Structure-Guided Discovery of Selective Antagonists for the Chromodomain of Polycomb Repressive Protein CBX7

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S Supporting Information

ABSTRACT: The chromobox 7 (CBX7) protein of the polycomb repressive complex 1 (PRC1) functions to repress transcription of tumor suppressor *p16^{INK4a}* through long noncoding RNA, *ANRIL* (antisense noncoding RNA in the *INK4* locus) directed chromodomain (ChD) binding to trimethylated lysine 27 of histone H3 (H3K27me3), resulting in chromatin compaction at the *INK4a*/ARF locus. In this study, we report structure-guided discovery of two distinct classes of small-molecule antagonists for the CBX7ChD. Our Class A compounds, a series including analogues of the previously reported MS452, inhibit CBX7ChD/methyl-lysine binding by occupying the H3K27me3 peptide binding site, whereas our Class B compound, the newly discovered MS351, appears to inhibit H3K27me3 binding when CBX7ChD is bound to RNA. Our crystal structure of the CBX7ChD/MS351 complex reveals the molecular details of ligand recognition by the aromatic cage residues that typically engage in methyl-lysine binding. We further demonstrate that MS351 effectively induces transcriptional derepression of CBX7 target genes, including *p16^{INK4a}* in mouse embryonic stem cells and human prostate cancer PC3 cells. Thus, MS351 represents a new class of ChD antagonists that selectively targets the biologically active form of CBX7 of the PRC1 in long noncoding RNA- and H3K27me3-directed gene transcriptional repression.

KEYWORDS: Chromodomain, polycomb repressive complex, antagonist, gene transcription

Methyl-lysine recognition by the chromodomain (ChD) represents a fundamental mechanism in the epigenetic control of gene transcription in chromatin.^{1–3} One premier example involves histone H3 lysine 27 trimethylation (H3K27me3), an absolutely conserved epigenetic mark that signals gene transcriptional repression in all eukaryotes.⁴ Two conserved polycomb repressive protein complexes (PRCs) carry out H3K27me3-directed gene transcriptional repression, in that PRC2 first establishes H3K27me3 modification at a target gene site by its lysine methyltransferase EZH2,^{5,6} and PRC1 follows by binding H3K27me3 via a ChD of its chromobox (CBX) protein, which results in chromatin condensation and target gene repression.^{5–8} Recent studies from our laboratory and others show that long noncoding RNAs participate directly in gene transcriptional repression by facilitating recruitment of the PRCs to their target gene loci in chromatin.^{9–11} Dys-regulation of PRC proteins is linked to cancer etiology. Particularly, elevated levels of CBX7 and EZH2 are directly correlated to decreased expression of tumor suppressor *p16^{INK4a}* in prostate cancer cells as compared to

normal prostate epithelial cells, whose transcriptional repression is directly controlled by PRCs.^{12,13} To fully understand the mechanistic underpinnings of the regulatory capacity of H3K27me3-mediated protein–protein and protein–RNA interactions in gene transcriptional repression in chromatin, we need effective small-molecule chemical compounds capable of modulating the functions of the ChD in the cellular context. Given the functional versatility of the CBX ChDs, particularly CBX7ChD that also binds noncoding RNAs, we refer such small-molecule chemical compounds as antagonists, rather than simply inhibitors.

Potent peptide-based antagonists have been recently reported for the CBX ChDs,^{14,15} but they commonly suffer poor cell permeability, preventing them for functional study *in vivo*. To tackle this challenge, we conducted a high-throughput screen of approximately 100,000 drug-like compounds and

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discovered a promising ChD antagonist, MS452, that is capable of displacing the Polycomb group repressor CBX7 from its target gene locus and inducing transcriptional derepression of *p16^{INK4a}* in human prostate cancer PC3 cells.¹⁶ While this compound represented the first-in-class small-molecule chemical antagonist reported for the ChDs, its potency and selectivity required optimization to increase its usefulness as a chemical probe¹⁷ to study the mechanism of H3K27me3-directed transcriptional repression in biology and to validate CBX7 as a potential drug target for the treatment of castration-resistant prostate cancer.

Developing potent small-molecule chemical antagonists for the ChDs is much more challenging than it is for the acetyl-lysine binding bromodomains (BrDs), whose chemical inhibitors have greatly advanced our understanding of the role of BrD proteins in gene transcriptional activation.¹⁸ This challenge is due to the fact that ChDs recognize lysine-methylated histones with residues located in much shallower and extended protein surfaces. Even the aromatic cage residues for the methyl-lysine recognition are not well positioned in the free state, a sharp contrast to the well-formed acetyl-lysine binding pocket found in the BrDs.¹⁹ Indeed, the structural flexibility of CBX7ChD agrees with the two distinct conformations our lead compound MS452 can adopt when bound to the CBX7ChD¹⁶ (Figure 1A). Specifically, while the

Figure 1. Structure-guided optimization of MS452 series antagonists for the CBX7ChD. (A) Crystal structures of CBX7ChD bound to MS452 (also known as MS37452¹⁶) in *cis* or *trans* conformation. Right, details of molecular interactions of *trans*-MS452 bound to CBX7ChD. (B) *In vitro* SAR study of MS452 and its chemical analogues.

dimethoxybenzene ("A" ring) and piperazine ("B" ring) moieties of MS452 bind in the same way in both conformations, interacting with Phe11, Trp32, and Trp35 in the Kme binding aromatic cavity, or sandwiched between Phe11 and Trp32, respectively, the methyl-benzene moiety ("C" ring) adopts a *cis* or *trans* conformation with respect to the dimethoxy-benzene, hinged at the carbonyl that connects the "C" and "B" rings. The methylbenzene "C" ring of *trans*-MS452 appears to bind more securely than *cis*-MS452 within the CBX7ChD, as the former, positioned similarly to the H3K27me3 peptide, interacts with His47, Val13, and Glu43, whereas the latter swings upward into the protein, disrupting the β 1 strand (Figure 1A). *trans*-MS452 is further bolstered by water-mediated hydrogen bonding of ligand's two carbonyl groups with His5 (from His-tag) and Tyr39 in the aromatic cavity that embraces the dimethoxybenzene "A" ring. Notably, MS452 binds the CBX7ChD about 3-fold and >10-fold stronger than the ChDs of CBX4 or CBX2/6/8, respectively, and has almost no binding to the ChDs of CBX1/3/5 (also known HP1 $\beta/\gamma/\alpha$).¹⁶ These differences between the two subgroups are most likely due to Trp35 that is replaced by a Phe in HP1ChDs, thus weakening binding to the "A" ring in the aromatic cage. The preferred MS452 binding for CBX7

over CBX2/4/6/8 is due to the collective role of Tyr39, Val13, and His47 of CBX7, in that Val13 is replaced by an Ala in CBX2/6/8, and His47 by an Asn in CBX2/4/6/8, resulting in reduced interactions and a loss of imidazole aromatic stacking with the "C" ring, respectively. Therefore, we reasoned that stabilization of the *trans* conformation of the tolyl "C" ring through introduction of additional hydrogen bonding or hydrophobic interactions with the CBX7ChD would likely improve ligand affinity.

Toward this goal, we synthesized a set of compounds with different moieties attached to the tolyl ring using a synthetic scheme as described in detail in the [Supporting Information](#). Specifically, we synthetically introduced functional groups to the "C" ring that interact with the protein residues engaged in H3K27me3 peptide binding (Supplemental Scheme 1). This synthetic route starts by generating 1-(2,3-dimethoxybenzoyl) piperazine from Boc-protected piperazine and 2,3-dimethoxybenzoic acid, which then reacts with bromoacetyl chloride to yield 2-bromo-1-[4-(2,3-dimethoxybenzoyl) piperazin-1-yl]-ethan-1-one;^{20,21} the latter further reacts with an R-group-containing phenol to produce a final product.²² Using this scheme, we have explored the introduction of an alkyl group at the outward-facing *meta* position of the "C" ring, which is supported by the observed hydrophobic interactions with a Leu in a peptide ligand at this location.²³ Indeed, we observed a significant gain in affinity with a methyl group at this site, i.e., MS508, MS528, and MS521 vs MS501, MS527, and MS504, respectively (Figure 1B). In addition, we introduced an *para*-amine group in the "C" ring by utilizing 4-amino-3-methylphenol or 4-amino-3,5-xyleneol instead of *m*-cresol as a starting material, which extends the molecule at this position with amide, sulfonamide, or urea moieties. Notably, MS521, containing a urea moiety added to the free amine of MS508 with the reaction by Fuchs et al.,²⁴ shows 7-fold improvement in affinity over MS452 for CBX7ChD ($K_i = 4.8 \mu\text{M}$ vs $33.1 \mu\text{M}$), as determined by a fluorescence anisotropy binding assay¹⁶ (Figure S1A). This improvement in affinity is possibly due to a hydrogen bond interaction between the urea moiety and backbone oxygen of His47. The full MS452-derived series of compounds and their *in vitro* binding affinities for the CBX7ChD can be found in the [Supporting Information](#) (see Supplemental Table S1).

To establish a cellular mechanism of action, we sought to determine the efficacy of our newly synthesized ChD antagonists on CBX7 activity in the transcriptional repression of the *INK4/ARF* locus in human PC3 cells. To our surprise, we observed that the *in vitro* structure–activity relationship (SAR) data of these ChD antagonists did not correlate to their cellular activity in transcriptional derepression of *p16^{INK4a}* in PC3 cells relative to the potency of MS452, and that other top-performing compounds *in vitro* proved unsuitable for cellular assays (i.e., due to poor solubility or cell permeability) (see Supplemental Figure S1B and Table S1). The overall low cellular activity of this family of compounds, even among those that could enter the cell effectively, led us to reexamine our ligand development strategy. Given the fact that the CBX7ChD function in transcriptional repression of the *INK4/ARF* locus requires its binding to H3K27me3 as well as the lncRNA *ANRIL*, this suggests that the conformation of the methyl-lysine binding pocket of the CBX7ChD is influenced by allosteric binding of *ANRIL*, making it difficult to conduct lead optimization solely based on *in vitro* SAR study of CBX7ChD binding, i.e., without the noncoding RNA context.

This notion is supported by our discovery and characterization of a structurally distinct iminobenzimidazole compound, MS351, which we discovered initially from an *in silico* screen. We observed that MS351 selectively binds to the CBX7ChD alone with modest affinity (estimated K_d of approximately 500 μM , as determined by NMR titration), but does not bind ChDs from other CBX proteins including CBX1/3/5 (HP1 β / γ / α) and CBX2/4/6/8 (Figure 2A and Supplemental Figure S2A),

Figure 2. Structure-enabled discovery of MS351 as a novel CBX7ChD antagonist. (A) NMR spectral characterization of MS351 binding to the CBX7ChD, as assessed by 2D ^1H - ^{15}N HSQC spectra of the ^{15}N -labeled CBX7ChD (200 μM) depicting chemical shift perturbations of protein residues upon addition of MS351 (200 μM). Residues exhibiting major perturbations are annotated. (B) Fluorescence anisotropy assay assessing relative binding affinity of CBX7ChD to RNA, H3K27me3 (aa 21–33), SETDB1-K1170me3 (aa 1165–1174), MS452, or MS351. A FITC-labeled loop C of ANRIL (10 nM) was used as an assay probe as described in detail in our previous study.⁹ (C) Dose-dependent effects of MS351 on transcriptional derepression of $p16^{\text{INK4a}}$ after the 12 h compound treatment (1–5 μM) in human prostate cancer PC3 cells. $HIF1a$, a non-CBX7 target gene, was used as a control in this study. (D) Upper panel, immunoblots of $p16^{\text{INK4a}}$ of mouse embryoid bodies (EBs) following 8 days of leukemia inhibitor factor (LIF) in the presence of MS351. Lower panel, $p16^{\text{INK4a}}$ mRNA level measured by qPCR with TaqMan probe set of RNA obtained from mouse embryonic stem cells treated with or without MS351. (E) Upper panel indicates enrichment of Cbx7 in mouse embryonic stem (ES) cells. Alignments were plotted on UCSC Genome tracks to depict chromosome 2 and enrichment along the axis of chromosome 2 directed from the centromere based on existing Cbx7 ChIP-Seq data.²⁵ Enrichments are valued as peak calls through alignments of redundant sequence reads using BOWTIE to create WIG files and plotted using GALAXY and cis-genome software algorithms. Lower left panel, qPCR to measure $Hoxd3$ and $Noggin$ mRNA following treatment of MS351 (0.5 μM) at the time points indicated. Lower right panel, direct ChIP analysis of Cbx7 and histone H3 at the transcript start site of mouse $Hoxd3$. $Gapdh$, a non-CBX7 target gene in mESCs, was used as a control for both qPCR and ChIP experiments.

or the bromodomains of BRD4 (Supplemental Figure S2B). Strikingly, our fluorescence anisotropy binding study reveals that in contrast to MS452, MS351 appears to have the unique ability to bind and form a ternary complex with CBX7ChD and a predicated hairpin RNA derived from ANRIL⁹ with affinity K_d of 23.8 μM (Figure 2B), which is a 21-fold higher than its affinity to the free protein. An increase of anisotropy signal was observed in our previous study when H3K27me3 was added to CBX7ChD in complex with the FITC-labeled ANRIL RNA, or when ANRIL RNA was added to CBX7ChD with the FITC-labeled H3K27me3 peptide;⁹ and this effect is even more pronounced with a SETDB1-K1170me3 peptide than the H3K27me3 peptide (Figure 2B and Supplemental Figure S2C) owing to SETDB1 peptide's higher affinity to the CBX7ChD than the H3K27me3 peptide.¹⁶ Additionally, MS351 enhanced RNA binding to CBX7ChD in a dose dependent manner, as shown in fluorescence anisotropy binding assay, whereas MS452 or the H3K27me3 peptide did not have such an effect (Supplemental Figure S2D). Notably, the ternary complex appears sensitive to the stoichiometry of the lysine-methylated peptide and ANRIL RNA, as an increasing peptide over a certain level causes a decrease of the anisotropy signal. This observation is consistent with a mutually allosteric influence of the H3K27me3 and ANRIL binding to the CBX7ChD in gene transcriptional repression, as reported in our previous study;⁹ it also indicates that MS351 likely blocks H3K27me3 binding to the CBX7ChD in its functionally active form, i.e., bound to RNA. Indeed, we observed that MS351 can effectively induce transcriptional derepression of $p16^{\text{INK4a}}$ in human prostate cancer PC3 cells in a dose-dependent manner at 1–5 μM , but not a non-CBX7 target gene $HIF1a$, as a control (Figure 2C).

We further established the cellular efficacy of MS351 (1–2 μM) against CBX7 activity in transcriptional repression of $p16^{\text{INK4a}}$ in mouse embryonic stem cells, as demonstrated by both mRNA transcript and protein expression levels (Figure 2D). Consistently, we confirmed that MS351 even at 0.5 μM effectively induced derepression of other CBX7 target genes including $Hoxd3$ and $Noggin$ in mouse ES cells and that $Hoxd3$ derepression is correlated well with MS351 treatment-dependent reduction of CBX7 occupancy at the $Hoxd3$ gene locus, as shown by ChIP analysis (Figure 2E). MS351 has almost no effects on transcriptional expression of non-CBX7 target genes such as $Gapdh$ in mouse ES cells. Collectively, our results confirm the cellular on-target activity of MS351 in disrupting H3K27me3-mediated CBX7 binding to target genes in chromatin such as the $INK4A/ARF$ gene locus likely in the context of noncoding RNA binding, resulting in transcriptional derepression of $p16^{\text{INK4a}}$ in human PC3 and mouse ES cells.

We next determined a crystal structure of the CBX7ChD/MS351 complex, which reveals that the main determinant of MS351 binding is likely its iminobenzimidazole core interacting with the aromatic cage residues Phe11, Trp32, and Trp35 that normally house the methyl-lysine of the bound H3K27me3 peptide (Figure 3A and Supplemental Table S2). This "B" ring is aligned nearly perfectly with Phe11 (which undergoes a side-chain rotation from its H3K27me3-bound form) forming π - π stacking interactions. The iminobenzimidazole is aligned perpendicular to the indole ring of Trp32, adding to the strength of MS351 interactions with the aromatic cage. The local conformational rearrangement of Phe11 is likely significant for the allosteric influence between the H3K27me3 and RNA binding, as this residue is located in the N-terminal segment connected to the $\beta 1$ - $\beta 1'$ strand where the key RNA

Figure 3. Molecular basis of MS351 recognition by the CBX7ChD. (A) Comparison of crystal structures of the CBX7ChD binding to MS351 (yellow) or H3K27me3 peptide (magenta) in the aromatic cage. The protein is colored in pink and cyan for the MS351 or H3K27me3-bound form, respectively. (B) Structural insights of MS351 interactions with Thr41 and Glu43 of β_3 strand that bridges the H3K27me3 binding aromatic cage and noncoding RNA ANRIL binding site involving residues located on the β_1' – β_2 – β_3 sheet. Note that Arg17 was identified to be one of the key residues essential for ANRIL binding.⁹ (C) Schematic illustration of the role of CBX7 of the PRC1 in noncoding RNA ANRIL and H3K27me3-mediated gene transcription, and the distinct modes of action of MS452 (blue hexagon) and MS351 (purple pentagon) in modulating CBX7 in this functional context.

binding residue Arg17 is located^{9,16} (Figure 3B). Additionally, the hydroxyl group in the linker between the “B” and “C” rings of MS351 is pointed toward the side-chain hydroxyl of Thr41 and carboxylate of Glu43 in the β_3 strand in CBX7ChD. Indeed, one enantiomer of MS351 with a distinct chiral configuration at this hydroxyl group, obtained after chiral chromatographic separation of MS351, appears to be more active than the other enantiomer in inducing derepression of $p16^{\text{INK4a}}$ in human PC3 (see Figure S3). Notably, Ser40 and Thr41 are two key residues that likely participate in mediating the allosteric influences between H3K27me3 and RNA binding, as Ser40 sits right between Trp32 and Arg17, whereas the side-chain hydroxyl group of Thr41 forms a strong 2.7 Å hydrogen-bond to the indole nitrogen of Trp32. As such, these residues were observed to exhibit major chemical shift perturbations upon binding to H3K27me3, RNA,^{9,16} or MS351.

Finally, our crystal structure suggests a direction for chemical optimization of MS351. For instance, a conformationally constrained linker, preferably with a chiral center as in MS351, connecting the iminobenzimidazole (“B” ring) and dichlorobenzene (“C” ring) moieties will likely enhance target interactions. Additionally, given the iminobenzimidazole ring is engulfed within the aromatic cage, certain substituent groups may optimize its interactions with the cage residues under the allosteric influence of noncoding RNA ANRIL binding on the other side of the protein. Importantly, because the molecular detail of the noncoding RNA ANRIL binding to the CBX7ChD remains elusive, our study strongly argues that cell-based SAR study of compound effects on transcriptional derepression of $p16^{\text{INK4a}}$, as shown here, is more functionally relevant to guide lead optimization than merely *in vitro* biochemical binding.

In summary, we report here the structure-guided discovery of two distinct classes of small-molecule antagonists for the CBX7ChD (Figure 3C). Class A MS452-derived compounds inhibit CBX7ChD/methyl-lysine binding by blocking H3K27me3 binding, whereas the Class B compound MS351 appears to be uniquely capable of inhibiting CBX7ChD binding to H3K27me3 when the protein is bound to RNA. Our study highlights the importance of developing selective small-molecule chemical antagonists for its target protein in a biologically active form. This is achieved not only by detailed structural analysis of ligand recognition *in vitro*, but also more importantly by cell-based SAR study of lead compounds in a functional context. This mechanism-guided strategy is demonstrated here in our discovery of MS351, which can effectively induce transcriptional derepression of CBX7 target genes including $p16^{\text{INK4a}}$ in mouse ES cells and human prostate cancer PC3 cells. Thus, MS351 represents a promising lead compound of a unique class of ChD antagonists that can be further optimized to selectively target CBX7ChD in its biologically active form in long noncoding RNA- and H3K27me3-directed gene transcriptional repression in biology and disease.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmmedchemlett.6b00042.

Materials and instruments used, synthesis, and spectroscopic details of compounds and biological assays (PDF)

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Author Contributions

^{||}These authors contributed equally to this study. C.R., S.G.S., M.J.W., and M.-M.Z. designed the experiments. C.R. performed biochemical and structural analyses as well as cellular characterization of the compounds in PC3 cells. S.G.S. conducted design, synthesis and SAR study of the compounds. M.M. did *in silico* chemical screening. K.Y., S.D.L., J.L., Y.R., and A.S.V. participated in various aspects of the biochemical and cellular characterization, as well as ligand design and synthesis. S.D.L., F.A. and M.W. performed cellular studies in mES cells. All authors contributed to the writing of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BrD, bromodomain; CBX7, chromobox 7; ChD, chromodomain; PRC1, polycomb repressive complex 1; H3K27me3,

trimethylated lysine 27 of histone H3; SAR, structure–activity relationship

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