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Rational Adaptation of L3MBTL1 Inhibitors to Create Small-Molecule Cbx7 Antagonists

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Chromobox homolog 7 (Cbx7) is an epigenetic modulator that is an important driver of multiple cancers. It is a methyl reader protein that operates by recognizing and binding to methylated lysine residues on specific partners. Herein we report our efforts to create low-molecular-weight inhibitors of Cbx7 by making rational structural adaptations to inhibitors of a different methyl reader protein, L3MBTL1, inhibitors that had previ-

ously been reported to be inactive against Cbx7. We evaluated each new inhibitor for Cbx7 inhibition by fluorescence polarization assay, and also confirmed the binding of selected inhibitors to Cbx7 by saturation-transfer difference NMR spectroscopy. This work identified multiple small-molecule inhibitors with modest (IC_{50} : 257–500 μ M) potency.

Introduction

One of the key mechanisms of controlling chromatin is through post-translational modifications (PTMs) on histones. These PTMs are called epigenetic marks, as they control gene expression without changing the underlying DNA sequence. Several PTMs have been identified on different residues of core histones including methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, glycosylation, and others (Figure 1a).^[1]

Writer, eraser, and reader proteins are three classes of proteins that add, remove, and recognize PTMs, respectively (Figure 1b). The orchestrated action of these protein classes defines chromatin structure and dynamics and influences gene expression by various mechanisms. Abnormalities and dysregulation of these protein classes are often associated with disease conditions.^[2] Pharmacological intervention of these protein classes has been a growing subject of research. Some of the writers (lysine methyltransferases and acetyltransferases) and erasers (histone demethylases and deacetylases) are validated therapeutic targets. Four different small-molecule inhibitors

targeting these protein classes have become FDA-approved drugs, and several more are in ongoing clinical trials.^[3] Readers, particularly acetyllysine reader proteins (bromodomains) have been successfully targeted with small-molecule inhibitors, and several of them are the subject of ongoing clinical trials for diverse malignancies, inflammation, atherosclerosis, and diabetes.^[3c,4] Despite the high abundance of structural data, increasing evidence for the participation of methyl readers in several diseases, and the predicted high druggability of methyl readers,^[5] the development of chemical inhibitors against them has lagged behind relative to other PTM-related protein classes. To date, a small subset of the over 200 methyl readers that are present in the human proteome have been targeted with chemical inhibitors. These include MBT (L3MBTL1, L3MBTL3),^[6] PHD (Pygo-2),^[7] Tudor domain (53BP1, Spindlin-1, UHRF1),^[8] chromodomains (Cbx4, Cbx6, Cbx7, Cbx8, CDYL),^[9] and WD40 domains (EED).^[10]

Methyl PTMs are generally installed by methyltransferase enzymes on side chains of basic amino acid residues [lysine (K), arginine (R), histidine (H)] of both histones and non-histone proteins. The side chains of lysine (K) residues can be mono-, di-, or trimethylated at the ϵ -amino group (Figure 2). Specific lysine methylation patterns, including the methylation status and position of a particular amino acid that is methylated, drive different biological outcomes such as activation or repression of gene expression.^[11] Multiple lines of evidence suggest that aberrant lysine methylation is linked to health conditions such as ageing and cancer.^[1c] In some of these cases, the biological outcomes require the recruitment of suitable reader proteins.^[12]

Methyllysine readers use a binding pocket called an “aromatic cage” to recognize the methylated lysine side chains.^[12a] The notable exceptions to this are PHD domains of CHD4 and TRIM33, which do not have an aromatic cage in their struc-

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Figure 1. Nucleosome, PTMs on H3 tail, and a cartoon representation of epigenetic writer, eraser, and reader proteins. a) Left: nucleosome, which is composed of DNA and a histone octamer. The histone octamer is made of two copies each of histone-3, histone-4, histone-2A, and histone-2B. Multiple histones have protruding tails that are the locations of several PTMs. Right: PTMs on the histone-3 tail are highlighted. PTMs including methylation (Me), acetylation (Ac), and phosphorylation (P) are specified on different lysine (K) and arginine (R) residues of histone-3. Methyl marks in green and blue indicate the positions associated with transcriptional repression and activation, respectively. General reader proteins of the PTMs are shown. b) Depiction of writer, eraser, and reader proteins on the H3 tail. The image of the nucleosome was generated with PDB ID 1AOI.^[14]

Figure 2. Different methylation states of lysine at physiological pH. Lysine (K) can be mono-, di-, or trimethylated on the ϵ -amino group.

tures.^[13] The size and composition of the aromatic cage of reader proteins play a critical role in accommodating different methylation states.

As the recognition of a specific methyl mark to a particular reader protein is crucial for a defined biological outcome, inhibiting reader proteins is a possible route to new therapeutics. Understanding the physiological basis for the recognition would be helpful to create inhibitors and chemical probes. High-quality chemical probes to a specific reader domain would reveal the target's functional roles in a cell of interest,

and validate its potential for therapeutic targeting with small molecules.^[15]

Chromobox homolog 7 (Cbx7) is a methyllysine reader protein that reads the H3K27me3 mark through its N-terminal chromodomain reader module. It belongs to the polycomb group of proteins, which play diverse roles in cancer and stem cell biology.^[16] Other human polycomb paralogs Cbx2, 4, 6, and 8 are also known to recognize the same marks. Cbx7 is the best-studied paralog and is a master controller of stem cell self-renewal and differentiation.^[17] Cbx7 expression is tissue- and tumor-specific, and it can be significantly up- or down-regulated in different malignancies.^[18,19]

The molecular basis of Cbx7 involvement has been best established in prostate cancer. At the molecular level, the tumor suppressor gene p16 (Ink4a locus) repression is initiated by a specific methyltransferase enzyme, EZH2, a PRC2 component, which trimethylates lysine 27 in histone-3 (H3K27me3) and creates a methylated site for Cbx7 to bind. Cbx7, a PRC1 component, uses its N-terminal chromodomain to bind H3K27me3 and the non-coding RNA ANRIL at distinct regions, which leads to transcriptional repression of the Ink4a locus.^[20] Methyllysine binding is key to the functions of Cbx7. The pro-growth effect of Cbx7 overexpression is neutralized in Cbx7 W35A mutants

that lose the ability to mediate Cbx7–H3K27me3 recognition.^[20] This led to the hypothesis that chemical inhibitors could be made to target the chromodomain of Cbx7 and compete for the formation of the Cbx7–H3K27me3 complex. These inhibitors should inhibit the multiple downstream biological consequences of Cbx7–H3K27me3 recognition.

Successful approaches to Cbx7 inhibition have been driven by peptide-based approaches^[9a,d–f,i] and high-throughput screening for small-molecule inhibitor approaches.^[9b,c] Small-molecule Cbx7 antagonists MS35472 and MS351 have been shown to de-repress the p16 gene in PC3 prostate cancer cells.^[9b,c]

Herein we report efforts to develop a class of small-molecule inhibitors for Cbx7 by adapting small-molecule antagonists of lethal (3) malignant brain tumor-like protein 1 (L3MBTL1). L3MBTL1 is an unrelated reader protein that selectively recognizes lower methylation states (Kme1, Kme2) of histones, and was the first methyl reader protein to be inhibited by small-molecule ligands.^[6a,d] Our cross-class approach was enabled by understanding the binding interfaces of Cbx7 and L3MBTL1 with their bound ligands (Cbx7–H3K27me3 and L3MBTL1–UNC669).

Results and Discussion

Design of Cbx7 antagonists

Analysis of co-crystal structures of bound Cbx7 and L3MBTL1 with their ligands suggests that the L3MBTL1 antagonists could be repurposed to target Cbx7 (Figure 3). Cbx7 has a wide and shallow binding site compared to the narrow and deep binding pocket of L3MBTL1. The Cbx7–H3K27me3 structure shows the Kme3 motif of the histone peptide in the aromatic cage of the protein (Phe11, Trp32, and Trp35), where binding is driven by a combination of cation– π interactions and the hydrophobic effect.^[21] The positive charge of the Kme3 group is stabilized by the carboxylate side chain of Glu43 (Figure 3a). Side chains that are upstream to Kme3 of the peptide engage in van der Waals and hydrophobic interactions with residues lining the β -groove of the protein. The peptide backbone participates in an extensive backbone-to-backbone β -sheet-like hydrogen-bonding network with residues (Val10–Phe11–Ala12) of the protein. The co-crystal structure of the inhibitor UNC669 with L3MBTL1 shows the pyrrolidine moiety of the inhibitor deeply buried in the protein's aromatic cage (Phe379, Trp382, and Tyr386), with binding mediated by van der Waals, cation– π , and salt-bridge interactions between the basic amine of the pyrrolidine and the side chain of Asp355. The 3-bromonicotinamide anchor of the antagonist stems out of the pocket through a conformationally rigid aliphatic linker and is solvent-exposed. We assume a similar binding mode for analogues UNC280, UNC280s, and UNC591 as that of UNC669 with L3MBTL1 (Table 1 and Figure 3b). The differences in the potencies toward L3MBTL1 are due to the length and type of aliphatic linker that is between the 3-bromonicotinamide anchor and the basic amine pyrrolidine.^[6c] Given the differences in the aromatic cages of L3MBTL1 and

Figure 3. Structural comparison of binding interfaces of Cbx7–H3K27me3 and L3MBTL1–UNC669. a) NMR-derived structure of H3K27me3-bound Cbx7, with an illustration of two important binding pockets of Cbx7: β -groove and aromatic cage. Left: Cbx7–H3K27me3 complex. Cbx7 is in surface representation, colored gray and the H3K27me3 peptide is in sticks, colored teal. The black arrows point to the β -groove, hydrophobic clasp, and an aromatic cage of Cbx7, and the residues of the peptide are annotated. Right: stick representation of the key residues of the protein portions that are circled at left. The backbone of the protein residues is removed for clarity. Yellow dotted lines represent the distance between trimethylammonium cation of the peptide and the acidic residues (Glu8/Glu43) of the protein. Blue dotted lines are hydrogen bonds. The crystal structure of the complex, PDB ID 2L1B, was used to generate the image. b) The left portion represents the bound structure of UNC669 with L3MBTL1; UNC669 is shown as sticks in cyan and protein as a surface in light-brown. The right shows a zoomed-in view of UNC669 interacting with binding pocket (aromatic cage) residues; PDB ID: 3P8H.

Table 1. Published binding data of L3MBTL1 inhibitors toward L3MBTL1 and Cbx7.

ID	Compound	IC ₅₀ [μ M] ^[a]	
		L3MBTL1	Cbx7
Entry 13 ^[6a]		17 $\pm$ 0.1	NA
UNC280 ^[6d]		46 $\pm$ 20	inactive
UNC669 ^[6d]		6 $\pm$ 0.5	inactive
UNC280s ^[6d]		25 $\pm$ 8 ^[b]	inactive
UNC926 ^[6e]		3.9 $\pm$ 1.1	> 30 (11%) ^[c]
UNC591 ^[6c]		NA	NA

[a] AlphaScreen assay was used to determine IC₅₀ values. Data are the average $\pm$ SD of at least two independent trials; NA: not available; inactive: IC₅₀ > 500 μ M. [b] UNC280s—our name for the sulfonamide analogue of UNC280—was not given a UNC designation in the original article; the published value is K_d. [c] For UNC926, only 11% inhibition was observed upon titration of 30 μ M compound into protein.

Cbx7, it is not surprising that none of the L3MBTL1 antagonists are active against Cbx7. Unlike L3MBTL1, Cbx7 does not have an acidic residue that is $\approx$ 4 Å from its aromatic cage residues;

consequently, tertiary ammonium-containing inhibitors cannot form salt-bridge interactions when they bind Cbx7. Cbx7 is known to preferentially bind quaternary ammonium ions over tertiary amines such as the pyrrolidine moiety of the L3MBTL1 antagonists.

On the basis of this structural analysis, we hypothesized that small-molecule inhibitors can be designed for Cbx7 by quaternarizing the basic amine of the L3MBTL1 antagonists to target the aromatic cage of Cbx7, while also adjusting the linker chemistry between the ammonium ion and aromatic elements of the inhibitors. Previously, similar design modifications on UNC280- and UNC280s-type scaffolds were applied to identify potent inhibitors for other methyl readers.^[6e, 8a] These studies did not yield potent inhibitors for Cbx7. We anticipated that the aromatic core of nicotinamide or arylsulfonamide could be positioned into the β -groove portion of Cbx7 using flexible aliphatic linkers such as those in entry 13, UNC280, UNC280s, and UNC591. With this in mind, nicotinamide and arylsulfonamide structures from UNC280 and UNC280s were chosen as scaffolds to repurpose them as Cbx7 antagonists. These scaffolds have three basic regions to explore: the basic amine, the aliphatic linker, and the aromatic core (Figure 4). A systematic plan involving quaternarization of the basic amine, altering the aliphatic linker, and optimization of the aromatic core on the both scaffolds was designed to achieve affinity toward Cbx7 (Figure 4). While being aware of the potential decrease in cell permeability that could be caused by quaternization, we were motivated by the chance to create small-molecule agents for an important target that has had mainly peptidic inhibitors published until now.

Figure 4. Design of small-molecule inhibitors of Cbx7 by adapting L3MBTL1 antagonists as scaffolds. a) Three possible areas of synthetic modifications on nicotinamide and sulfonamide scaffolds. b) Inhibitor design based on rigid linker nicotinamide scaffold.

Synthesis

The synthesis of the first set of nicotinamide analogues was achieved using commercially available 5-bromonicotinic acid (1). The reaction with 5-aminopentanol using HBTU in DMF provided the amide compound with a free pendant alcohol group (compound 2), which was then converted into the corresponding mesylated compound 3 by treating with methane-

sulfonyl chloride (MsCl) under basic conditions.^[6d] The mesylated compound 3 was used as the precursor material to make a series of cationic analogues 3A–H by reacting it with variety of cyclic, aliphatic, and aromatic tertiary amines (Scheme 1a). The same amide coupling conditions did not produce 6-bromonicotinamide pendant alcohol 5 from the corresponding 6-bromonicotinic acid 4, but a change to the coupling reagents EDCI/HOBt in DMF provided the compound in 20% yield (Scheme 1a). The mesylation product 6 and the corresponding quaternarized products 6E and 6F were prepared using the same conditions that were developed to prepare 5-bromonicotinamide acid analogues (Scheme 1a).

The synthesis of quaternarized sulfonamide analogues was started with a nucleophilic displacement of 3-bromoarylsulfonyl chloride (7) with 5-aminopentanol to make 8, followed by mesylation to produce a common intermediate 9. The series of quaternarized analogues 9A–F were prepared by reacting 9 with a variety of tertiary amines (Scheme 1b).^[6d] The same protocols were applied to make similar analogues of 2-bromo and 4-bromo regioisomers. Mesylation products of 3-bromo, 2-bromo, and 4-bromoarylsulfonamide analogues (9, 12, and 15, respectively) were isolated as pure products and hence the following quaternarization reactions were carried out without purification.

To investigate the effects of the flexibility of the aliphatic linker on the potencies of inhibitors, a small set of rigid-linker compounds UNC669 (17) and 19 were synthesized. The rigid linker 4-(1-pyrrolidinyl)piperidine (16) needed to make 17 was commercially available, and coupling it with 5-bromonicotinic acid (1) was conducted in a similar manner as above, resulting in a 91% yield (Scheme 1c).^[6d] To make compounds like 19, the methylated version of the rigid linker was necessary. The methylated rigid linker (18) was prepared through Boc protection on the secondary amine of 4-(1-pyrrolidinyl)piperidine, which allowed quaternarization with methyl iodide (CH_3I) on the nitrogen of the pyrrolidine (Scheme 1d). Workup of the reaction before CH_3I addition was not necessary, as the reaction contents do not interfere with the formation of the methylated product. Once methylated, product formation was confirmed by mass spectrometry on a sample of the reaction, the crude product was then Boc-deprotected with TFA/ CH_2Cl_2 , affording 18 over three steps in 93% yield. The reaction of 6-bromonicotinamide acid (4) and 18 successfully produced 19 with a 24% yield (Scheme 1d).

To explore the SAR of the aromatic core of the nicotinamide and sulfonamide scaffolds, the bromine was substituted with groups of different lipophilicities (phenyl and pyrimidinyl groups). Suzuki coupling reaction conditions between 6-bromonicotinic acid and corresponding arylboronic acid reaction partners were optimized (Scheme 2a). Purification attempts on these arylated nicotinic acids 20 and 23 were unsuccessful due to their high polarity and further synthetic steps were carried out on the crude materials. A similar synthetic approach was used to make compounds 22F and 25F (Scheme 2a). To prepare Suzuki-coupled sulfonamide analogues, the bromoarylsulfonamide with a free alcohol group was coupled with appropriate boronic acids to give 26, 28, and 30 (Scheme 2b). Sub-

Scheme 1. Synthetic routes to prepare modifications of the basic amine and altering the aliphatic linker of nicotinamide/sulfonamide scaffolds: a) 5-aminopentanol, HBTU, triethylamine, DMF, RT, 17 h (for the synthesis of **5**, EDCI-HCl and HOBt reagents were used instead of HBTU); b) MsCl, triethylamine, CH₂Cl₂, 0 °C → RT, 3 h; c) MeCN/THF, 70 °C, 17 h; d) 5-aminopentanol, triethylamine, CH₂Cl₂, RT, 3 h; e) HBTU, triethylamine, DMF, RT, 17 h; f) Boc-anhydride, CH₂Cl₂, 24 h, RT; g) diethyl ether, CH₃I, RT, 17 h; h) TFA, CH₂Cl₂, 2 h, RT; i) **4**, triethylamine, DMF, 5 min, RT.

Scheme 2. Synthesis of Suzuki-coupled products of nicotinamide/sulfonamide scaffolds: a) pyrimidine-5-boronic acid/phenylboronic acid, 2 N Na₂CO₃, [Pd(PPh₃)₄], THF, H₂O, 100 °C, 17 h; b) 5-aminopentanol, HBTU, triethylamine, DMF, RT, 17 h; c) MsCl, triethylamine, CH₂Cl₂, THF, 0 °C → RT, 3 h; d) MeCN/THF, isoquinoline, 70 °C, 17 h; e) R⁵-B(OH)₂, 2 N Cs₂CO₃, [Pd(PPh₃)₄], toluene/H₂O, 100 °C, 4 h; f) MsCl, triethylamine, CH₂Cl₂, 0 °C → RT, 3 h; g) K₂CO₃, benzyl bromide, DMF, RT, 17 h.

sequent steps were similar to those above to make compounds **27F**, **29F**, and **31F**.

N-Benzoylation of the sulfonamide was also carried out to increase the hydrophobicity of the compounds. N-Benzoylation of the sulfonamide nitrogen in 3-pyrimidinylarylsulfonamide ana-

logue **33F** was achieved by deprotonating the sulfonamide NH group of **30** with K₂CO₃, followed by addition of benzyl bromide to yield **32** (Scheme 2c). The corresponding mesylated product **33** and quaternarized product **33F** were prepared as described above.

Binding studies

We established a competitive FP assay to evaluate the binding affinities of the Cbx7 inhibitors. All biochemical assays in this paper use Cbx7 chromodomain, which has been shown to reproduce the binding activity of full-length Cbx7.^[9d] A direct titration of Cbx7 chromodomain into FITC-H3K27me3 provided a dissociation constant with a $K_d \approx 7.3 \mu\text{M}$ (Figure 5a). Next, we evaluated the binding affinities of synthesized inhibitors by establishing a competitive FP assay (Figure 5b). Inhibitors with a potency $\text{IC}_{50} > 7.3 \mu\text{M}$ can be measured by this FP assay.^[9a,22]

Figure 5. Determination of IC_{50} binding data of selected antagonists for the disruption of Cbx7 chromodomain–H3K27me3 complex by competitive FP assay. a) Binding curve of the direct titration of Cbx7 into FITC-H3K27me3 (FITC- β A-AARKme3SAPY-NH₂). b) Overlay of FP curves of inhibitors (**3F** and **9F**) and control (**17**). [Cbx7] = $10 \mu\text{M}$. Curve fitting was performed with GraphPad Prism. K_d determined for Cbx7–H3K27me3 is $7.3 \pm 1.25 \mu\text{M}$, and IC_{50} values for compounds **3F**, **9F**, and **17** were determined to be 1.3, 0.52, and $> 2 \text{ mM}$, respectively.

Tables 2–5 show the IC_{50} values for disruption of Cbx7 chromodomain–H3K27me3 for all compounds that were made. The first compounds in the series **3A** and **9A**, which are quaternarized versions of existing MBT antagonists UNC280 and UNC280s, demonstrated weak binding ($> 1000 \mu\text{M}$) to Cbx7. We also resynthesized and tested UNC280 and UNC280s themselves and confirmed that they have very weak activity against Cbx7 ($> 2500 \mu\text{M}$, see Figure S1). Next, we evaluated other quaternarized (basic amine modification) compounds (see Scheme 1a,b for synthesis and Table 2 for the binding data). The six-membered cyclic amine in compound **3B** decreases the binding, presumably because it is too bulky to be accommodated by the aromatic cage of Cbx7. Aliphatic amine modifications to both nicotinamide/sulfonamide scaffolds such as those in **3C**, **3D**, and **9D** showed no binding to Cbx7 even at the highest concentration tested ($> 2000 \mu\text{M}$). Aromatic ammonium modifications such as the pyridinium in **3E** and **9E** or the quinolinium in **3G** and **9G** of the nicotinamide and sulfonamide families showed weaker binding than the corresponding analogues **3A** and **9A**. The isoquinolinium modification on amide scaffold as in **3F** registered a small deterioration in binding to Cbx7, while the sulfonamide **9F** became the strongest binder in the sulfonamide series of inhibitors. Although the aromatic tertiary amine **3H** was the most potent among the nicotinamide series of inhibitors, its IC_{50} value is near to its highest concentration tested ($630 \mu\text{M}$). Hence this was not considered as a reliable result, and therefore this modification was not used in further steps to improve the potency of the nicoti-

Table 2. Binding data for compounds toward Cbx7–H3K27me3 as a function of various basic amine modifications on 3-bromonicotinamide/aryl-sulfonamide scaffolds.

R	$\text{IC}_{50} [\mu\text{M}]^{[a]}$	
	3	
A	930 ± 200	1720 ± 460
B	2870 ± 670	NA
C	$> 2100^{[b]}$	NA
D	$> 3500^{[b]}$	$> 2230^{[b]}$
E	1470 ± 350	2460 ± 790
F	1300 (960–1900) ^[c]	520 (400–670) ^[c]
G	1080 ± 350	NA
H	570 ± 90^b	NA

[a] Binding affinity values were determined by FP assay and are the mean of three independent trials; NA: not applicable, as the compound was not synthesized. [b] Values are the tested compound concentration against Cbx7; no binding was observed for these compounds within the concentration range tested. [c] Data for these two compounds were better fitted with asymmetric errors, which provide uncertainties as a 95 % confidence interval (CI) instead of a standard error.

namide framework. Additional molecules with different quaternarized basic amines on both scaffolds were synthesized, and all of them showed a weak binding profile, with IC_{50} values $\geq 2000 \mu\text{M}$ (see Supporting Information Figure S3).

Next, we studied the compounds that were designed to evaluate the influence of linker length and flexibility on the potencies of the ligands. Analogues of **3A**, **3F**, **9A**, and **9F** were synthesized with altered linker length between the aromatic core and the basic amines to smaller (four carbon) and longer (six carbon) chain lengths. None of these compounds showed significant improvement in their binding efficiency (binding data not shown). In contrast to L3MBTL1 ligands, rigidifying the linker between the aromatic core and the basic amine is detrimental for the potency of the ligands to Cbx7. The most potent L3MBTL1 compound UNC669/**17** and a quaternarized version of the *para* regioisomer **19** showed no binding affinity against Cbx7 (Table 3).^[6e] The lack of binding of UNC669/**17** to Cbx7 emphasizes the rationale of the inhibitor design of using lengthy aliphatic linker-containing scaffolds, and the results were consistent with previous observations from an AlphaScreen assay (Figure 4 and Table 1).^[6d] Overall, these results suggest that five carbon aliphatic chain linker is optimal for optimization efforts with both scaffolds. All the inhibitors in Tables 2 and 3 from both families are weak binders of Cbx7. To improve the potencies of the inhibitors we sought to find out what ligand moieties are in contact with the protein using saturation-transfer difference (STD) NMR and then capitalize further optimization efforts on the ligand moieties that are in contact with the protein

Table 3. IC₅₀ binding data of the rigid linker compounds toward Cbx7–H3K27me3.

ID	Compound	IC ₅₀ [μM] ^[a]
17/UNC669		> 4000
19		> 2370

[a] Binding affinity values were determined by FP assay and are the mean of three independent trials.

STD NMR is a ligand-based NMR technique that focuses on a ligand's proton signals and provides insight on what portion of the ligand binds to the protein.^[23] The prerequisite for STD NMR is that the binding under study should be in equilibrium with weak to medium (10^{-3} to 10^{-8} M) affinity,^[23] which made STD NMR^[23,24] ideal to study the binding of the current ligands to Cbx7 chromodomain.

STD NMR data of representative compounds **3D**, **3A**, **3F**, **9A**, and **9F** confirm that some of the portions of the ligands are interacting with protein (Figure 6). From these data, qualitative information pertaining to important binding elements were obtained by comparing the portions that do or do not interact closely with the protein within each ligand and also from comparison of STD NMR data between different ligands. The STD NMR spectra of **3F** and **9F** showed high STD signals for all the protons in the molecules, which indicates that all protons are in proximity to the residues of the protein. This could be because the aromatic isoquinolinium cation heads are directing the ligands toward the aromatic cage of Cbx7 through favorable π – π stacking and cation– π interactions and bringing the whole ligand closer to the protein. Consequently, all portions of the ligand signals appear in STD NMR (Figure 6a,b). In contrast, the STD NMR spectra of **3D**, **3A**, and **9A** indicate that when the nicotinamide/sulfonamide is attached to aliphatic cationic heads via aliphatic linkers, the cationic heads as well as aliphatic linkers make fewer or less favorable contacts with protein residues which results in low STD intensities (see STD signals of the aliphatic portions of the compounds and peaks in red box in Figure 6). This is perhaps because aliphatic cation heads lack π – π -stacking ability and are less prone to make cation– π interactions with the protein due to an inductive donation from alkyl groups to the positively charged nitrogen atom.

By comparing the STD NMR spectra of sets **3D** and **3A** with **3F**, and **9A** with **9F**, the relative intensities of the protons that are related to aliphatic linkers were high for **3F** and **9F**. These STD NMR observations are consistent with FP data of **3F** and **9F** which showed that these compounds are comparatively strong binders (Table 2). In summary, these STD NMR results provide structural confirmation that compounds arising from the quaternization approach on nicotinamide/sulfonamide scaffolds bind to Cbx7, and shed light on the importance of aromatic cationic head groups.

Next, we wanted to evaluate one of the STD NMR studied ligands, **9F**, to understand if it occupied the natural binding site of Cbx7, by performing competition STD NMR experiments with a known Cbx7 binder. For the competition study, an antagonist entry **62** was chosen because it binds Cbx7 with an IC₅₀ value of 6 μM under the same FP assay conditions, and has a K_d value of 0.2 μM, as determined by isothermal titration calorimetry.^[9a] Entry **62** is a close analogue of a compound that occupies the natural binding partner site of Cbx7 (H3K27me3) as determined by X-ray crystallography and 2D-HSQC NMR spectroscopy. STD NMR spectroscopy with entry **62** was performed, and the results indicated that almost all portions of the compound are in proximity to Cbx7 (spectra 40 in Supporting Information). A competition titration was carried out with a single-point titration of entry **62** into a mixture of **9F**–Cbx7, and the concentrations of both ligands were kept the same (≈ 2 mM; Figure 7). Contrary to expectations, competition STD NMR results showed a very small decrease in the intensity of the peaks of **9F** after addition of entry **62** into the mixture (Figure 7). In comparison with the aromatic core and isoquinolinium group of **9F**, an even lesser decrease in signal intensities was observed for the linker region (protons 8, 9–12, aliphatic region; Figure 7a). These results are probably because: 1) compound **62** competes and pushes **9F** elsewhere onto the protein surface, and/or 2) the concentration of both ligands used for the experiment, 2 mM, are far above their corresponding IC₅₀ values and therefore at this high concentration of compounds, both could have significant binding to Cbx7.

Based on the STD NMR results presented above, the choice of ammonium ion groups were limited to the pyrrolidinium and aromatic ammonium ion (isoquinolinium and others) containing cation heads as basic amine modifications in further optimization efforts on the nicotinamide/sulfonamide scaffolds. Other aromatic ammonium cation heads for basic amine modifications were considered (Table 2).

The optimization of the aromatic core was planned in two parts. First, the goal was to find suitable regioisomers of both scaffolds, and second, to substitute the bromine with suitable replacements by Suzuki coupling. Comparisons of binding data of compound sets such as **3E** vs. **6E**, **3F** vs. **6F**, **9E** vs. **15E**, and **9F** vs. **15F** indicated that regioisomers at the *para* position of both scaffolds slightly improved the potency of the ligands and suggested that bromine in the *para* position makes a specific interaction with protein (Tables 3 and 4). From these results, nicotinamide analogues are slightly more potent than sulfonamide analogues, and generalized selectivity across both nicotinamide and sulfonamide families of regioisomers is *para* > *meta* > *ortho*. We envisioned that substituting bromine with lipophilic groups such as phenyl and pyrimidinyl would better engage the ligand into the β -groove and consequently increase the potencies of the ligands. Accumulated data indicated that the isoquinoline group is a better basic amine modification than the others, so all subsequent compounds included isoquinoline in that position.

The binding data in Table 5 show that Suzuki coupling of ligands did not improve binding efficiency but gave insight into the tolerated positions for both scaffolds. Suzuki couplings,

Figure 6. Qualitative comparison of STD NMR spectra of compounds **3D** and **3A** with **3F**, and **9A** with **9F**. a) Overlay of reference NMR spectra with corresponding STD NMR spectra of the nicotinamide analogues **3D**, **3A**, and **3F**. Red boxes highlight the less intense STD signals of the corresponding ligand. These less intense signals are from adjacent CH₂ protons to the amide NH, and the nitrogen atom of cation head. b) Overlay of reference NMR spectra with corresponding STD NMR spectra of the sulfonamide analogues **9A** and **9F**. Protons of the compounds are labeled on the structures. Peaks labeled as 4/8, for example, means the peak is either related to protons at C4 or at C8. The reference spectra are shown in brown and STD spectra in green. All spectra are overlaid with respect to D₂O residual peak at 4.79 ppm. Control STD NMR spectra of representative ligands in the absence of protein showed no peak intensity. The peak at 2.5 ppm represents residual solvent, [D₆]DMSO.

Figure 7. Competitive STD NMR titration of the **9F**-Cbx7 chromodomain with a potent inhibitor **entry 62**. a) Overlay of STD NMR before (blue) and after (red) addition of the second inhibitor **62** to the mixture, **9F**-Cbx7 chromodomain. Aromatic (7.1–9.7 ppm) and aliphatic (1.1–4.6 ppm) regions of the spectra. b) Overlay of reference spectra of **9F** (bottom) and **entry 62** (middle), and STD spectra of the mixture of both components and Cbx7 chromodomain (top). All spectra are overlaid with respect to D_2O residual peak at 4.79 ppm. Peak 2.5 ppm represents residual solvent.

both phenyl and pyrimidinyl substitutions to the *para*-nicotinamide scaffold, **22F**, and **25F**, respectively, decreased binding ≥ 2 -fold relative to the corresponding bromonicotinamide parent **6F**. This suggests that at the *para* position, the nicotinamide scaffold cannot tolerate bigger groups. Contrary to *para*-nicotinamide substitutions, both phenyl (**27F**) and pyrimidinyl (**29F**) additions to the *para*-sulfonamide scaffold were accommodated with similar affinity to the corresponding parent **15F**. However, pyrimidine *meta*-substitution to sulfonamide (**31F**) decreased binding by > 3 -fold. Benzylation on the sulfonamide

NH was performed to increase hydrophobicity of the ligand, and the product **33F** showed > 5 -fold enhancement in binding and was the most potent inhibitor among all small-molecule inhibitors tested in this work. This improvement in the binding arose from the benzyl group as supported by STD NMR data. STD spectra of **33F** showed that the benzyl group of the molecule has larger STD signals (see Supporting Information). Compound **33F** was further tested against other Cbx chromodomains, using a pan-Cbx FP probe that we reported elsewhere.^[9h] We found immeasurably weak IC_{50} values ($> 2500 \mu M$) for

Table 4. Binding data of regioisomers of bromonicotinamide/arylsulfonamide scaffolds toward Cbx7–H3K27me3.

Fragment			IC_{50} [μM] ^[a]
		6	12	15
A		NA	1960 ± 520	NA
E		1290 ± 340	1600 ± 390	> 2000
F		420 ± 100	880 ± 270	430 ± 30
G		NA	780 ± 280	590 ± 30

[a] Binding affinity values were determined by FP assay and are the mean of three independent trials; NA: not applicable, as the compound was not synthesized.

Table 5. Binding data for compounds with multiple modifications on nicotinamide and sulfonamide scaffolds toward Cbx7–H3K27me3.

General structure	ID	R	IC_{50} [μM] ^[a]
6F	Br	420 ± 100
22F		850 ± 260
25F		> 1700
15F	Br	430 ± 30
27F		480 ± 80
29F		470 ± 50
9F	Br	520 (400–670) ^[b]
31F		1680 ± 930
33F		257 ± 20 ^[c]

[a] Binding affinity values were determined by FP assay and are the mean of three independent trials. [b] Data for the compound were better fitted with asymmetric errors, providing uncertainties reported as a 95% confidence interval (CI). [c] The IC_{50} value for **33F** is an average of two data sets from different batches of compound/FP probe/protein: a total of six replicates.

Cbx1 and Cbx6, and an IC_{50} of 1000 μM for Cbx8 (Figure S3). We caution against direct quantitative comparison of such IC_{50} values, as each Cbx has an intrinsically different affinity for the common probe ligand.

While any finding of weak-binding fragments and ligands raises concerns about nonspecific binding, we are reassured by the SAR that we do observe. The various Suzuki-coupled products show different results for *para* and *meta* substitutions. Also, the more hydrophobic/higher surface area biaryl products are not globally more potent, and are in fact generally less potent than their less hydrophobic aryl bromide analogues.

Overall, the effects of multiple modifications of the left hand, right hand, and linker elements on the both scaffolds are minimal, representing shallow SARs of the scaffolds. No single modification or a combination of modifications produced inhibitors with a potency of < 100 μM . The best single change was N-benylation on the sulfonamide which improved binding > 6-fold relative to its parent compound **9A** and af-

forded the most promising compound of all. Given the shallow SAR and overall low potency of these compounds, it is unlikely that doing further modifications to these both scaffolds would drastically increase the binding of inhibitors to the desired extent.

Conclusions

Non-peptidic small-molecule inhibitors have pharmacological advantages over peptide-based inhibitors and can be translated into clinical candidates in a shorter timeframe.^[25] With this in mind, we designed and synthesized small-molecule inhibitors for Cbx7 by adapting inhibitors of other methyllysine-binding domain containing protein L3MBTL1. The first series of compounds on both nicotinamide/sulfonamide scaffolds were synthesized by substituting different quaternarized amines as cationic heads. All these inhibitors exhibited weak binding inhibitory potential, > 500 μM , against Cbx7 (Table 2). To ascertain the binding elements of these inhibitors at the Cbx7 bind-

ing interface, we performed STD NMR of representative compounds **3D**, **3A**, **3F**, **9A**, and **9F** from both families. From both STD NMR and FP results, we optimized both scaffolds further resulting in compound **33F** with an IC_{50} of 257 μM , the most potent small-molecule inhibitor of the series.

The potency of compounds arising from this work is modest, but this work offers some lessons for Cbx inhibition. The conversion of tertiary ammonium ions in inhibitors that target Kme1/Kme2 readers into quaternary ammonium ions is effective at introducing some potency against Kme3-reading proteins. This work reports the first STD NMR data for chromodomain-containing proteins. STD NMR is useful for guiding inhibitor optimization in this class of proteins, where aromatic cage-targeting groups and groups that target the remainder of the reader proteins binding site offer distinct contributions to overall potency. State-of-the-art Cbx inhibitors fall into two groups: high-potency peptides and peptidomimetics, and low-potency small-molecule ligands. On an optimistic note, those low-potency small-molecule ligands include compounds like MS351, which has a 500 μM affinity in solution but which shows cellular activities at only 5 μM .^[9c] The lack of significant traction gained by the approaches in this paper, and by the approaches of others, makes it clear that the creation of potent, low molecular weight, non-peptidic inhibitors of Cbx proteins will continue to be challenging.

Experimental Section

Chemistry

Bromonicotinic acids, bromoarylsulfonyl chlorides, and other reagents used in the synthesis of small-molecule inhibitors were purchased from Sigma-Aldrich except boronic acids, which were purchased from TCI America. Any commercial materials used as received. Solvents for reactions and column/HPLC purifications, and deuterated solvents for NMR were purchased either from Thermo Scientific or Sigma-Aldrich.

^1H and ^{13}C NMR spectra were recorded using a Bruker AV300 spectrometer at 300.27 MHz and 75.50 MHz, respectively. Chemical shifts in ^1H and ^{13}C NMR spectra were expressed in parts per million (ppm) relative to trimethylsilane (TMS) and were referenced to residual protons of NMR solvents, CDCl_3 at 7.26 for δH and 77.6 for δC ; D_2O at 4.79 for δH ; CD_3OD at 3.30 for δH and 49.00 for δC ; and $[\text{D}_6]\text{DMSO}$ at 2.50 for δH . ^1H NMR data presented as: chemical shift, multiplicity (s=singlet, d=doublet, dd=doublet of doublet, ddd=doublet of doublet of doublet, dt=doublet of triplet, m=multiplet, brs=broad singlet), coupling constant J in Hertz, integration. Low-resolution electrospray ionization mass spectrometry (LR-ESI-MS) data were collected using a Finnegan LCQ-Trap. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data were acquired on Thermo Scientific Orbitrap Exactive Plus.

All compounds that were tested for in vitro binding were purified by HPLC. The crude compound of interest was dissolved in a mixture of HPLC solvents (0.1 % TFA in MeCN and 0.1 % TFA in H_2O), filtered through a syringe filter to remove un-dissolved particles and loaded onto a preparative HPLC column. Preparative reversed-phase HPLC was done on Alltech Apollo or Phenomenex Luna columns (C18, 10 μm particle size, 250 mm $\times$ 22 mm) using a gradient of 0.1 % TFA in H_2O and 0.1 % TFA in MeCN (90:10 to 10:90, $\text{H}_2\text{O}/$

MeCN). Pure fractions that were related to the product were combined and lyophilized. Any products purified by HPLC are isolated as their TFA salts. Compounds were characterized by minimum one of the following methods: LR-ESI-MS or HR-ESI-MS, ^1H NMR, and ^{13}C NMR.

Synthesis of all compounds and their characterization data can be found in the Supporting Information.

Protein expression and FP assay of the compounds

Expression of chromodomain of Cbx7 and its purification, and conducting FP experiments and data analysis of the compounds were performed in similar manner as described in the published reports.^[9a,f] Addgene plasmid 25241 for CBX7, deposited by C. Arrow-smith, Structural Genomics Consortium, Toronto (Canada), and encoding for Cbx7 chromodomain residues 7–62, was used for all biochemical binding studies.

Competitive FP analysis of compounds binding to Cbx7 was used to determine IC_{50} values with FITC-H3K27me3 (FITC- $\beta\text{A-AARKme3S-APY-NH}_2$) as the competitive binding probe. Compound was dissolved in distilled H_2O . The assay was carried out in NUNC black 96-well plates using the following buffer: 20 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 0.01 % Tween. The probe was at a constant concentration of 500 nM. Cbx7 protein was at a constant concentration of 10 μM . Averaged S and P values, minus values for buffer control wells, for each reading were determined by Microsoft Excel. Millipolarization units (mP) were calculated using the formula $[(S-P)/(S+P)] \times 1000$. For competitive FP, values of [inhibitor] and corresponding mP on x and y axes, respectively, were graphed and fitted with curve-fitting function in XLfit (add-in in Microsoft Excel) that uses the equation $C + [(BL_{\text{max}} \times x)/(K_d + x)]$. For direct FP, values of [Cbx7] on the x axis were used instead of values of [inhibitor]. S and P are parallel ($I_{\parallel}$) and perpendicular emissions ($I_{\perp}$), respectively. BL_{max} is the range of y values (mP values) the curve takes; K_d is the x ([Cbx7] (=10–20 μM) for direct FP and [inhibitor] for competitive FP, respectively) at which half the maximum value of y is attained. C is the y value when $x=0$. Where required, data analysis of poorly saturated curves was done with asymmetric errors. Values of [inhibitor] and corresponding mP on x and y axes, respectively, were graphed using GraphPad Prism 8 and fitted using a sigmoidal curve function from which IC_{50} values were extrapolated. The equation used was: $y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{x - \text{LogIC}_{50}})$. Errors are reported as 95 % confidence intervals.

STD NMR

Sample preparation: Buffer pD 7.8 (534 μL), inhibitor (6 μL of stock solution 200 mM in $[\text{D}_6]\text{DMSO}$), and Cbx7 (60 μL of stock solution 300 μM in buffer D_2O) were added to an NMR tube. Subsequently, the ^1H STD NMR spectrum was recorded at 600 MHz. Final volume of the sample in the tube 600 μL , and final concentrations of inhibitor, protein, and DMSO were 2 mM, 30 μM , and 1 %, respectively. All the solutions were kept cold throughout sample preparation.

For each inhibitor, a sample was prepared without protein, buffer pD 7.8 (594 μL) and inhibitor (6 μL of stock solution 200 mM in $[\text{D}_6]\text{DMSO}$) were added to an NMR tube. The ^1H NMR spectrum was recorded as reference, and ^1H STD NMR spectrum was recorded to check that there were no signals in the absence of protein.

^1H STD NMR spectroscopy: ^1H STD NMR experiments were performed on a Varian Inova 600 MHz spectrometer equipped with a

5 mm indirect detection probe head, at a temperature of 10 °C. Selective saturation was achieved by a train of Gauss-shaped pulses of 50 ms each, separated by a 0.1 ms delay. A number of 60 selective pulses were applied, leading to a total length of the saturation train of 3 s. The on-resonance irradiation on the protein was performed at a chemical shift of 0 or −2 ppm, and the off-resonance irradiation was set to −25 ppm, where no protein signals were present. NMR spectra were multiplied by an exponential line broadening function of 1 Hz prior to Fourier transformation. All spectra were recorded with a 20 ms spin-lock pulse, which minimizes the background protein resonances. The “DPFGSE sculpted solvent suppression” was enabled. The data were acquired interleaved with blocks of four scans. The spectra were subtracted manually in MestReNova.

For competition STD NMR titrations, entry **62** (6 μL of a 200 mM stock solution in [D₆]DMSO) was added to the NMR tube that contained inhibitor **9F** (6 μL of a 200 mM stock solution in [D₆]DMSO), buffer pD 7.8 (534 μL), and Cbx7 (60 μL of stock solution 300 μM in buffer D₂O). Final volume of the sample in the tube 606 μL, and final concentrations of **9F**, competitor **62**, protein, and DMSO were 1.98 mM, 1.98 mM, 30 μM, and 1%, respectively.

Abbreviations

AlphaScreen: amplified luminescent proximity homogeneous assay screen; CHD4: chromodomain-helicase-DNA binding protein 4; chromodomain: chromatin organization modifier domain; Cbx7: chromobox homolog 7; EDCI: *N*-(3-dimethylaminopropyl)-*N*¹-ethylcarbodiimide hydrochloride; EZH2: enhancer of zeste homolog 2; FDA: US Food and Drug Administration; FITC: fluorescein isothiocyanate; FP assay: fluorescence polarization assay; H2A: histone-2A; H2B: histone-2B; H3: histone-3; H3K9me3: trimethylated lysine 9 on the histone-3 tail; H3K27me3: trimethylated lysine 27 on the histone-3 tail; H4: histone-4; HBTU: *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate; HOBt: hydroxybenzotriazole; HR-ESI-MS: high-resolution electrospray ionization mass spectrometry; L3MBTL1: lethal (3) malignant brain tumor-like protein 1; PDB: Protein Data Bank; PHD: plant homeodomain; PRC1: polycomb repressive complex-1; PRC2: polycomb repressive complex-2; PTM: post-translational modification; SAR: structure-activity relationship; STD NMR: saturation-transfer difference NMR; TRIM33: tripartite motif-containing 33 protein.

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Conflict of interest

The authors declare no conflict of interest.

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