Proteome Analysis

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Identification of Covalent Bromodomain Binders through DNA Display of Small Molecules**

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Abstract: *The regulation of transcriptional programs by epigenetic readers (bromodomains) has been linked to the development of several pathologies. Notably, it has been implicated in the regulation of cellular growth and evasion of apoptosis, in cancer as well as in inflammation. The discovery of small-molecule probes to dissect the role of bromodomains is thus important. We demonstrate that specific cysteine residues conserved across the bromodomains can be harnessed for covalent trapping. We report the discovery of two small molecules that form a covalent bond with cysteine residues conserved across the bromodomain family, analyze the subset of bromodomains that can be addressed through covalent binding, and show proteomic analyses enabled by the enrichment of bromodomains from native lysates.*

Bromodomains are important actors in epigenetic regulation. Bromodomains are small (about 110 amino acids) epigenetic reader domains that dock onto chromatin bearing acetylated lysine histone tails and thereby modulate transcriptional programs, which results in phenotypic changes. The human proteome encodes 61 bromodomains, which despite a low degree in overall sequence homology, share a conserved fold of four α helices (Z, A, B, and C; see below).[1] These four helices are joined by highly variable linkers between helices B and C, and helices Z and A. A large hydrophobic cavity that binds acetylated lysine within the target proteins provides an attractive pocket for the development of small molecule inhibitors.[2] However, druggability within the bromodomain family varies quite considerably[3] and inhibitors have so far been described for only a small number of bromodomain targets.[1,4–7] Bromodomain-containing proteins are involved in a variety of biological functions and act, amongst other things, as regulators of transcription or as parts of chromatin remodeling complexes.[8] It is therefore not surprising that these proteins are involved in a variety of human diseases, including cancer and inflammatory diseases, and that specific molecules for pharmacological intervention, as well as further studies on their biological and pathophysiological roles, are warranted. The usefulness of potent and selective inhibitors, like the BET-selective inhibitor (JQ1),[9] has opened new avenues for therapeutic strategies and the first BET inhibitors have entered clinical trials. These discoveries have spurred parallel efforts to discover novel inhibitors for this interesting protein family.[2] While selective inhibitors are crucial to explore the biology of a specific target, tool compounds with a wider specificity profile are also important to explore the involvement of a whole protein family. The first broad-spectrum inhibitor for bromodomains has recently been described (http://www.thesgc.org/chemical-probes/bromosporine) and provides a useful tool compound for exploring the biology of these reader domains, as well as for establishing functional in vitro and cellular assays. Taking cues from activity-based profiling[10] and the successful use of covalent inhibitors to terminally inactivate kinases,[11–17] targeting cysteines on bromodomains may provide a general entry into covalent probes. Herein, we report the discovery of two small molecules that broadly engage bromodomains through covalent interactions, analyze the distribution of cysteines across bromodomains, and demonstrate the utility of these probes to enrich bromodomains for proteomic analysis.

As a starting point in the identification of new bromodomain-targeting probes, we performed an affinity screen with P300/CBP-associated factor (PCAF) using a library of PNA-encoded small-molecule fragments combinatorially displayed on a DNA microarray.[18–21] We identified 25 fragments that were used for the synthesis of a 625-member focused library by growing the 25 fragments with 25 diverse synthons (See the Supporting Information).[22–23] As shown in Figure 1, the library was screened against PCAF, thereby leading to the identification of compounds **8a1b** and **19a1b**. The fragment encoded by **1b** is ethacrynic acid, an FDA-approved drug used to treat high blood pressure, which is known to react with glutathione S-transferase (GST).[24] The fragments encoded

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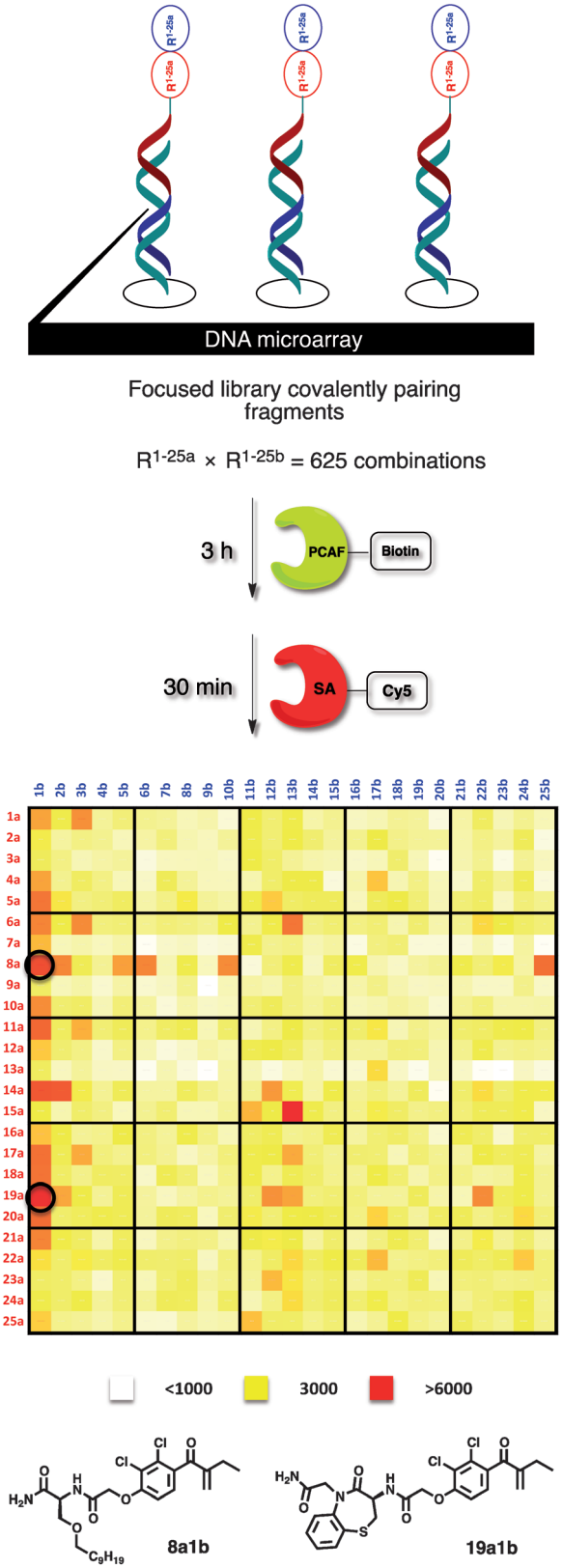

Figure 1. Screening of PCAF at 100 nM against a 625-membered focus library (see the Supporting Information for fragment structures). Spots represent the average fluorescence intensity of 24 measurements for each compound (SD < 5%). Compounds in each row have the same first building block and compounds in each column the same second building block.

by **8a** and **19a** are serine residues modified with an alkyl chain and a thiazepine, respectively. It is interesting to note that the combination of **8a** and **1b** has some pharmacophore overlap with two previously reported PCAF inhibitors in which a functionalized phenyl ring is substituted with an alkyl chain.[25–26] Furthermore, several bromodomain ligands have been identified with a diazepine motif reminiscent of fragment **19a**.[2] Mindful that fragment **1b** might engage PCAF through a covalent interaction, both compounds were resynthesized as biotin conjugates (**8a1b**-biotin, **19a1b**-biotin) to establish the nature of their interaction with PCAF and other bromodomains.

Incubation of **8a1b**-biotin with purified PCAF followed by SDS-PAGE analysis of the protein clearly indicated the formation of a covalent adduct. To assess the selectivity of the PCAF interaction with respect to a crude proteome, the same reaction was performed with a dilution of PCAF (0.15 to 2.8 μM) into crude lysates (Figure 2A). PCAF was clearly

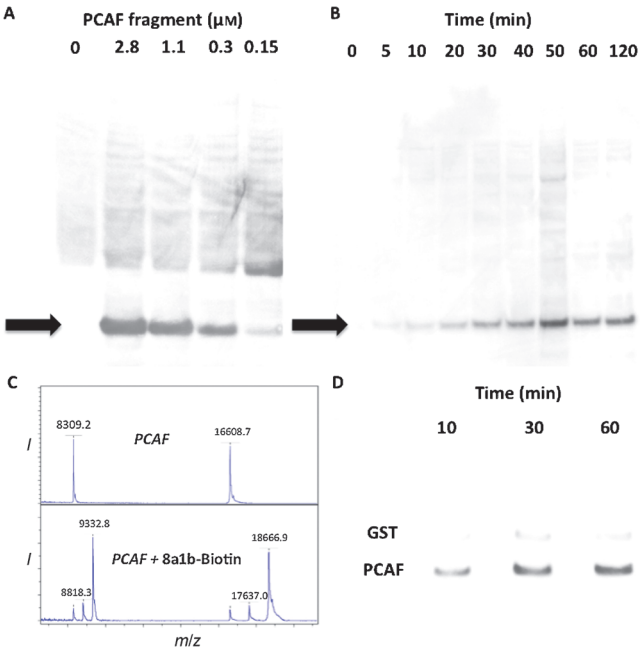

Figure 2. A) Labeling of PCAF with **8a1b**-biotin at decreasing dilution in crude lysates. PCAF was diluted from 2.8 μM to 0.15 μM in 10 μg of HeLa cell extract and treated with of **8a1b**-biotin (2 μM). B) Reaction time course for PCAF (0.4 μM) and **8a1b**-biotin (2 μM). C) MALDI analysis of the reaction between PCAF and **8a1b**-biotin. PCAF fragment (MW = 16608); 5 μM PCAF + 5 μM **8a1b**. D) Selectivity of **8a1b**-biotin for PCAF versus GST. 400 nM Protein was incubated with 2 μM **8a1b**-biotin followed by denaturing SDS-PAGE and western Blot with SA–HRP detection. SA = streptavidin, HRP = horseradish peroxidase.

labeled down to the lowest tested concentration (150 nM). A time course of the reaction between **8a1b**-biotin and PCAF under the same conditions indicated full conversion at 30 min (Figure 2B). MALDI analysis of the reaction product indeed showed the mass for the covalent adduct (Figure 2C). Based on the reported reactivity of ethacrynic acid with GST, we performed competition experiments for the labeling of PCAF versus GST. When both proteins were incubated at equimolar

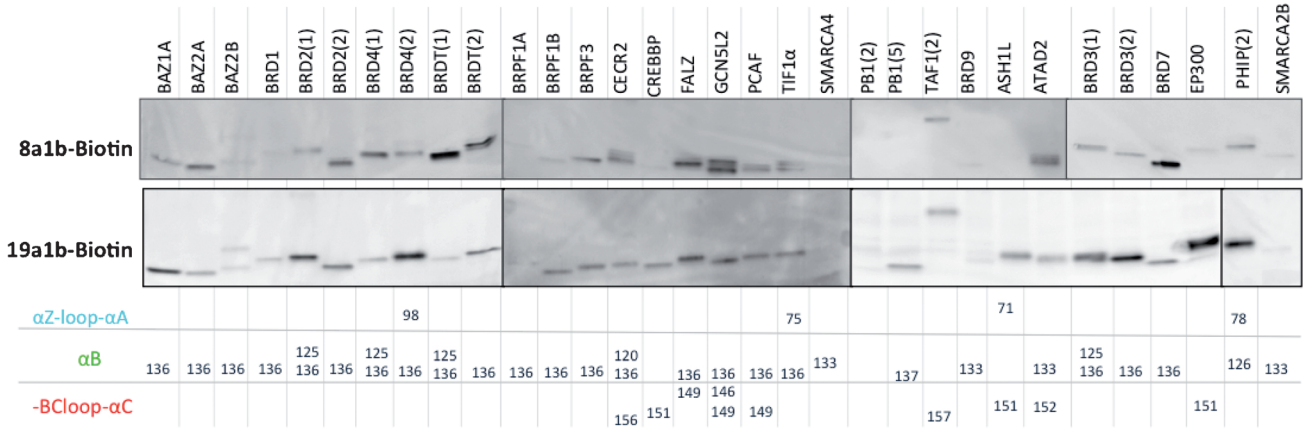

Figure 3. Labeling of 32 bromodomains with **8a1b**-biotin (top)and **19a1b**-biotin (middle), and positioning of cystein residue according to BRD4 numbering (bottom). Each bromodomain was incubated at 0.4 μM in presence of 10 μg HEk293 cell extract and 2 μM of probe for 30 min followed by denaturing SDS-PAGE and western Blot with SA–HRP detection (See Figure S2 for full gels).

concentrations (400 nM) with an excess of **8a1b**-biotin, PCAF was preferentially labeled at a ratio of more than 10:1 (Figure 2D). Furthermore, addition of a 5-fold molar excess of ethacrynic acid relatively to **8a1b**-biotin did not abrogate the labeling of PCAF with **8a1b**-biotin (Figure S1 in the Supporting Information).

We next compared the reactivity of **8a1b**-biotin and **19a1b**-biotin across a panel of 32 bromodomains (Figure 3) and analyzed the positioning of cysteine residues across human bromodomains by using a previously reported structure-guided sequence alignment[1] (Figure 4). Out of 61 bromodomains, 55 contain one or more cysteine residues distributed across three areas in the vicinity of the substrate binding site: the loop joining αZ and αA (cyan); the top of the αB helix (green), and the αC helix (red); see Table S1 in the Supporting Information for full sequence analysis. The most conserved cysteine residue is C136 (based on the numbering for BRD4),[1] which is present in 23 of the bromodomains. Within the tested panel, 20 bromodomains share the cysteine residue at position C136 (based on BRD4 numbering), 5 others have unique cysteine residues at a different position (C133, C137, C151, and C157), and 1 bromodomain does not possess a cysteine residue (PB1 (2), negative control). As shown in Figure 3, the negative control PB1(2), which lacks a cysteine residue, was not labeled, whereas other bromodomains were labeled to various extents with subtle differences between **8a1b**-biotin and **19a1b**-biotin. The fact that SMARCA2B, BAZ1A, PB1(5), CREBP, and TAF1(2), which have a single cysteine residues at positions 133, 136, 137, 151, and 157, respectively, all reacted with either **8a1b** or **19a1b** establishes the reactivity of each of these positions. While the extent of labeling varied across the tested bromodomains, these differences are not surprising considering that variations of the binding pocket must impact the affinity for the ligand and positioning of the cysteine trap. For instance, **19a1b** reacts faster with CREBBP than **8a1b**, whereas the opposite selectivity is observed with BRD7 (Figure 5), thus illustrating the potential to achieve selectivity for a given cysteine position with different pharmacophores.

We then assessed the utility of the probes to enrich bromodomains for proteomic analysis. HeLa cell lysates were supplemented with different amounts of recombinant PCAF

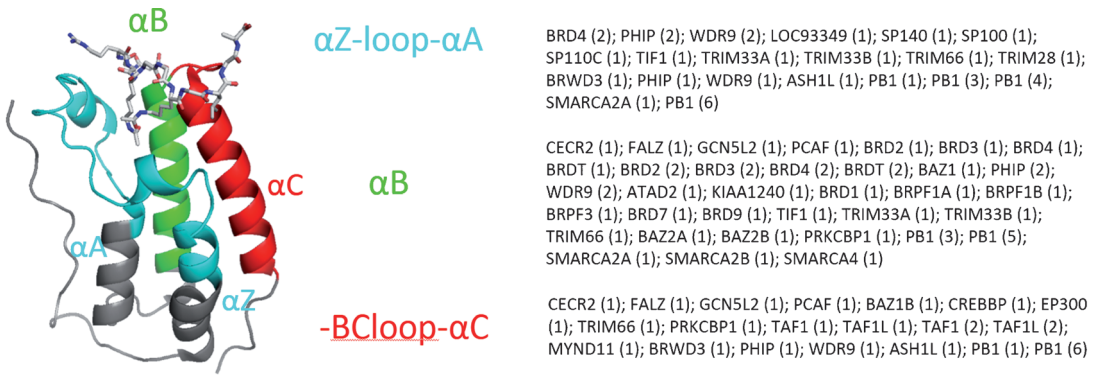

Figure 4. Positioning of the cysteine residues within the three loops of bromodomains. Left: Structure of BRD4 (1) in complex with a bis-acetylated lysine peptide (PDB ID: 3UVW) as a representative bromodomain structure showing the loop between the αZ and αA helices (cyan; the length and structure of this loop varies across the bromodomain family); the αB helix (green), and the BC loop and αC helix (red; the length of the BC loop varies across the bromodomain family).

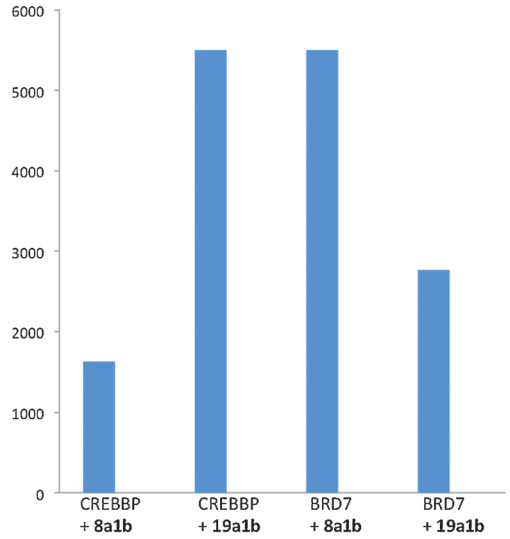

Figure 5. Quantification of CREBBP and BRD7 labeling with **8a1b** and **19a1b**. The labeling reactions were performed in parallel with the same condition as before (Figure 3).

bromodomains and treated with **8a1b**-biotin. The labeled proteins were enriched by using streptavidin-conjugated beads and then digested and analyzed by LC–MS/MS. PCAF was detected at all tested dilutions, but not in the control lacking **8a1b**-biotin. Moreover, endogenous PCAF in native non-supplemented lysates was also enriched by **8a1b**-biotin (Figure S3). This is noteworthy because endogenous PCAF is present in HeLa cells at very low concentrations,[27] thus establishing that the probe can enrich a low-abundance bromodomain through covalent trapping. Furthermore, the same experiment performed by preincubating the lysate with a 50-fold excess of **8a1b** abrogated the detection of PCAF. Aside from PCAF, TRIM28, BAZ1B, and SMARCA4 were also enriched from native HeLa extracts (Table S2). We next compared the performance of probes **8a1b**-biotin and **19a1b**-biotin in enriching bromodomains present in MV4-11 cells based on the fact that the growth of this cell line is inhibited by JQ1, a BRD4 ligand.[9,28] Gratifyingly, BRD4 was enriched by both probes along with 9 other bromodomains (TRIM28, TRIM33, SMARCA2, SMARCA4, ATAD2, BAZ1A, BAZ1B, PHIP, PB1, and GCN5L2), but not by a reduced analogue of **8a1b**-biotin lacking the Michael acceptor (Table S2). Moreover, one other bromodomain (BRWD3) was enriched exclusively by **19a1b**-biotin, thus highlighting the potential to modulate target selectivity through different pharmacophores. Small molecules that target GCN5L2 may find application in treatment of acute lymphoblastic leukemia (ALL)[29] and **19a1b** represents an excellent starting point for the development of selective GCN5L2 inhibitors. Furthermore, pretreatment of MV4-11 lysates with JQ1 led to a reduction in BRD4 enrichment by **8a1b**-biotin relatively to other bromodomains. This result is consistent with the results of a competition assay performed with purified protein (Figure S4). Finally, it must be noted that when using the probes, several other proteins were enriched that do not contain bromodomains, such as ANAPC7, ACOT7, TK1, ZAK, MAP2K7, and ALDH1A3. These proteins typically contain functional cysteine residues[30] and are also known targets of Michael-acceptor-based kinase inhibitors.[31]

In conclusion, DNA display of small molecule libraries was used to identify ethacrynic acid derivatives that form a covalent adduct with members of the bromodomain family. We established that at least five distinct positions for cysteine in the human bromodomain family (C133, C136, C137, C151, and C157) can be engaged covalently. This suggests that at least 40 out of the 61 bromodomains can be addressed with covalent inhibitors. While the identified compounds are relatively promiscuous across the tested bromodomains, we have demonstrated that different pharmacophores can bias the selectivity of covalent trapping. To the best of our knowledge, this is the first report of covalent bromodomain inhibitors. We have also demonstrated the suitability of **8a1b**-biotin and **19a1b**-biotin for the enrichment of bromodomains from crude lysates for proteomic analysis. Since bromodomains frequently act as part of larger complexes (e.g., SMARCA2/4 as part of the BAF or PCAF complex), these compounds could be useful tools for studying the role of these critical proteins in proteomic analysis. In light of their covalent engagement, the reported compounds are complementary to broad-spectrum bromodomain ligands such as bromosporin. We anticipate that the findings reported herein will inspire further development of covalent inhibitors through the combinination of specific pharmacophores with a Michael acceptor or other pharmacologically suitable cysteine traps. It is noteworthy that ethacrynic acid has recently been identified in a high throughput screen for EP300 inhibition[32] and that its butyl ester derivative was reported to induce apoptosis in leukemia cells through a pathway independent of GST inhibition.[33]

Keywords: activity-based proteomic profiling · bromodomains · covalent inhibitors · microarrays

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