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Improved methods for targeting epigenetic reader domains of acetylated and methylated lysine

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Abstract

Responsible for interpreting histone post-translational modifications (PTMs), epigenetic reader proteins have emerged as novel therapeutic targets for a wide range of diseases. Chemical probes have been critical in enabling target validation studies and have led to translational advances in cancer and inflammation-related pathologies. Here, we present the most recently reported probes of reader proteins that recognize acylated and methylated lysine. We will discuss challenges associated with achieving potent antagonism of reader domains and review ongoing efforts to overcome these hurdles, focusing on targeting strategies including the use of peptidomimetic ligands, allosteric modulators, and protein degraders.

Introduction

Proper regulation of gene transcription by chromatin regulatory factors is essential to nearly every aspect of normal biology in multicellular organisms. Consequently, growing research has demonstrated the importance of the dysregulation of this network of transcription factors, chromatin remodelers, and histone post-translational modifications (PTMs) in diseases ranging from cancer to autoimmune and neurological disorders [1–3]. However, complex genetic association data and uncertain disease etiology has created a need for chemical tools to both investigate and pharmacologically perturb the function of these proteins. Potent, selective, and cell-permeable chemical probes have numerous advantageous over genetic methods of protein-target manipulation as they provide temporal resolution, domain-specific antagonism, and, most excitingly, the potential for therapeutic development [4]. In this review we discuss recent advancements in chemical probes targeting ‘readers’ of histone PTMs, which are responsible for binding to a specific PTM to elicit a downstream biological output. Due to their central and highly regulated role in gene transcription,

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Declaration of interest

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the development of validated probes for these targets has been of great interest in the last 20 years and led to the progression of small molecules to the clinic (Table 1, ClinicalTrials.gov).

Despite growing evidence validating the importance and druggability of some readers, the development of antagonists toward these proteins has overall been slow, in part due to the nature of their endogenous binding modes. Typically, reader domains interact with their histone binding partners through extended protein-protein interactions (PPIs) that can be difficult to replicate with drug-like small molecules [5]. Additionally, while some readers show high affinity and specificity for their histone targets, others do not, binding with affinities in the mM range in *in vitro* assays [6]. Finally, it has been shown that some readers bind their substrates through an induced-fit binding mode, where binding to the PTM induces a substantial conformational change in the reader domain to engender high-affinity binding [5,7]. This can make structure-based and rational design of antagonists challenging. Recent developments in the field have therefore focused on various targeting strategies in addition to traditional small molecule antagonism. Here, we discuss the use of peptides and peptidomimetic ligands, allosteric modulators, and targeted chemical degraders as alternative approaches to block the function of reader domains.

Acyl-lysine readers

Acetyl-lysine (Kac) is a common histone PTM typically associated with active transcription [5]. By neutralizing the positive charge of lysine on histone tails, Kac weakens the interaction between histones and the negatively charged DNA and thereby promotes a more open and accessible chromatin state. Furthermore, readers of Kac often act as transcriptional coactivators that recruit transcription factors and other effectors such as RNA polymerase II to these relaxed chromatin sites to initiate and maintain active transcription [8]. Accordingly, dysregulation of these proteins is implicated in a wide array of diseases including cancer [8,9].

Bromodomains.

Bromodomains (BRDs) are structurally conserved Kac reading modules found in 46 human proteins, separated into eight subfamilies according to sequence similarity (Figure 1A) [10]. In these proteins, Kac is recognized by a hydrophobic cavity formed from a left-handed bundle of four α helices (αZ, αA, αB, and αC) connected through interhelical ZA and BC loops. Binding to Kac is typically stabilized through a direct hydrogen bond with a conserved asparagine and a water-mediated hydrogen bond with a conserved tyrosine residue [8]. Despite the modest affinity of BRDs to isolated acetylated peptides *in vitro*, numerous highly potent nanomolar antagonists and chemical probes have been developed for this class, making BRDs an exceptionally well-studied and successfully targeted class of reader proteins. The profound biological effects observed with (+)-JQ1 and I-BET762, the first-in-class chemical probes targeting the bromodomain and extra-terminal (BET) subfamily of BRDs, established the tractability of targeting reader modules and fueled the rapid development of new antagonists to investigate the therapeutic relevance of the BET proteins [5,11,12]. Since the discovery of (+)-JQ1 and I-BET762 ten years ago,

>30 chemical probes for the BET family and >20 chemical probes for non-BET BRD proteins (*e.g.* KAT2A/B, CERC2, CREBBP/EP300, ATAD2A/B, BRD1, BRPF1/3, BRD7/9, TAF1/1L, PBRM1, SMARCA2/4, and BAZ2A/B) have been developed as discussed in previous reviews [9,13,14].

Domain selectivity within the BET family.

In recent years, in addition to the ongoing chemical probe efforts in this area, there has been a renewed interest in developing BET antagonists with selectivity for only one of the two tandem BRDs, BD1 or BD2, that are present in BET family members BRD2, −3, −4, and −T. *In vitro* evidence supporting unique roles for BD1 and BD2 highlights the need for selective BD1 and BD2 chemical probes to deconvolute the associated biological activity of these domains [15]. Although pan-BET antagonists that target BD1 and BD2 have been efficacious in oncology and inflammation-related clinical trials, several on-target and dose-limiting adverse events have been observed, with thrombocytopenia and gastrointestinal toxicity now associated as severe safety signals of pan-BET antagonism [9]. Intriguingly, the early BD2-biased RVX-208 is the only BET antagonist to have advanced to phase III clinical trials, in this case for type II diabetes mellitus, coronary artery disease, and cardiovascular disease (Table 1), suggesting that selective antagonists may increase the tolerability profile of these compounds [16,17].

Using a “bump-and-hole” chemical genetics approach, pioneered by the Shokat lab, in which subtle, structurally nondisruptive mutations of individual BET BRDs were paired with orthogonal “bumped” analogs of pan-BET antagonist I-BET762, the BD1 domain of BRD4 was shown to be required for chromatin binding, while BD2 appeared less essential [18–20]. More recent cellular studies using a “clickable” BD2-biased molecular probe and chromatin immunoprecipitation confirm that BRD4 is bound to chromatin through its BD1 domain [21]. Meanwhile, the interactions of BRD4-BD2 with transcription factors including P-TEFb and TWIST support its unique role in recruiting non-histone proteins to target genes [22,23]. In contrast, BD1 of BRD3 has been shown to bind the hematopoietic transcription factor GATA1, further emphasizing the complex biological roles of these domains and the need for selective antagonists to better understand their function [24,25]. With >90% sequence identity at the Kac binding pocket of BET BRDs, developing isoform-selective antagonists is a formidable task [10]. Due to the lower conservation between BD1 and BD2 across isoforms, selective antagonism of these domains can more readily be achieved. By taking advantage of specific residue differences and alternate dynamics of the ZA and BC loops of BD1 and BD2, several domain-selective pan-BD1 (binding BD1 of BRD2, −3, and −4) and −BD2 (binding BD2 of BRD2, −3, and −4) chemical probes have been developed within the last year (Figure 1A) [15].

The first reported pan-BD1 antagonist, LT052, is a nanomolar binder of BRD3, 4, and T-BD1 (AlphaScreen IC_{50} = 88-357 nM) that is 138-fold selective for BRD4-BD1 over BRD4-BD2 and 202-fold selective for BRD3-BD1 over BRD3-BD2 [26]. An unexpected loss in BRD2-BD1 binding and selectivity was observed with an IC_{50} of 9 μM and only 2-fold BD1/2 selectivity. In a dual luciferase reporter gene assay, LT052 was more potent in downregulating NF-κB activity compared to BD2-biased RVX-208 eliciting an 11- versus

2-fold change relative to the model. LT052 was also shown to be efficacious in a rat model of acute gout arthritis. Together, these results suggest that BD1 antagonism has superior anti-inflammatory activity than BD2 antagonism. However, separate studies using superior pan-BD2 antagonists report contrasting roles of BD2 in models of inflammatory and autoimmune disease (*vide infra*), underlining the need for follow-up studies.

Developed from the pan-antagonist I-BET151, GSK778 (iBET-BD1) exhibits potent pan-BD1 activity (TR-FRET IC_{50} = 40–158 nM) and 100 to 158-fold selectivity for BRDT- and BRD4-BD1 over their respective BD2 domains [27,28]. Lower selectivity was observed for the BD1 domains of BRD2 and −3 over their corresponding BD2 domains (40- and 25-fold, respectively). X-ray co-crystal structures of GSK778 bound to BRD4-BD1 and BRD2-BD2 attribute its BD1 selectivity to the “Asp/His switch,” which refers to a key residue difference between the BD1 and BD2 domains (PDB IDs: 6SWN and 6SWO; Figure 1B–C) [27]. When bound to BRD4-BD1, the 3-methylene pyrrolidine substituent of GSK778 extends toward the BD1-specific Asp144 and another nearby aspartic acid, Asp145, rotates to engage the pyrrolidine nitrogen through a water-mediated hydrogen bond. In BRD2-BD2, the corresponding BD2-specific residue, His433, adopts an “in” conformation that restricts the rotation of the analogous nearby Asp434, thus accounting for the loss in affinity. Similar rationale has been used to explain the affinity differences of other domain-selective antagonists, with the Asp/His switch, as well as Lys/Pro and gatekeeper residues Ile/Val BD1/BD2 structural differences shown to be responsible[16,17,26,29–33]. Encouragingly, GSK778 was efficacious *in vivo* in oncology and inflammation models [27,28].

During the development of ATAD2 chemical probe GSK8814, several compounds with submicromolar potency and >60-fold selectivity for BRD4-BD1 over BRD4-BD2 were identified and progressed to pan-BD1 antagonist, GSK789 [29]. With >1000-fold selectivity over all BET BD2 domains and >500-fold selectivity over other non-BET BRDs, GSK789 is the most selective pan-BD1 antagonist developed to date. However, GSK789 was not progressed to an *in vivo* probe due to suboptimal pharmacokinetics [29].

The first reported pan-BD2 chemical probe with superior selectivity compared to BD2-biased RVX-208 is ABBV-744, currently in early-stage clinical trials for acute myeloid leukemia (AML) and myelofibrosis [30,31]. Encouraging preclinical *in vivo* efficacy and reduced toxicity compared to pan-BET antagonist ABBV-075 suggests that ABBV-744 may also have enhanced tolerability in these trials [30]. With low nanomolar BET-BD2 potencies (TR-FRET IC_{50} = 1–5 nM) and >252-fold selectivity for the BD2 domains of BRD2, −3, and −4 over their respective BD1 domains [31], ABBV-744 has achieved a tremendous leap in selectivity compared to the first BD2-biased antagonist RVX-208, which exhibited a modest 11- to 45-fold BD1 selectivity [16,17].

Recently, pan-BD2 and -BD1 selective antagonists were characterized alongside one another in a series of *in vitro* and *in vivo* phenotypic assays [28]. Specifically, GSK046 (iBET-BD2) was utilized as a pan-BD2 ligand which exhibits nanomolar potency for its respective BD2 targets and an impressive selectivity over BD1 domains (>1200-fold for BRD4 and ~40–398 for BRD2, −3, and −T) and other non-BET BRDs [32]. GSK046 was shown to be ineffective at displacing chromatin-bound BET proteins, while pan-BD1 antagonist GSK778 showed

similar efficiency as pan-BET antagonist I-BET151, supporting previous reports that BD1 plays a more significant role in localizing BET proteins to chromatin [28]. Additionally, while GSK778 phenocopied I-BET151 in terms of anti-proliferative effects on a range of human cancer cells, GSK046 was less effective. Instead, a unique effect of BD2-selective antagonism was revealed with GSK046 affecting the induction of gene expression more so than the expression of steady-state genes, in contrast to GSK778 [28].

Although effective in *in vivo* inflammation studies, GSK046 was further optimized, leading to the development of an improved pan-BD2 antagonist, GSK620, with retained potency and high selectivity (80 to 316-fold for BD2/1) along with improved pharmacokinetic properties [33]. GSK620 was highly effective in a collagen-induced arthritis model in rats, outperforming the pan-BET antagonist I-BET151 in terms of reduced joint swelling and overall disease score, again supporting the advantage of domain-selective BET antagonism. Chemically related pan-BD2 antagonist GSK549 has slightly improved potency (TR-FRET $IC_{50} \approx$ 16-63 nM) and superior selectivity (~501 to 1260-fold for BD2/1), although its pharmacokinetic properties are subpar [33].

GSK973 retains the excellent BD2 selectivity of GSK549 (>1000-fold selective against all BD1 domains) and is also suitable *in vivo*, with the added benefit of a unique chemical composition relative to other pan-BD2 antagonist and a negative control compound for further studies [34].

In summary, these potent pan-domain-selective chemical probes have only recently been disclosed and future efforts, as well as clinical studies, will enable further investigations into the individual roles of BET BD1 and BD2 domains, as well as additional insight regarding the efficacy and tolerability profiles of BET domain-selective antagonism.

Proteolysis-targeting chimeras.

In addition to traditional small molecules, alternate modalities for achieving potent and selective antagonism, including bivalent antagonists and proteolysis targeting chimeras (PROTACs), have hugely impacted the development of BRD antagonists [35–38]. PROTACs are bivalent molecules that link a ligand for a protein-of-interest (POI) to an E3 ligase-targeting ligand in order to recruit ubiquitin-proteasome machinery to a POI and induce targeted degradation of the protein. Extensive reviews have summarized the advantages of degraders over their more conventional small-molecule counterparts, which include (1) prolonged efficacy due to target removal rather than occupancy-driven target antagonism, (2) improved selectivity due to the formation of cooperative ternary complexes (POI:Degrader:E3), and (3) reduced off-target effects due to the catalytic mechanism of these compounds and the lower doses that are required for efficacy [39–41]. Chemical degradation is an especially important strategy for targeting reader domain containing proteins/protein complexes because the reader domain itself may not be the critical functional module in terms of disease phenotype.

Despite the ongoing success in selectively targeting BD1 and BD2 domains within BRDs outlined above, limited progress has been made in selectively targeting individual BET family members with conventional small molecule antagonists. Due to the potential

functional redundancy of the BET proteins, there is much interest in developing chemical tools to better decipher the roles of these related proteins. FL-411, ZL0420, and ZL0454 are the most successful compounds reported thus far, with submicromolar to low nanomolar potency for BRD4 and 50 to 200-fold selectivity against other BET BRDs [42,43]. PROTACs offer another way to engineer potent and selective—even isoform-specific (e.g. BRD4 alone)—BET antagonists. For example, the JQ1-based PROTAC MZ1 demonstrates selective degradation of BRD4 over BRD2 and BRD3 despite equipotent binding of JQ1 to the three proteins [44]. This selectivity profile was rationalized by the extensive protein-protein interactions observed in the X-ray co-crystal structure of the BRD4:MZ1:VHL ternary complex [45]. Since this initial report, inducing shape complementarity at the interface between a POI and E3 ligase to achieve potency and selectivity has become a common design strategy of PROTACs [46–48], with similar successes achieved by AT1 [45], as well as macroPROTAC-1, the first cyclic PROTAC targeting BRD4 [49].

Beyond the BET family, several PROTACs targeting BRDs including BRD7/9, TRIM24, PCAF/GCN5, and SMARCA2/4 have also been developed [50–54]. Interestingly, the TRIM24 degrader, dTRIM24, demonstrates unique potency on acute leukemia cells compared to conventional small molecule antagonists that had no functional output [51]. The success of dTRIM24 demonstrates that chemical degradation is especially applicable for targeting chromatin regulatory complexes containing reader domains when ligands for these domains do not lead to similar phenotypic activity as protein knockout experiments ([51]). Chemical probes and degraders for additional BRDs will undoubtedly progress understanding of biological mechanisms and therapeutic opportunities within this target class.

YEATs domain.

Lysine crotonylation is a more elusive PTM than acetyl-lysine, with only four crotonyl-lysine (Kcr) readers currently known to be encoded by the human genome: YEATS proteins ENL (MLLT1), AF9 (MLLT3), YEATS2, and GAS41 [55]. Similar to BRDs, dysregulation of these reader domains impacts transcriptional programs affecting oncogenic gene expression in aggressive cancer types [56,57]. Unlike BRDs, the open-ended and tunnel-like binding cavity of YEATS domains enables the recognition of Kac as well as larger acyl-marks such as Kcr [58]. However, the unique **π-π-π** stacking interaction between two conserved aromatic residues and the conjugated crotonyl-amide group of Kcr enhances its binding affinity over Kac by 2- to 5-fold [55,58]. SGC-iMMLT is the first small molecule chemical probe of a YEATs domain that is equipotent and selective for ENL (ITC K_d = 129 nM) and AF9 (ITC K_d = 77 nM). In MV4;11 AML cells, SGC-iMLLT downregulated pro-oncogene c-Myc and altered the expression of several other target genes, supporting previous ENL knockdown/out studies [59]. The submicromolar tripeptide antagonist, XL-13m, had a slightly improved 5-fold selectivity for ENL over AF9 and, likewise, antagonized the leukemia gene signature in MOLM-13, MV4;11, and HEL cell lines [60]. Effective targeting of the YEATs domain is an emerging area for probe discovery and future progress in probe potency and selectivity, as well as degrader development, may reveal new therapeutic opportunities.

Methyl-lysine readers

Methyl-lysine (Kme) is the most versatile histone PTM. Lysine can be mono-, di-, or trimethylated (Kme1/2/3) across a range of sites along the flexible histone tails. Unlike acetylation, lysine methylation does not alter the charge of the amino group, but rather, its effect on hydrophobicity and size is enough to potently and specifically recruit Kme reader proteins to the PTM [7]. Through their own catalytic functions or the subsequent recruitment of effector proteins, Kme readers translate their recognition of the methylation signal into a diverse set of downstream biological outcomes [5].

Kme readers recognize their histone targets through a cage comprised of 2–4 aromatic residues that form cation-π interactions with the lysine methylammonium group. The size and composition of the aromatic cage plays a key role in dictating selectivity for its substrate [5,7]. Typically, readers of lower methylation states (Kme1/2) have smaller cages with one of the aromatic cage residues replaced by a negatively charged aspartic or glutamic acid. This charged residue forms a hydrogen bond with the N^+-H donor present in these methylation states while making binding of Kme3, which lacks this hydrogen bond donating capability, energetically unfavorable with Kme1/2 readers [61]. In addition, this larger PTM is often sterically occluded from these smaller binding pockets. Further specificity for a particular methylated lysine is imparted by some Kme readers through interaction with the surrounding residues. This is typically found in readers that utilize a surface-groove binding mode, where the aromatic cage is accessible from the surface of the protein, allowing the flanking histone residues to make important contacts outside of the Kme binding pocket [62]. Surface-groove Kme readers have been difficult to target using traditional small molecules due to the extensive protein-protein interaction (PPI) surface created by the histone peptide with its reader. On the other hand, Kme readers that utilize a cavity-insertion binding mode often have deep binding pockets that retain localized interactions between the aromatic cage and the lysine methylammonium group, making them more amenable for small molecule binding.

Small-molecule antagonists.

Using targeted screening and ligand-based design, our lab developed UNC1215, the first chemical probe for a Kme reader protein, targeting the MBT domains of L3MBTL3 [63]. UNC1215 binds L3MBTL3 with a K_d of 120 nM and results in a cellular EC_{50} of 50–100 nM. UNC1215 has continued to demonstrate its utility as a chemical probe and has been widely used to investigate the biology of L3MBTL3 [64,65], but its use in disease-related biology has been limited. Despite the apparent druggability of cavity-insertion reader domains and this initial success, the development of small-molecule antagonists for Kme reader proteins has been slow relative to those for Kac readers. Recent advances in small molecule discovery have been concentrated largely on the WD40 domain of EED, resulting in the potent antagonists EED226 [66] and A-395 [67] from Novartis and AbbVie/SGC, respectively. Interestingly, co-crystallization of EED226 with EED suggests that the high binding affinity of this compound is dependent on the rearrangement of an aromatic cage tryptophan residue upon antagonist binding, a phenomenon that could not have been rationally predicted (Figure 2A) [66]. It is therefore unsurprising that many small molecule

successes for Kme reader targets have been discovered by unbiased, industry-scale high-throughput screening.

The application of fragment-based drug discovery (FBDD) to the development of Kme reader antagonists has created new opportunities for small molecule discovery. FBDD uses sensitive biophysical techniques such as NMR or surface plasmon resonance (SPR) to detect weak binding of small fragment molecules (<20 non-hydrogen atoms) to the protein of interest [68]. From there, these fragments can be grown through rational design or linked together to create larger, more potent molecules. FBDD is particularly amenable to Kme reader antagonism for two key reasons: (1) the small fragments can bind the shallow binding pockets that occlude binding of larger molecules and (2) linking and growing these fragments creates peptide-like interactions with the protein of interest that can mimic the endogenous PPIs while maintaining drug-like properties. FBDD efforts toward Kme reader domains have led to the development of high-quality chemical probes for previously undruggable families including the PWWP and Tudor domains. BI-9321, which targets PWWP1 of NSD3, was developed through FBDD collaborations with the Structural Genomics Consortium (SGC) (Figure 2B). [69]. Using a small fragment library of less than 2,000 fragments, Böttcher *et al.* discovered weak binders of $NSD3_{PWWPI}$ through NMR and differential scanning fluorimetry (DSF). Fragment hits that showed binding to the histone binding pocket by two-dimensional $^{1}H/^{15}N$-transverse relaxation optimized spectroscopy (TROSY) NMR and NMR K_ds <2 mM were progressed to structural analysis by co-crystallization. Subsequent optimization using a combination of virtual screening based on the validated fragment hits and ligand-based rational design resulted in the highly potent and selective chemical probe, BI-9321. With an ITC K_d of 445 nM and cellular EC_{50} of 5.5 μM, BI-9321 had a selective effect on proliferation of cancer cell lines sensitive to NSD3 loss-of-function mutations (MOLM-13 and RN2), likely through an observed downregulation of MYC. BI-9321 has demonstrated the therapeutic potential of single PWWP domain antagonism, and has already paved the way for other antagonists of this family (See: https://www.thesgc.org/chemical-probes/UNC6934). In a similar method, the first-in-class Tudor domain antagonist for Spindlin1, VinSpinIn, linked previously discovered SPIN1 binders, A-366 and EML631, to create a bidentate, or dual-domain, ligand that interacts with Tudor domains 1 and 2 of the protein (Figure 2C) [70]. While A-366 demonstrates high *in vitro* potency toward SPIN1 (IC_{50} = 186 nM), the fragment was originally developed for the methyltransferase G9a (IC_{50} = 3 nM) and would therefore have poor SPIN1 selectivity [71]. Using structure-based design, A-366 was modified for potent and selective binding of SPIN1 Tudor 2. However, the optimized compound showed high toxicity in cells at concentrations >3 μM, leading Fagan *et al.* to develop a new series of bidentate ligands. Bidentate antagonists have the potential not only for increased selectivity through elongated interactions, but also for increased functional efficacy in cells by targeting multiple functional domains within the protein. Fagan *et al.* again utilized rational design to combine and optimize the previously discovered EML631, which targets SPIN1 Tudor 1 [72], with the optimized Tudor 2 antagonist to create VinSpinIn. VinSpinIn demonstrated high *in vitro* and cellular potency (IC_{50} = 33 nM, EC_{50} = 270 nM), excellent selectivity (no significant off-target binding to 16 related Kme/Rme reader domains and 33 methyltransferases including G9a), and, importantly, no observed toxicity.

While VinSpinIn demonstrated only moderate effects on cell proliferation, the well-characterized probe will be exceptionally useful for investigation of SPIN family protein biology in normal and disease-relevant contexts.

Peptides and peptidomimetics.

While small molecules are often seen as the preferred mode of antagonism due to their drug-like properties, peptides and peptidomimetic ligands can be especially useful tool compounds due to their high affinity and selectivity combined with their generally low off-target effects and toxicity [73]. Peptide and peptidomimetic ligands are of particular interest for Kme reader antagonists as the natural histone binding partners provide useful starting points for ligand development. In particular, peptide-based ligands have the potential to replicate and improve upon the surface-groove binding modes of Kme readers that can be difficult to target by small molecules.

To date, the most successful examples of Kme reader peptidomimetics have targeted the CBX family of chromodomains within the Polycomb Repressive Complex 1 (PRC1) which bind H3K27me3 in a surface-groove binding mode. Initial efforts toward development of small molecule antagonists of CBX proteins led to MS452 [74] and MS351 [75], which demonstrated moderate binding to CBX7 *in vitro* (MS452 K_d = 29 μM; MS351 K_d ~500 μM) but lacked cellular efficacy likely due to limited solubility at the required concentrations [76]. Development of UNC4976 and its predecessor, UNC3866, therefore exemplify the utility of peptidic ligands for these readers by demonstrating high potency, selectivity, and cellular activity against CBX7 (UNC4976 K_d = 59 nM; UNC3866 K_d = 97 nM) (Figure 2D) [76,77]. UNC3866 and UNC4976 make use of a few key design strategies that have been essential for the development of potent, cell permeable, and proteolytically stable peptidic ligands. First, both peptides contain a hydrophobic lysine mimic at the Kme3 site which both increases engagement with the aromatic cage through hydrophobic interactions and allows for removal of the quaternary amine that can be detrimental to cell permeability. Additionally, the peptides are ‘capped’ with a methyl ester on the C-terminus and a non-aminoacid, aromatic N-terminus. While the C-terminal cap provides added permeability relative to an amide and the potential for intracellular trapping by esterase conversion to the corresponding acid, the N-terminal cap has been optimized to improve potency of the peptide [78]. It is worth noting that both UNC3866 and UNC4976 show acceptable permeabilities in the chloroalkane penetration assay (CAPA) with CP_{50} values in the low micromolar range [76]. In related studies, through a DNA encoded library approach the Dykhuizen and Krusemark labs discovered SW2_110A, which binds the chromodomain of the closely-related CBX8 with a K_d of 800 nM and shows moderate anti-proliferative effects in MLL-AF9 transformed leukemia [79]. Similar to UNC3866 and UNC4976, SW2_110A swaps the Kme3 for a more hydrophobic tertiary amine (diethyl-lysine) and includes a heterocyclic aromatic N-terminal cap. With these modifications, SW2_110A demonstrated modest permeability by CAPA with a CP_{50} of 26 μM. Continued work on CBX antagonists is ongoing in our lab and others through a variety of approaches [80].

Allosteric modulation within a multicomponent complex.

Kme readers play key scaffolding and modulating roles in larger complexes binding to nucleic acids and/or regulating catalytic domains responsible for propagating these marks. Recent discoveries have highlighted the importance of exploiting allosteric interactions to achieve cellular potency and efficacy with Kme probes [81].

The aforementioned UNC4976 is a good case study for allosteric modulation. While UNC3866 and UNC4976 exhibit similar K_d values *in vitro,* UNC4976 demonstrated a 14-fold increase in cellular potency (EC_{50} = 3.2 μM) from UNC3866 (EC_{50} = 42 μM). Lamb *etal.* proposed that this discrepancy arises from the unique ability of UNC4976 but not UNC3866 to increase the affinity of CBX7 for DNA while simultaneously antagonizing H3K27me3 recognition, thereby further diminishing the specific binding of CBX7 to H3K27me3 sites. In this way, UNC4976 acts both as a competitive antagonist of H3K27me3 and as a positive allosteric modulator (PAM) of CBX7 binding to DNA, increasing its overall efficacy in cells. This PAM mechanism was first proposed by the Zhou lab in the characterization of MS351 [75].

One of the most prolific uses of allosteric regulation has been through the use of EED antagonists as allosteric modulators of EZH2. EED functions as a reader of H3K27me3 in the Polycomb Repressive Complex 2 (PRC2), which also propagates the repressive mark through the methyltransferase EZH2. Binding of H3K27me3 to EED induces a conformational change in EZH2 that allosterically activates its catalytic activity. Accordingly, positive and negative modulators of EED have been discovered that allosterically regulate EZH2 activity. As previously mentioned, EED226 is a potent antagonist of EED, which recently led to the development of MAK683, the first small molecule targeting a Kme reader that has progressed into clinical trials (Table 1). Like EED226, binding of MAK683 in the H3K27me3-binding pocket is a very high affinity interaction (AlphaScreen IC_{50} = 26 nM) which prevents a conformational change in EZH2 required to activate PRC2 catalytic activity. Importantly, these EED antagonists have been shown to be effective against EZH2 mutants that are resistant to EZH2 inhibitors as a first line treatment [66]. Allosteric binders have also been used by our lab to develop selective allosteric agonists of the mutant EED-I363M, which results in PRC2 loss of function and plays a driving role in myeloid dysplasia. Excitingly, rational design led to UNC5635 and UNC5636, which are capable of compensating for the loss of EZH2 activation in this mutant. In a catalytic assay, UNC5635 and UNC5636 showed agonism of PRC2-EED-I363M but not PRC2-EED-WT activity, demonstrating their selective activity toward the disease-relevant mutant [82].

PROTACs revisited.

Finally, additional methods to target EED and the PRC2 complex have recently been achieved through the development of PRC2-targeted PROTACs. Namely, UNC6852 [83] and PROTAC1/PROTAC2 [84] potently target EED but have also been shown to degrade all three core components of the PRC2 complex: EED, EZH2, and SUZ12. This large-scale degradation is highly effective in antagonizing PRC2 activity, as re-establishment of PRC2

function requires re-synthesis of all three components. Both groups showed significant anti-proliferative effects toward PRC2-dependent cancer cell lines.

Conclusions

Chromatin regulation is a complex network consisting, in part, of histone PTMs and the readers that translate them. The development of chemical probes for readers offers tools to map these networks and has enriched our understanding of normal and disease biology. As shown with (+)-JQ1, these efforts have led to translational discoveries targeting the BET family, with Kme reader EED antagonists also moving into the clinic. Future success regarding BET antagonism will rely on selective modulation of individual BRD domains, BD1 and BD2. As epigenetic research has grown outside the BET and Kme reader family, so has the number of chemical probes targeting non-BET BRDs and YEATS domains. Among Kme readers, antagonists with new binding modalities and mechanisms-of-action are being actively pursued to overcome inherent challenges of targeting this class. In particular, this review highlights the lack of chemical probes targeting the large subset of Kme readers making up the PHD family, as well as the smaller BAH, ADD, Ankyrin, and chromo-barrel families. Probes for these families have remained elusive due to their shallow binding pockets and limited chemical space explored for hit discovery. New techniques such as DNA-encoded libraries applied to reader domains will hopefully provide new opportunities for ligand discovery for these challenging targets. Overall, chemical probe development will continue to play a leading role in the investigation of the therapeutic potential and biological role of epigenetic readers.

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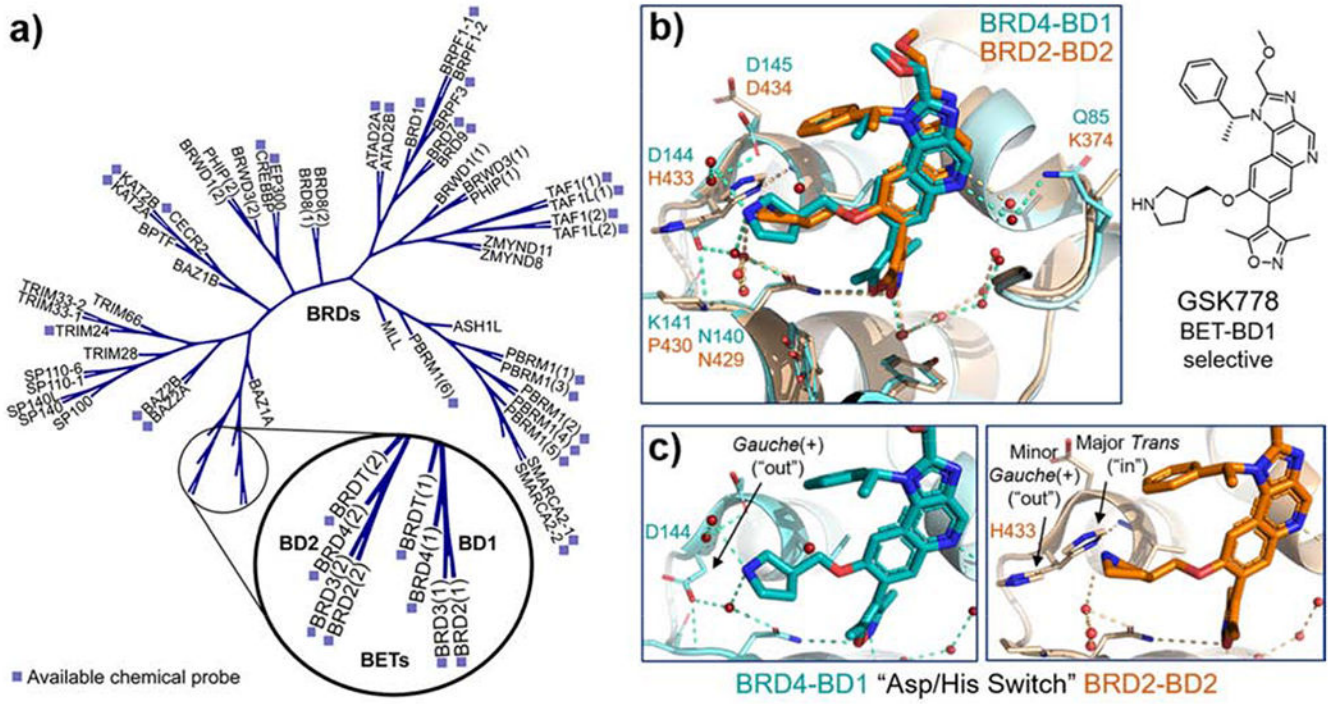


Figure 1.

Developing selective chemical probes for the BET subfamily. **(a)** Phylogenetic tree of bromodomains, with available chemical probes noted; the BET subfamily and the divergence of its first and second bromodomains, BD1 and BD2, are highlighted (adapted from chromohub.thesgc.org); **(b)** BET BD1-selective GSK778 bound to BRD4-BD1 (in cyan, PDB ID: 6SWN [27]) overlayed with GSK778 bound to BRD2-BD2 (in orange, PDB ID: 6SWO [27]); **(c)** (Left) Isolated view of GSK778 bound to BRD4-BD1 highlighting the *gauche*(+), or "out", conformation of BD1-specific residue Asp144; (right) isolated view of GSK778 bound to BRD2-BD2 highlighting the major *trans,* or "in," conformation of BD2-specific residue His433.

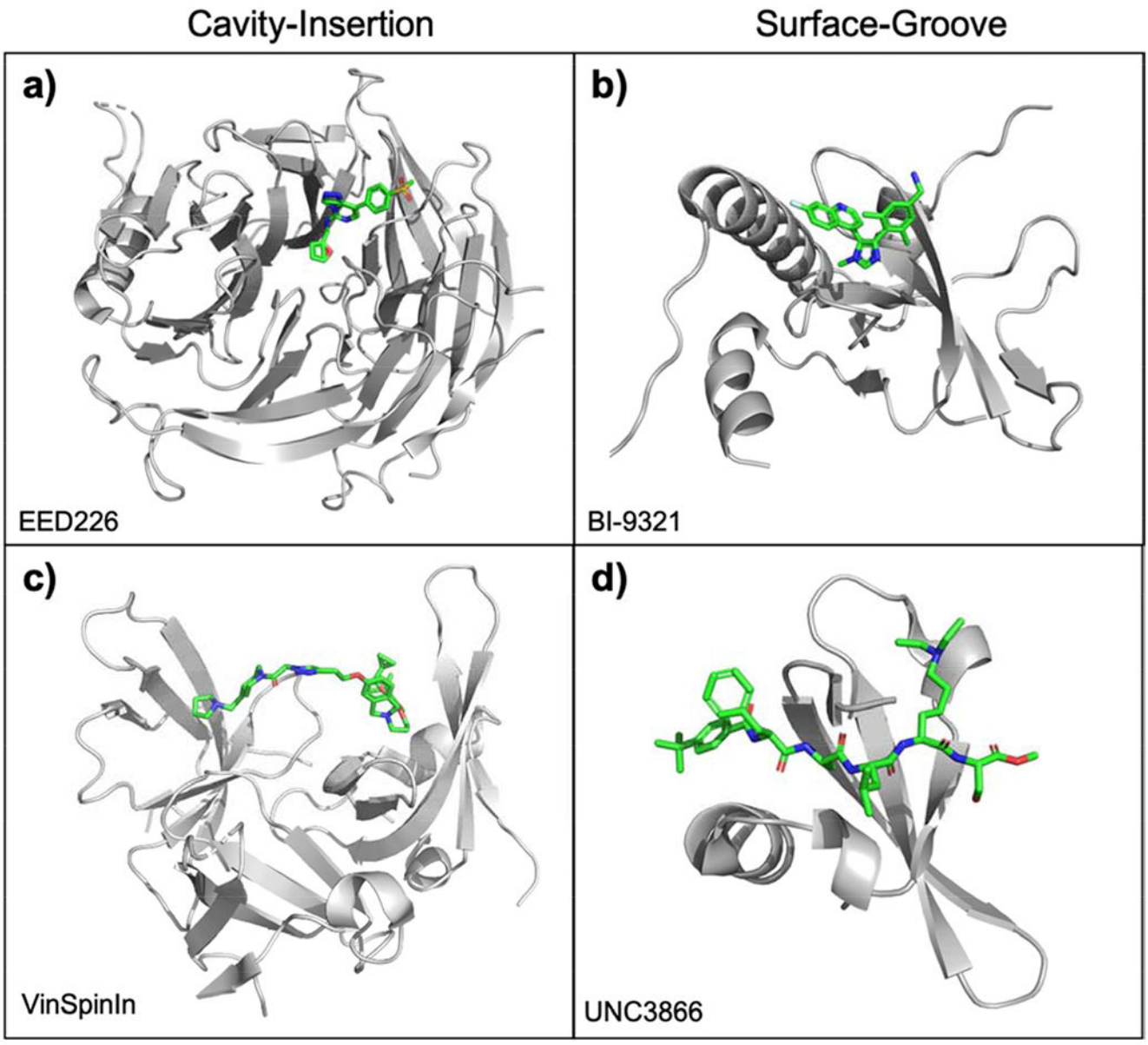


Figure 2.

Representative co-crystal structures of Kme reader antagonist binding. **(a)** EED226 inserts into EED (PDB ID: 5WUK); **(b)** BI-9321 utilizes fragment linking to engage in multiple contacts throughout $NSD3_{PWWP1}$ (PDB ID: 6G2O); **(c)** VinSpinIn binds Tudor domains 1 and 2 of SPIN1 through a cavity-insertion binding mode (PDB ID: 6I8B); **(d)** UNC3866 creates an extended PPI with CBX7 chromodomain (PDB ID: 5EPJ).

Table 1

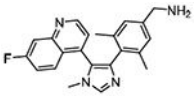
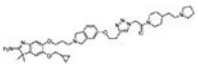
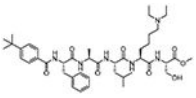
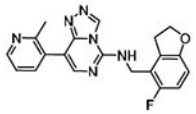
Antagonists of epigenetic reader domains in clinical trials

Antagonist	Antagonist Class	Target	Disease State	Phase(s)	NCT Number(s)
ABBV-075	Kac Reader	BET	Advanced Cancers	I	NCT02391480
			Myelofibrosis	I	NCT04480086
ABBV-744	Kac Reader	BET (BD2)	AML	I	NCT03360006
			Myelofibrosis	I	NCT04454658
BMS-986158	Kac Reader	BET	Pediatric Tumors Lymphomas	I	NCT03936465
			Advanced Cancers	I-II	NCT02419417
FT-1101	Kac Reader	BET	Hematologic Malignancies	I	NCT02543879
GS-5829	Kac Reader	BET	Solid Tumors Lymphomas	I	NCT02392611
			Breast Cancer	I-II	NCT02983604
			Castration-Resistant Prostate Cancer	I-II	NCT02607228
GSK2820151	Kac Reader	BET	Solid Tumors	I	NCT02630251
GSK4027	Kac Reader	PCAF/GCN5	COPD Emphysema Chronic Bronchitis	IV	NCT00175565
I-BET762 (GSK525762)	Kac Reader	BET	Hematologic Malignancies	I II	NCT03925428 NCT01943851
			Castration-Resistant Prostate Cancer	I	NCT03150056
			Solid Tumors	I I II	NCT02259114 NCT03925428 NCT03266159
			Breast Cancer	II	NCT02964507
			Carcinoma	I I-II	NCT01587703 NCT04116359
INCB054329	Kac Reader	BET	Hematologic Malignancies Solid Tumors	I-II	NCT02431260
INCB-057643	Kac Reader	BET	Myelofibrosis	I	NCT04279847
			Solid Tumors	I-II	NCT02959437
MAK683	Kme Reader	EED	DLBCL	I-II	NCT02900651
MK-8628 (OTX015)	Kac Reader	BET	Hematologic Malignancies	I	NCT01713582
			Advanced Cancers	I	NCT02259114
			GBM	II	NCT02296476
PLX51107	Kac Reader	BRD4	AML Myelodysplastic Syndromes	I	NCT04022785
			Hematologic Malignancies Solid Tumors	I	NCT02683395
RO6870810	Kac Reader	BET	AML Myelodysplastic Syndromes	I	NCT02308761
			Multiple Myeloma	I	NCT03068351
			Solid Tumors	I	NCT01987362
			Ovarian Cancer TNBC	I	NCT03292172
			B-cell Lymphomas	I	NCT03255096
RVX-208	Kac Reader	BET	Diabetes	II III	NCT01728467 NCT02586155

Antagonist	Antagonist Class	Target	Disease State	Phase(s)	NCT Number(s)
			Fabry Disease	I-II	NCT03228940
			Kidney Failure	I-II	NCT03160430
			Cardiovascular Diseases	II I-II II III II	NCT01058018 NCT00768274 NCT01423188 NCT02586155 NCT01067820
SYHA1801	Kac Reader	BRD4	Solid Tumors	I	NCT04309968
ZEN003694	Kac Reader	BET	Castration-Resistant Prostate Cancer	I I-II I-II II	NCT02705469 NCT04145375 NCT02711956 NCT04471974
			TNBC	II	NCT03901469

Table 2

Representative chemical probes of Kme readers with unique binding modes and mechanisms

Kme Reader	Chemical Probe		
	Name	Modality	Structure
NSD3 (PWWP)	BI-9321	Small Molecule Cavity Binder	
SPIN1 (Tudor)	VinSpinIn	Bidentate Small Molecule	
CBX7 (CBX)	UNC3866	Peptidic Surface-Groove Binder	
EED (WD40)	MAK683	Allosteric Modulator	
EED (WD40)	UNC6852	PROTAC Degrader