and Table 1). The compounds **2** and **3** do not show a significant thermal shift on five non-BET bromodomains belonging to five different families: CREBBP, BAZ2B, BRD1, TAF1(2), and SMARCA4 except for compound **3** on CREBBP (Fig. S4). These results indicate that they are selective over non-BET families.

Compound **1** bears an isoxazole ring, known as an acetyl-lysine mimic,[7,25,26] while the other compounds represent three novel acetyl-lysine mimics. Interestingly, compound **4** is diazepam, a drug firstly marketed as Valium to treat anxiety, insomnia, and symptoms of acute alcohol withdrawal. Following the discovery of JQ1,[27] derivatives of its Markush structure have been extensively examined in industry,[28] and benzodiazepine drugs have also been screened in academia.[29] However, compound **4** has not been reported to inhibit the BET family. Thus, our finding may suggest diazepam as a starting point for development of potent BET inhibitors.

The predicted binding mode of both compounds **2** and **3** in BRD4(1) is characterized by a lipophilic sandwich of their bicyclic core between residues Val87, Leu92, Leu94 and Tyr97 on one side, and Phe83 and Ile146 on the other side of the binding pocket (Fig. 3 and S5). The carbonyl oxygen of both compounds is engaged in hydrogen bonding interactions with the highly conserved Asn140, a typical feature among bromodomain inhibitors, as most of them are acetyl-lysine mimics.[6–9,27,28,30,31] Water molecules present at the bottom of the pocket are not displaced. Compound **2** is predicted to further occupy the hydrophobic WPF shelf, which is an important region for ligand design to gain potency. In contrast, compound **3** does not form interaction with the WPF

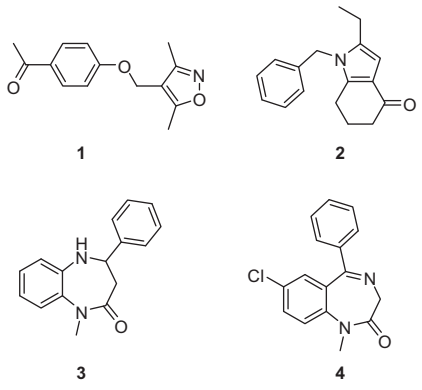

Figure 2. BRD4(1) inhibitors identified by fragment-based high-throughput docking.

Table 1
Activity of four compounds identified by fragment-based virtual screening

	Thermal shift assay[a] (°C)			Binding assay[b]	In silico prediction		
	ΔT_m	ΔT_m	ΔT_m	IC_{50} (% inhibition)	ΔG[c]	Rank[d]	Rank[e]
1	1.2	1.9	3.0	ND (35%)	−6.6	4188	299
2	2.6	ND	3.7	7.0 μM (99%)	−6.6	3830	636
3[f]	1.7	2.8	4.3	7.5 μM (99%)	−6.8	2937	465
4[g]	1.1	1.4	1.6	ND (43%)	−7.0	1741	616

ND: Not determined.
[a] Protein concentration of 2 μM and ligand concentration from left to right of 50 μM, 100 μM, and 200 μM. Shown are average values of two replicates and the standard deviation was smaller than 0.2 °C.
[b] Competition binding assay with effective IC_{50} values from 10 doses starting at 50 μM and using a factor of 2 for each dilution. Percentage inhibition at 50 μM concentration of compound are in parentheses.
[c] Binding affinity (kcal/mol) predicted by the scoring function.[22]
[d] Rank by predicted binding affinity among 665,184 compounds.
[e] Rank by predicted LE. This rank does not take clustering into account.
[f] Racemic mixture as purchased.
[g] Diazepam (marketed as valium).

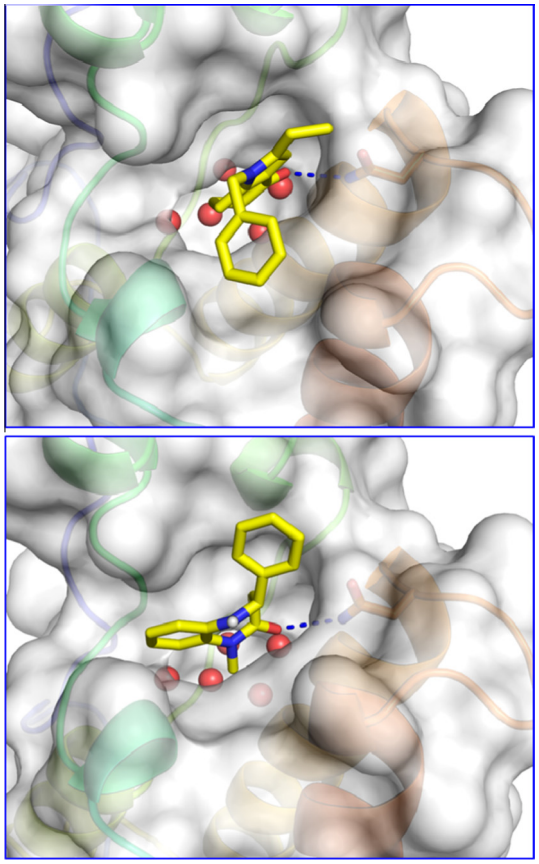

Figure 3. Docking pose of compound **2** (top) and **3** (bottom) in the recognition site of BRD4(1).

shelf, and its phenyl ring points upward mainly interacting with the ZA loop. Such tight interactions of both compounds in the recognition pocket may account for their very favorable LE (0.37 kcal/mol per heavy atom), a measure of how efficiently a ligand binds to a biomolecule.

Distinguishing hits from false positives is a big challenge in high-throughput screening in silico as well as in vitro. Analysis of ten HTS campaigns from Pfizer suggested that hit selection based on ranking by primary screen activity values is inefficient.[32] Although the binding affinity of the compounds **1**–**4** is predicted rather accurately by the scoring function, there are too many false positives which make the rank of the active compounds quite large (Table 1). The van der Waals interaction energy in the scoring function is dominant, and it positively correlates with the molecular size, therefore, a higher false positive rate by the scoring function[22] can be expected for larger compounds. There are two regimes for the 4826 compounds remaining after docking, scoring, and filtering (Fig. 4). For compounds with 15–22 non-hydrogen atoms the predicted binding affinity is constant (and slightly more favorable than the threshold of −6.5 kcal/mol used for filtering) while the LE deteriorates with increasing compound size (Fig. 4). In the size range between 22 and 28 heavy atoms the LE is constant (and very close to the threshold of 0.3 kcal/mol per non-hydrogen atom)[33] while the predicted binding affinity improves monotonously. By LE, compounds **1**–**4** are ranked 299, 636, 465, and 616, respectively, among 665,184 compounds docked. These relatively high LE ranks indicate that clustering by chemical diversity and filtering by MD simulations are very useful for removing false positives in the final phase of a high-throughput docking campaign.

In summary, we have discovered two novel chemotypes of antagonists of acetylated peptide binding to BRD4(1) by a fragment-based high-throughput docking approach. Given their selectivity against non-BET bromodomains and favorable LE of 0.37 kcal/mol per heavy atom, both compounds may inspire medicinal chemists to further develop them into candidate lead

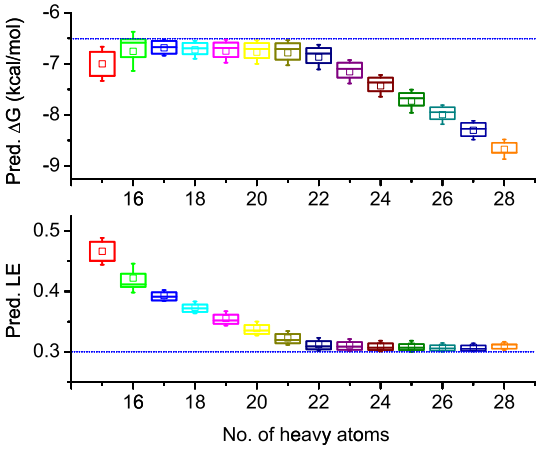

Figure 4. Distribution of predicted binding affinity (top) and predicted LE of the 4826 compounds from high-throughput virtual screening of BRD4(1) inhibitors. The bottom and top of each box are the first and third quartiles, and the band inside the box is the median. The whisker represents one standard deviation above and below the mean value shown as square at the center of the box. The horizontal lines illustrate the thresholds used to reduce the number of compounds upon high-throughput docking.

compounds for the treatment of hematological malignancies and solid cancers for which BRD4 is a therapeutic target.

Availability of software: The program used for docking is available from the corresponding authors.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.04.017.

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