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CHEMICAL GENETIC AND EPIGENETICS:

Chemical probes for methyl lysine reader domains

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Abstract

The primary intent of a chemical probe is to establish the relationship between a molecular target, usually a protein whose function is modulated by the probe, and the biological consequences of that modulation. In order to fulfill this purpose, a chemical probe must be profiled for selectivity, mechanism of action, and cellular activity, as the cell is the minimal system in which 'biology' can be explored. This review provides a brief overview of progress toward chemical probes for methyl lysine reader domains with a focus on recent progress targeting chromodomains.

Introduction

Advances in understanding the regulation of chromatin accessibility via post-translational modifications (PTMs) of histones have rejuvenated drug discovery directed toward modulation of transcription as the opportunities for pharmacological intervention are significantly better than direct perturbation of transcription factors [1–3]. Chemical biology is poised to play a central role in advancing scientific knowledge and assessing therapeutic opportunities in chromatin regulation. Specifically, cell penetrant, high-quality chemical probes that influence chromatin state are of great significance [4,5]. The advantages of a small molecule driven approach to modulating chromatin biology are numerous: temporal resolution; mechanistic flexibility (targeting a specific activity of a protein as opposed to ablating them all via DNA editing and RNA-interference techniques)[6]; ease of delivery; and most significantly, a small molecule tool has the potential to provide an immediate transition to a drug discovery effort, possibly cutting years off the time between target validation and therapeutic intervention [7,8].

While the enzymes that perform PTMs on histones are an important and precedented class of druggable targets [1,9], the biological consequences of many PTMs result from their recruitment of regulatory machinery via protein-protein interactions (PPI) directly facilitated by the PTM [10]. The binding domains involved in PTM recognition on chromatin are

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referred to as “readers”. We and others have been focused on exploration of the chemical biology of readers of methyl-lysine (Kme) as this PTM plays a central role in chromatin regulation and more than 200 Kme reader domains within several protein families occur within the human proteome, making this a large and relatively unexplored target-class for probe discovery [11–21].

Probe Validation

Characterization of selectivity and cellular target engagement are both essential and challenging aspects of probe validation [7,16]. In the case of the enzymes that regulate chromatin state, a knockdown of the target by siRNA, shRNA or gene editing directly perturbs a PTM that can be readily monitored at either a global level or at a specific gene locus [1,9]. For Kme readers genetic manipulations tend to result in biochemical or phenotypic outcomes that are less easily attributed to specific biochemical changes at the level of chromatin. Additionally, since most Kme readers occur in the context of multi-domain and hence multi-functional proteins, there is no *a priori* basis to expect that pharmacologic antagonism of the Kme reader function will be equivalent to the removal of the whole protein in which it is embedded.[6] For this reason, initial assessments of chromatin reader antagonism have frequently relied upon the effect of the probe on the localization or mobility of a tagged version of its reader target expressed in a cell of interest. This approach has been applied to bromodomains [22,23] and Kme readers in our own work [19]. While changes in target localization gives a readout that is both proximal to chromatin and logically attributable to the likely mechanism of action of the ligand, this phenomenology is difficult to relate to any specific biological function of the endogenous reader and does not directly establish a molecular pathway connection to phenotypic effects [15]. New technologies to assess cellular target engagement could have a significant impact on validation of Kme reader probes [24].

Selectivity assessment is perhaps the most important aspect of chemical probe characterization, and unfortunately, one that is often lacking in the literature [4,7,25]. While single-target specificity is not an absolute requirement, sufficient profiling data to confidently attribute *in vivo* effects to the *in vitro* profile of a probe are essential. We have attempted to address this for Kme readers (in collaboration with the Bedford lab at MD Anderson) by evaluating the binding of biotinylated versions of Kme reader probes to a nitro-cellulose membrane upon which hundreds of potential chromatin-associated effector domains have been spotted [26]. Binding is then observed with a streptavidin-dye conjugate and positive results are followed up via quantitative measurements in solution by isothermal titration calorimetry (ITC) [13,18]. In addition to assessing selectivity versus Kme reader proteins, probes must be profiled versus the enzyme families that modify lysine (PKMTs, lysine demethylases), as activity here would be likely to confound interpretation of both chromatin biochemical readouts and phenotypic outcomes. There is also a chemical logic for screening against these targets since Kme reader probes may mimic the substrates of these enzymes. Profiling versus general pharmacology panels is also performed in order to create a more complete assessment of potential off-target activities. While this data cannot rule out contributions from unexamined or unknown protein off-targets to a probe’s activity, it does support the case for specificity when cellular target-engagement has also been proven.[7]

Additionally, in the absence of comprehensive profiling data against all possible cellular targets, the use of a close structural analogue as an inactive control compound that lacks biochemical target activity is critical in order to establish a correlation between on-target *in vitro* activity and cellular effects [25].

Chemical Strategies

Kme binding sites are generally made up of an aromatic cage involving 3 to 4 aromatic amino acids, and often an acidic residue to hydrogen bond to the Kme cation in the case of mono- and dimethyllysine (Kme1,2) recognition, or simply to balance the charge in the case of Kme3 [27,28]. The Patel lab introduced a useful division of Kme readers into “cavity insertion” versus “surface groove” binders [29], and subsequent work toward chemical probes has been informed by this ontology and confirmed its relevance to ligand design. Table 1 illustrates chemical strategies and principles applied thus far to the discovery of Kme reader antagonists.

Our initial work focused on ligands for the MBT domains which utilize a cavity insertion recognition mode and led to the successful design of the first chemical probe for Kme readers, UNC1215 [19]. However, the relatively high affinity of UNC1215 for L3MBTL3 (K_d = 120 nM) depends upon a unique 2:2 dimer-binding mode that may not be accessible or relevant to other Kme reader targets. Based on our current experience, L3MBTL3 is the only MBT domain that elicits this recognition mode [30], although we continue to work toward Kme reader ligand engagement via dimerization, particularly in cases where this binding mechanism may be relevant *in vivo*, such as 53BP1 [14]. While we and others have reported weak Kme ligands discovered by high throughput screening (HTS) of diversity libraries [31–35], no hits from HTS have so far been optimized to potent chemical probes and our own experience suggests that many HTS hits are false positives that fail to confirm in biophysical assays. Kme cavity insertion binders have been largely targeted via traditional small molecule strategies (select inhibitors shown in Table 2) and this work has been summarized previously [15,36], while more recent progress toward Kme reader probes has resulted from ligand discovery efforts towards surface groove binding Kme readers and will be reviewed herein.

Peptidomimetic Chromodomain Inhibitors

Cavity insertion binding events between Kme readers and their cognate substrates are predominantly driven by localized interactions between the methyl-ammonium and a deep protein cleft, making them candidates for intervention with drug-like small molecules, whereas surface groove interactions cover a much larger area and rely on contacts with a number of adjacent residues in addition to Kme [29]. Consequently, in recent efforts targeting chromodomains, which are characterized by an induced fit, surface groove interaction with methylated histone substrates, we and others have employed peptide mimetic discovery strategies and scaffolds for the successful development of chromodomain inhibitors [17,18,37]. These efforts have resulted in inhibitors of the polycomb (Pc) chromodomains, well known for their participation in Polycomb repressive complex 1 (PRC1) [38]. PRC1 is one of the major chromatin regulatory complexes involved in

repression of gene transcription, and as such, plays a central role in differentiation and development [39,40]. The CBX chromodomains facilitate PRC1-mediated transcriptional repression by targeting the complex to the H3K27me3 mark. Consequently, PRC1 chromodomain chemical probes could be employed to explore the therapeutic potential of CBX chromodomains, investigate pharmacologic synergy with inhibitors of the H3K27me3 methyltransferase, EZH2 [41], and understand the precise roles of CBX target genes in different cancer types.

There are five human proteins that belong to the polycomb chromodomain family: CBX2, -4, -6, -7, and -8; all of which share a high level of sequence identity within their chromodomains, suggesting that development of selective inhibitors could be a challenge. Ligand design must also take into account the shallow aromatic cage that binds Kme3 and the continuous β-sheet-like interaction that includes a sequence of hydrogen bonds with the histone backbone; therefore development of peptidomimetic ligands has been pursued, despite the anticipated difficulties in cell permeability.

Hof and co-workers reported the first inhibitors of a chromodomain, CBX7, derived from a five-residue peptide sequence (FALKme3S) from the protein SETDB1, which was reported to have slightly higher *in vitro* affinity for CBX7 than the H3K27me3 sequence itself [17,42]. They performed a systematic study in which each amino acid of the parent peptide was substituted with both natural and unnatural residues, and the affinity of these and subsequently derived ligands for CBX7 was determined by fluorescence polarization and ITC. Iterative ligand design was further guided by X-ray crystallography and 2D NMR structural studies of ligands bound to CBX7. Upon evaluation of the tolerance of each site to modification, select compounds bearing multiple well-tolerated substitutions were prepared. This ultimately led to compound **64** (Figure 1a), which binds CBX7 with a K_d of approximately 200 nM and bears a number of modifications relative to the FALKme3S sequence. The most significant potency enhancement results from a benzoic amide at the N-terminus. The affinity of compound **64** for three other chromodomains was determined; resulting in 10-fold selectivity over CBX8, equal potency for CBX4, and no measurable affinity for the HP1 family chromodomain, CBX1.

More recently, the Hof group has elegantly demonstrated the ability of peptidomimetics to achieve selectivity within the family of polycomb CBX chromodomains. Structural knowledge of the features broadly required for CBX binding led to modification of the residue two amino acids from the Kme3 site (−2 position) which resulted in enhanced CBX selectivity [37]. Fluorescently labeled ligands were profiled against CBX2, -4, -6, -7, -8, and the HP1 paralog CBX1 via direct fluorescent polarization (FP) titrations. Inclusion of an isopropyl group in compound **5** at the (−2) position resulted in 90-, 20-, 18-, 6-, and 7-fold selectivity for CBX6 over CBX1, -2, -4, -7, and -8, respectively, accompanied by only a 3-fold loss in potency for CBX6 (K_d = 0.9 μM) relative to the analogous alanine containing compound (Figure 1a). This reveals that subtle modifications to this ligand scaffold are able to greatly diminish CBX7 binding and clearly demonstrates that occupying binding sites other than the Kme3-binding aromatic cage can modulate potency and selectivity.

Concurrent to the efforts of the Hof group, we initiated a program to discover high-quality chemical probes of the polycomb CBX chromodomains based on their known disease relevance, guided largely by molecular dynamics simulations, cocrystallization studies, and structure-activity relationships. This led to the design and characterization of UNC3866 (Figure 1), which binds the chromodomains of CBX4 and CBX7 most potently (K_d ~100nM), and is 6- to 18-fold selective as compared to the other polycomb CBXs [18]. Knowledge of the activity profile of a molecule is essential in order to associate its cellular effects with the modulation of a specific molecular target(s); therefore, UNC3866 was evaluated against >250 other protein targets including Kme readers, bromodomains (acetyllysine reader proteins), protein methyltransferases and demethylases, and GPCRs (G protein coupled receptors), among others (Figure 2). This revealed that in addition to the known polycomb targets, UNC3866 binds three members of the CDY family of chromodomains (CDY1, CDYL1b, and CDYL2). Importantly, UNC3866 was also shown to engage the intact PRC1 complex, antagonize PRC1 chromodomains in cells, exhibit a high level of stability in cells, and inhibit PC3 cell proliferation (a known CBX7 phenotype), supporting further investigation of PRC1 chromodomain antagonists as oncology therapeutics. The replacement of the quaternary amine of the native peptide ligand with an unnatural tertiary amine mimetic was a key achievement in the development of a cellularly active ligand, despite its relatively low permeability, and signifies that a quaternary amine is not essential for inhibition of Kme3 reader proteins. The design and concomitant use of an inactive negative control compound, differing from UNC3866 by addition of a single methyl group, further supported the characterization of UNC3866 as the first high-quality chemical probe for a chromodomain. Taken together, our progress toward cellularly active, peptidic chromodomain antagonists and the Hof's group demonstration of selectivity tuning within the PRC1 chromodomains bodes well for further breakthroughs in this area.

Conclusions

The pursuit of well validated chemical probes for Kme reader proteins represents an emerging area of chemical biology that will create new understanding of chromatin regulation and prove useful in validation of Kme readers implicated in disease. As this target-class is well represented in databases of proteins genetically altered in cancer and neurological diseases [43], the opportunity for translational impact is promising. While peptidic probes seem most likely to deliver high-affinity and selectivity for groove-binding Kme readers, such as chromodomains, progress toward more drug-like compounds for these domains could be spurred on by target validation studies with peptide-like chemical probes. [44,45]

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Highlights

- Methyl lysine reader domains are a major class of chromatin regulatory modules that are being explored for therapeutic relevance.
- Chemical probes are molecules that are suitable for target validation studies in cells based on their potency, demonstrated target engagement, and selectivity.
- Recent progress toward methyl lysine reader domain chemical probes has resulted in potent, cellularly active and selective ligands for the chromodomains of the polycomb repressive complex 1.

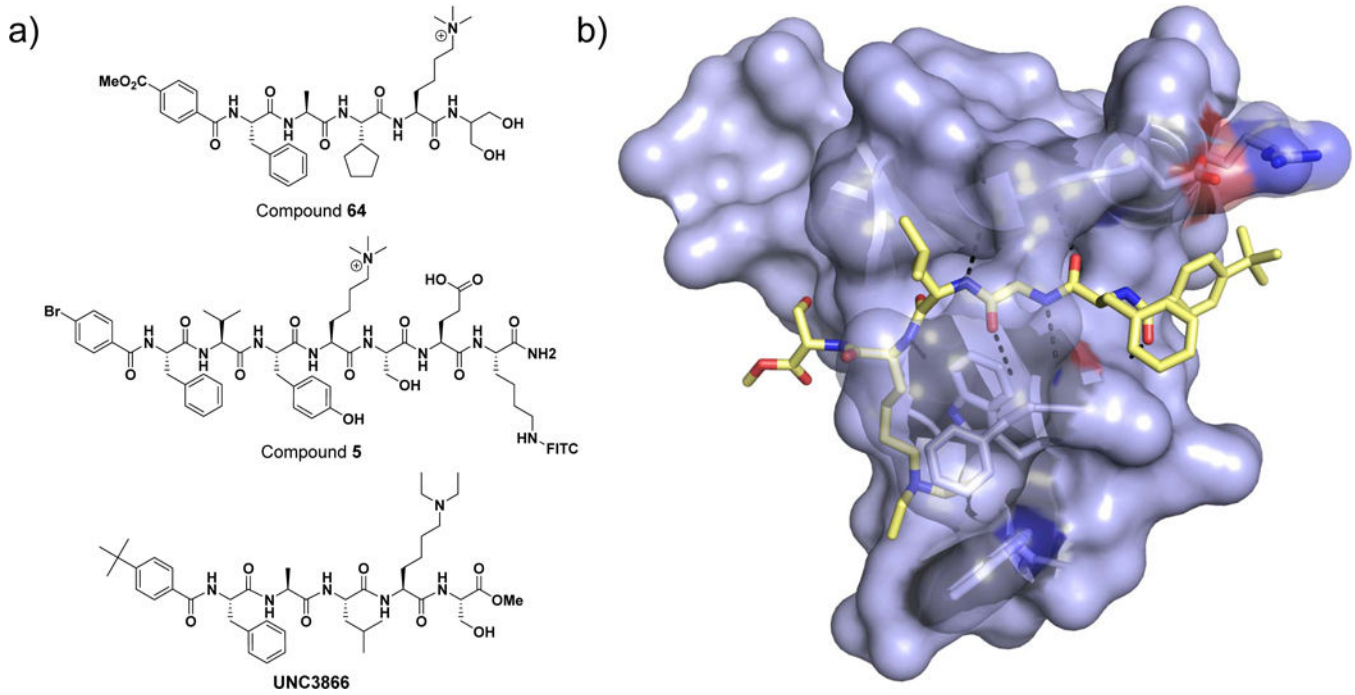
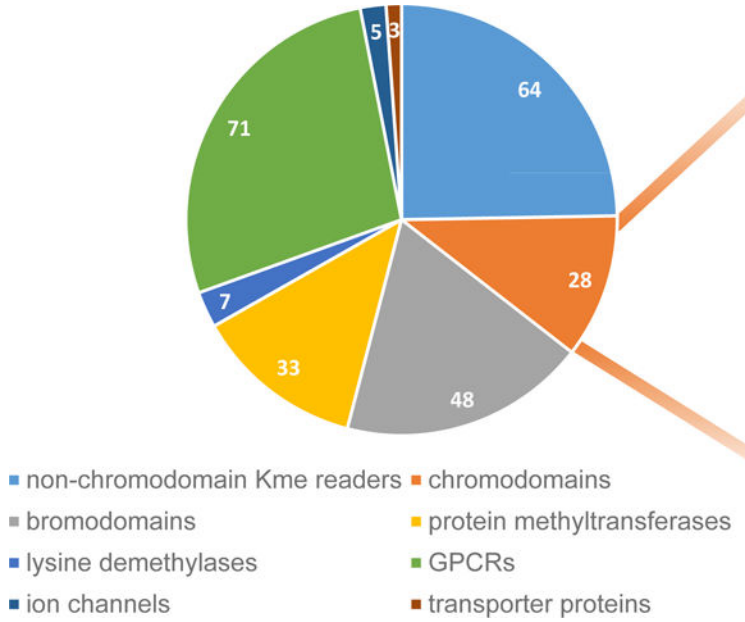


Figure 1. Peptidomimetic chromodomain ligands

a) Chemical structures of peptidic chromodomain inhibitors. b) Co-crystal structure of chemical probe UNC3866 bound to CBX7 (pdb 5EPJ). The protein surface is shown in light blue and UNC3866 in yellow. Hydrogen bonds between the protein and UNC3866 are shown by a black dotted line.



Chromodomain Target	UNC3866 K_d (μM)
CBX2	1.8 ± 0.21
CBX4	**0.094 ± 0.017**
CBX6	0.610 ± 0.0078
CBX7	**0.097 ± 0.0024**
CBX8	1.2 ± 0.021
CDY1	6.3 ± 0.92
CDYL1b	0.91 ± 0.076
CDYL2	0.85 ± 0.076

Figure 2. Selectivity profile of UNC3866

UNC3866 antagonizes the Kme reading function of the Polycomb CBX and the CDY families of chromodomains and is highly selective over >250 other protein targets evaluated. The affinity of UNC3866 for its chromodomain targets was determined by ITC.

Table 1

Chemical strategies and principles applied to the discovery of antagonists for cavity insertion methyllysine binders and surface groove methyllysine binders.

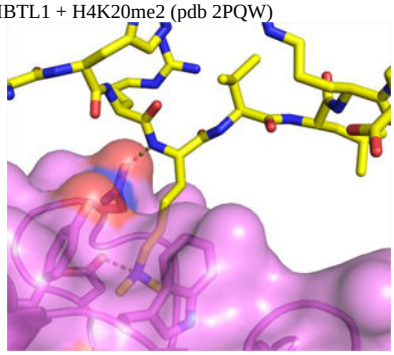
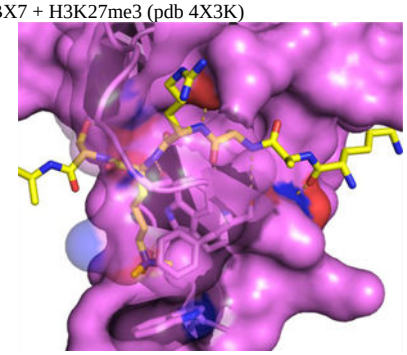
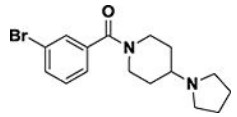
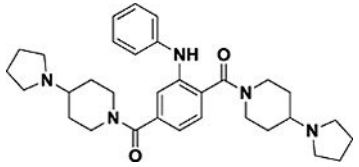
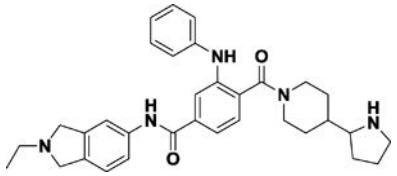
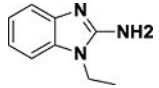
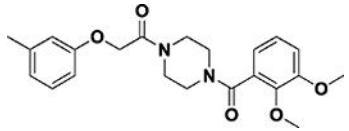
	Cavity Insertion Binders	**Surface Groove Binders**
Hit Discovery & Screening Strategies	Screen focused small molecule or fragment libraries Employ target class cross screening Utilize structure-based design for hit optimization	Apply structure-based design Screen peptide or peptoid libraries
Design Principles	Exploit cation-π and H-bonding interactions in aromatic cage Utilize conformational constrained alkyl amines	Use available Kme peptide SAR Target binding sites adjacent to aromatic cage Introduce unnatural amino acids, Kme mimics, and non-peptidic features
Major Challenge	Fragment-like size may result in low affinity	Low cell permeability may decrease overall utility
Example	L3MBTL1 + H4K20me2 (pdb 2PQW) 	CBX7 + H3K27me3 (pdb 4X3K) 

Table 2

Select small molecule, non-peptidomimetic inhibitors of methyllysine reader proteins that engage the aromatic cage where methyllysine binds.

Inhibitor	Structure	Potency (Assay)	Target(s)	Peptide Binding Mode	Ref.
UNC926		L3MBTL1 IC_{50} = 3.9 μM L3MBTL1 IC_{50} = 3.9 μM (Alphascreen)	L3MBTL1, L3MBTL3 (MBT domains)	Cavity insertion	21
UNC1215 *		Kd = 0.12 μM (ITC)	L3MBTL3 (MBT domain)	Cavity insertion	19
Compound **56**		Kd = 0.35 μM (ITC)	L3MBTL3 (MBT domain)	Cavity insertion	13
CF16		K_d = 7.3 mM (NMR)	Pygo2 (PHD finger)	Surface groove	34
MS37452		K_d = 29 μM (NMR)	CBX7 (Chromodomain)	Surface groove	31

* designates a high-quality chemical probe.

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