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Research Overview

Peptide Technologies in the Development of Chemical Tools for Chromatin-Associated Machinery

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ABSTRACT Discerning a mechanistic understanding of the cause-and-effect relationships between chromatin post-translational modifications (PTMs) and DNA accessibility for replication, transcription, and repair is an elusive goal being pursued using molecular and cellular biology, biochemistry, and more recently chemical inhibition. Chemical intervention of the chromatin-associated complexes that regulate PTM maintenance and chromatin structure faces numerous challenges due to the broad surface-groove interactions between many of these proteins and histones; yet, the increasing interest in understanding chromatin-modifying complexes suggests tractable lead compounds will be critical for elucidating the mechanisms of chromatin dysregulation in disease states and validating the druggability of these domains. Peptides and peptidomimetics afford several advantages to efficient inhibitor development including a rational starting point, modular assembly, and retention of secondary structure. Numerous peptide technologies have been employed in the chromatin field to characterize substrate interactions, evaluate ligand selectivity, and optimize potent peptidomimetic inhibitors. We describe the progress and advantages of these efforts, and provide a perspective on their implications for future chemical probe and drug discovery efforts. Drug Dev Res 78 : 300-312, 2017. © 2017 Wiley Periodicals, Inc.

Key words: chromatin; peptide; post-translational modification; microarray; chemical biology

INTRODUCTION

Innovative chemical strategies to interrogate protein function have irrevocably altered how we understand biological systems. Chemical tools contribute to an understanding of target-specific biology, but the extent to which they do so is fully dependent on the quality of the chemical tools [Bunnage et al., 2013; Frye, 2010; Arrowsmith et al., 2015]. Ultimately, high-quality chemical probes must have nanomolar or better target affinity, well-characterized selectivity, and activity in cells. These probes do not replace modern genetics or molecular biology, nor is that the intent. Instead, they complement these approaches by disrupting (enhancing or inhibiting) a specific function or interaction of the target protein. In contrast, genetic mutations and small interfering RNA (siRNA) knockdowns or gene knockouts may ablate several activities of the target and can prevent participation in, or the formation of, larger protein complexes. The chemical probe is intended to exquisitely elucidate the role of an individual protein modality in a temporally resolved and dose-dependent manner [Frye, 2010].

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The stringent standards for chemical probe status require a significant upfront investment. While early stage hit discovery often focuses on screening diverse and purchasable compound libraries, the chemical space occupied by these libraries tends to be biased toward pockets of more traditional, "druggable" targets (i.e., enclosed and hydrophobic) [Hopkins and Groom, 2002]. Often designed around aromatic scaffolds, the inherent "drug-like-ness" and lack of conformational complexity within these libraries frequently proves inadequate for inhibition of common protein–protein interactions (PPIs) [Tsomaia, 2015]; yet, constructing libraries of structurally diverse small molecules with complex, three dimensional features would be high risk, time consuming, and expensive [Hann and Oprea, 2004]. In contrast, peptidic and peptidomimetic ligands can adopt unique 3D conformations, naturally mimicking PPIs and promoting induced-fit binding through specific hydrogen bond contacts of the amide backbone and complimentary side group functionalities.

The low-cost and ease of peptide synthesis, accompanied by a variety of technological advances in screening, position peptidic ligands as an intriguing alternative to traditional small molecules for PPI inhibitor discovery [Lam et al., 1997; Smith and Petrenko, 1997; Frank, 2002; Takahashi et al., 2003]. Moreover, when a PPI has been structurally characterized, peptide optimization may proceed from a rational starting point. For instance, structural characterization of the interaction between an α-helix of p53 and MDM2 [Kussie et al., 1996] spurred extensive efforts in peptide and peptidomimetic ligand development for MDM2. Novartis employed combinatorial chemistry, structure-activity relationship (SAR) studies, and structure-based design (SBD) to evolve a low affinity 12-mer peptide of p53, first into a cellularly active, sub-micromolar 12-mer ligand, and then into a single-digit nanomolar, non-natural peptide inhibitor of MDM2 [Chène et al., 2000; Garcia-Echeverria et al., 2000; Sakurai et al., 2006; Furet et al., 2012]. Peptide SAR subsequently contributed to an understanding of the PPI hotspots between p53 and MDM2 that were found to be targetable by small molecules [Ding et al., 2005; Grasberger et al., 2005; Vassilev et al., 2004]. This and related examples demonstrate the significant impact peptide inhibitors can have as tools for therapeutic validation and as stepping stones toward small molecule discovery efforts [Sattler et al., 1997; Friberg et al., 2012].

Despite the many benefits of peptide ligand discovery, it is important to note some of the challenges that may need to be overcome when venturing into peptide optimization. Peptides suffer from poor physicochemical properties often due to their polar backbone and charged side groups [Tsomaia, 2015]. For instance, the hydrogen bond donation capacity of the amide proton has been shown to be a significant contributor to the poor membrane permeability of small peptides [Kwon and Kodadek, 2007; Tan et al., 2008]. Peptide ligands selected as starting points for optimization may also incur issues of low affinity and poor selectivity, making preliminary assay development and characterization challenging. Additionally, natural peptides are often substrates for proteases, and in the case of in vivo studies, peptides can face rapid clearance in the liver and kidneys [Tsomaia, 2015].

Fortunately, a range of strategies have been developed to overcome poor peptide properties. First, truncation studies to determine the minimal peptide sequence required for binding and the use of an alanine scan [Cunningham and Wells, 1989] can both provide insight into the potential interaction hot spots of the PPI and reduce the total molecular weight of these ligands, which is crucial if cellular penetration is desired. Next, replacing natural amino acids with non-natural ones simultaneously provides an opportunity for potency enhancement and reduces susceptibility to protease degradation [Gentilucci et al, 2010]. For example, D-amino acids are a commonly applied, non-natural substitution in peptide optimization. Additionally, backbone N-methylation is able to both rigidify the peptide conformation and improve permeability [Biron et al., 2008]. Instituting conformational constraints is one strategy to improve affinity by reducing the entropic penalty of binding [Khan et al., 1998], and this premise is often applied in the development of macrocyclic or stapled peptide inhibitors [Bock et al., 2013]. Lastly, the availability of structural information for a specific target can help guide which of these approaches for improving ligand permeability may be most applicable.

Burgeoning efforts to better understand chromatin biology suggests a need for chemical tools to help dissect the function of critical PPIs among chromatin-associated proteins. Higher-order packaging of DNA into chromatin within the nucleus is dynamically regulated and requires exquisite coordination between the factors that direct this packaging and those that require access to the underlying DNA for replication, transcription, and repair [Wang et al., 2007]. The core repeating unit of chromatin, known as the nucleosome, contains approximately 147 base pairs (bp) of DNA wrapped nearly twice around an octamer of histone proteins. Extensive efforts in chromatin-based research have shown that the deposition of post-translational modifications (PTMs) on histone and non-histone proteins is an essential mechanism for recruiting the factors that regulate replication and transcription [Strahl and Allis, 2000; Bode and Dong, 2004; Huang and Berger, 2008; Benayoun and Veitia, 2009; Delmore et al., 2011;

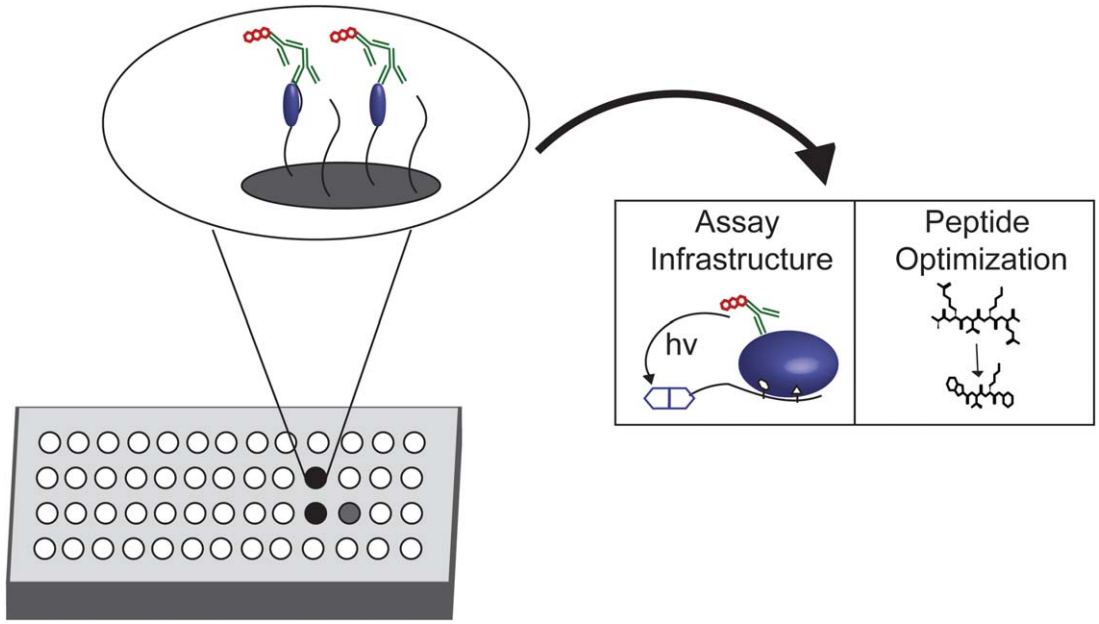

Fig. 1. Microarray screening platform. Preliminary microarray profiling facilitates substrate and selectivity characterization along with spurring subsequent assay development and inhibitor optimization. [Color figure can be viewed at wileyonlinelibrary.com]

Sadakierska-Chudy and Filip, 2015]. Commonly described PTMs of histone tails include methylation, ubiquitinylation, sumoylation, acetylation, crotonylation, butyrylation, ADP-ribosylation, and phosphorylation, although others have been discovered and many more are likely to exist [Andrews et al., 2016].

Although an individual PTM might appear minor in the context of the totality of chromatin regulation, these marks are central to the complex biology of epigenetics. The simplistic view of this regulation may be summarized as follows: PTMs are deposited and removed by distinct enzymes (referred to as "writers" and "erasers," respectively) and ultimately interpreted by proteins that bind to the mark and mediate a biological effect ("readers"). Many of these writers, readers, and erasers intricately connect with one another to coordinate their functions, trigger activities of the chromatin machinery, and indirectly regulate gene transcription. The diversity of both the types of modifications and their locations yields an overwhelming number of potential messages [Ernst et al., 2011] Despite rigorous examination, a knowledge gap persists in our understanding of how mechanistically many of these marks elicit a biological response, and chemical probes can play a central role in closing this gap. Herein, we detail current efforts in peptidic and peptidomimetic tool development for the chromatin PTM regulatory machinery. We focus on available technologies for the preliminary assessment of peptide–protein interactions, the evolving field of peptidomimetic inhibitor discovery against these proteins, and the implications of these studies for future small molecule efforts.

PRELIMINARY CHARACTERIZATION OF PEPTIDE–PROTEIN INTERACTIONS

Prior to initiating a program to optimize a peptidic inhibitor for a target of interest, a thorough knowledge of the substrate preferences for that target can be hugely insightful. Profiling protein–peptide interactions via microarray platforms has had an enormous impact on the characterization of chromatin-associated protein ligands. Beyond simply better understanding the binding preferences of a protein, the extensive sequence selectivity data that microarrays provide constructs a strong preliminary SAR profile that may rapidly allow peptide truncation and ligand optimization (Fig. 1). Moreover, a known peptide ligand for the pocket of interest enables the development of assays to efficiently screen compound collections or conduct follow-up peptide SAR.

Microarray chips with libraries of histone peptides affixed to a solid surface are capable of probing protein affinity and selectivity with minimal cost associated and material per chip [Uttamchandani and Yao, 2008]. Importantly, peptide microarrays are typically amenable to libraries of hundreds of peptides, or even more with the appropriate infrastructure, but significant synthetic effort and time are required to synthesize, purify, and

characterize the individual peptides for each library [Rothbart et al., 2012]. An exceptional example of such an approach comes from the Strahl group. They developed histone peptide microarrays labeled with >4000 peptides [Rothbart et al., 2012]. In brief, biotinylated histone peptides decorated with variable PTMs are synthesized and printed on a streptavidin coated glass slide. Next, the slide is coated with a His- or glutathione S-transferase (GST)-tagged target reader protein followed by the addition of a fluorescently-labeled secondary antibody. Fluorescence confirms that the target protein is bound and indicates a positive protein–peptide interaction. Although spotting final peptides onto chips for screening is relatively straightforward, careful design of the peptide library and optimization of screening conditions are critical to yield the information of interest.

When seeking to assign an endogenous ligand for less well-characterized reader proteins, peptide libraries require greater diversity in both the PTMs and histone sequences. (Scott B. Rothbart et al., 2012) For instance, the BAH domain of ORC1 was thought to associate with chromatin based upon the reported function of an orthologous domain in budding yeast [Kuo et al., 2012]. To assess the validity of this hypothesis, an 82 histone peptide library was probed with GST labeled $ORC1_{BAH}$ and significant fluorescence was observed for only H4K20me2-containing peptides. This subsequently led to insights into a genetic disease that features mutations adjacent to the ORC1 methyllysine (Kme) binding site [Kuo et al., 2012]. In contrast, when seeking to better profile the binding selectivity of a reader domain with a known substrate, a diverse library designed around the substrate sequence might be more informative and help to probe combinatorial PTM effects [Rothbart et al., 2012].

In another example, the Strahl lab used their peptide libraries to characterize the multivalent engagement of UHRF1's tandem tudor domain (TTD) and plant homeodomain (PHD) with histone H3, residues 1–10 [Rothbart et al., 2013]. The TTD had previously been shown to bind preferentially to H3 lysine 9 trimethylation (H3K9me3) [Rothbart et al., 2012], but secondary profiling of the dual domain construct showed synergism between TTD binding and the PHD domain's recognition of residues 1–4 of H3. Moreover, the array profile demonstrated that several PTMs on these four H3 N-terminal residues disrupted UHRF1 binding. The extensive combinations of PTMs available in the library afforded rapid classification of UHRF1 binding preferences, and demonstrated the utility of peptide microarrays for elucidating multivalent binding of chromatin-associated proteins. Recent efforts to exploit multivalency in ligand development for chromatin reader proteins indicate that inhibiting these broader interfaces is a tractable strategy [Tanaka et al., 2016; Waring et al., 2016]. Consequently, profiling these types of multidomain proteins via peptide arrays might shift preliminary ligand discovery efforts to focus on disrupting both pockets to exploit the synergy of such an interaction.

An analogous peptide array strategy applies "SPOT" synthesis to assess protein target binding to histone PTMs. In this approach, a peptide library can be synthesized directly on a cellulose membrane using standard peptide synthesis methodology and Fmoc-protected amino acids [Nady et al., 2008; Muller and Muir, 2015]. Following synthesis, SPOT arrays are incubated with epitope tagged proteins of interest and analyzed for peptide binding by either fluorescence-based or immunological detection [Nady et al., 2008; Muller and Muir, 2015]. The SPOT blot platform offers certain advantages over conventional peptide microarrays. First, direct synthesis on the cellulose membrane eliminates the need for purification of individual biotinylated peptides necessary for the assembly of microarrays [Toepert et al., 2003; Nady et al., 2008; Muller and Muir, 2015]. Second, thousands of modified peptides can be quickly and efficiently synthesized in parallel on a single SPOT array enabling the inclusion of any of the 100 plus histone marks in a singular or combinatorial fashion [Muller and Muir, 2015]. Importantly, screening unpurified peptides in this strategy does raise concerns regarding the potential build-up of synthetic by-products and the risk of false positive results, but this issue can be addressed via follow-up off-cellulose orthogonal assays to characterize the interaction.

Among the most prominent applications of SPOT array-based screening is the interrogation of chromatin-associated proteins to identify modification-specific and sequence-specific histone interactions. One impressive study describes a global, systematic analysis of bromodomain specificity for acetylated lysine-containing peptides [Filippakopoulos et al., 2012]. Numerous SPOT arrays were assembled, ranging from arrays decorated with singly acetylated peptides to acetylated peptides flanked by neighboring PTMs to polyacetylated peptides. These studies demonstrated the preference of bromodomains for poly-acetylated peptides over mono-acetylation [Filippakopoulos et al., 2012]. Another comprehensive study involved characterizing the sequence specificity of the Kme reader chromobox homolog (CBX) proteins for their cognate methylated peptides [Kaustov et al., 2011]. Using two SPOT arrays spanning histone H3 residues 3–15 and 21–33 with H3K9me3 or H3K27me3, respectively, peptides were assembled such that every residue in the sequence was systematically mutated to each of the twenty natural amino acids, yielding a total of 260 peptides per array. Representative members of both the HP1 (CBX3) and Polycomb (CBX7, CBX8) sub-families

of CBX proteins were evaluated for their binding specificity, providing a wealth of peptide SAR. While CBX3 bound only H3K9me3 peptides, CBX8 was selective for H3K27me3 peptides and CBX7 bound methylated peptides on both arrays. Conserved recognition of an "ARKme3S" motif present in both sequences was shared across all CBX proteins, yet little sequence specificity was observed for other regions of the peptides [Kaustov et al., 2011]. The specificity determinants characterized by this study were critical to the development of potent peptidomimetic CBX inhibitors and ultimately led to a chemical probe for the Polycomb CBXs [Simhadri et al., 2014; Stuckey et al., 2016].

While peptide microarrays have been most extensively applied to reader proteins of PTMs, they are certainly also amenable to substrate profiling of histone modifying-enzymes. Thermofisher Scientific, Millipore and Epicypher have all developed effective profiling platforms for these enzymes which can deliver vast amounts of information regarding substrate selectivity and the contextual effects of other PTMs. Similarly, rigorous examination of detection conditions for these platforms is critical [Rothbart et al., 2012; Cornett et al., 2016], but these approaches can rapidly inform efforts aimed at understanding protein function and selectivity.

Assigning endogenous ligands to chromatin-associated proteins can illuminate novel biology and lay a solid foundation for future peptide-to-probe optimization. Peptide microarrays yield information on consensus binding sequences and can provide initial peptide SAR, help to inform peptide truncation plans, identify key residues for modification, and suggest hotspot residues. Moreover, knowledge of the endogenous ligand or a more potent analog may facilitate construction of an assay infrastructure to pursue more extensive screening campaigns. Thus, such peptide technologies inform biological hypotheses and can help to rapidly launch chemical probe development efforts.

PURSUING PEPTIDIC PROBES OF KME READERS

Although early efforts in peptidic ligand development against chromatin-associated proteins included inhibitors of the histone deacetylase (HDAC) enzyme class [Montero et al., 2009; Olsen and Ghadiri, 2009], this work followed early successes in small molecule inhibitor development. Thus, we focus here on the development of peptidomimetic ligands targeting the less well-studied Kme readers to illustrate many of the advantages of utilizing peptidic starting points for the efficient targeting of PPIs in the study of chromatin regulation. Our own early small molecule screening campaigns for Kme reader proteins were met with some limited success, yet led to the first-in-class Kme reader chemical probe, UNC1215 [James et al., 2013]. However, the broad surface-groove binding modes of many Kme readers classify them as "undruggable" [Hopkins and Groom, 2002]. Bearing in mind that such exposed interfaces are historically rife with failed attempts at small molecule drug discovery, we and others pursued peptidomimetic optimization as a rational alternative for probe discovery.

In the development of Kme reader inhibitors, we were faced with a set of peptide-specific challenges to overcome. First, many Kme readers bind methylated histone peptides with affinities between 10 and 40 μM, and such low potencies can be problematic for assay development and early SAR [Botuyan et al., 2006; Guo et al., 2009; Wigle et al., 2009; Kaustov et al., 2011]. Second, many Kme readers of interest recognize the trimethyl-lysine (Kme3) mark. Quaternary amines generally have poor passive membrane permeability, limiting their application in cellular studies [Stuckey et al., 2016]. Additionally, the peptide backbone is likely to limit permeability due to the hydrogen bond donating capacity of the amide protons [Goodwin et al., 2001; Tan et al., 2008]. Third, the length of many methylated histone peptides known to interact with Kme reader domains is typically 10–20 residues [Guo et al., 2009; Xu et al., 2010; Kaustov et al., 2011; Qin et al., 2013]. Optimization efforts thus need to address truncating these ligands while improving their affinities and properties, as histone peptides are also generally poly-cationic and contain other undesirable functional groups for cellular efficacy (guanidine, primary amides, etc.). Finally, derivatization of the endogenous histone ligand may result in inhibitors that are naturally non-selective and bind multiple reader proteins.

Targeting the Polycomb CBX proteins, all of which contain a conserved N-terminal chromodomain responsible for Kme3 binding, exemplifies many of the challenges associated with Kme peptide optimization, but also demonstrates the successful use of peptidomimetic inhibitors to expand the ligandable proteome [Di Croce and Helin, 2013]. The five Polycomb CBX proteins (CBX2, −4, −6, −7, and −8) recognize H3K27me3 and serve as critical, but mutually exclusive, components of canonical Polycomb Repressive Complex 1 (PRC1), a key regulator of transcriptional programs. CBX7 is perhaps the best studied Polycomb paralog, and efforts have been focused on ascertaining its potential as a therapeutic target due to its oncogenic role in several cancers and its effects in mediating embryonic stem cell self-renewal [Bernard et al., 2005; Yap et al., 2010; Klauke et al., 2013; Shinjo et al., 2014]. We and others, therefore, sought a chemical toolbox to probe CBX, or more specifically CBX7, function. Like the majority of

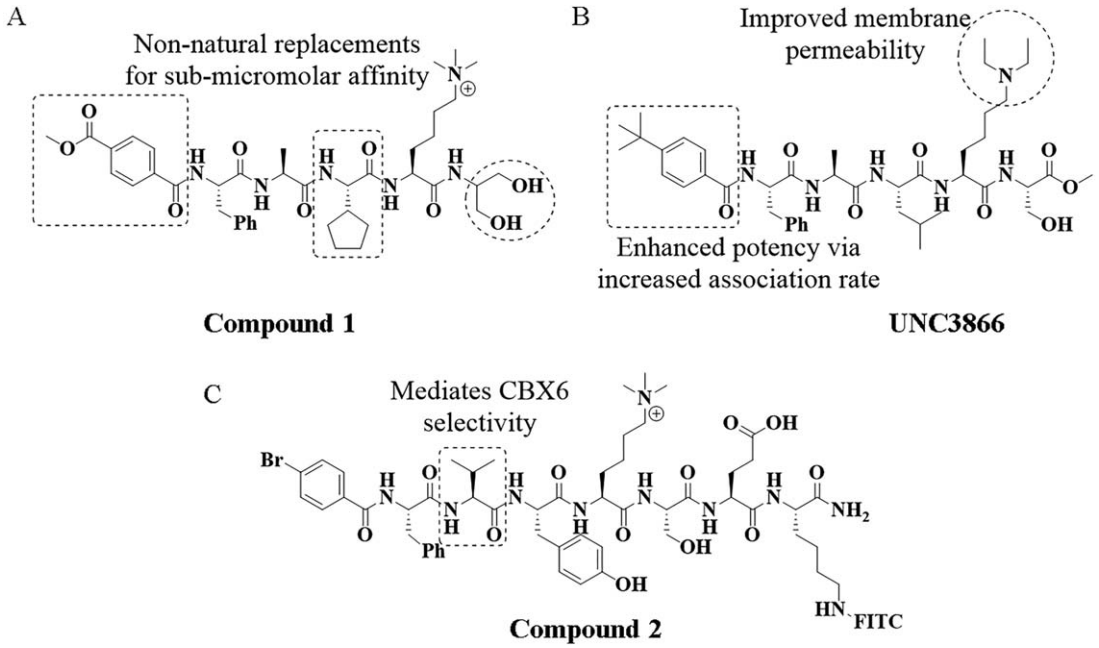

Fig. 2. CBX peptidomimetic inhibitors. (**A**) A preliminary peptidomimetic ligand of the PRC1 CBXs demonstrated nanomolar in vitro potency and was achieved via truncation and incorporation of non-natural modifications. (**B**) Replacement of the Kme3 with diethyl-lysine and optimization of the N-terminal cap furnished a cellularly active chemical probe, UNC3866. (**C**) Replacement of the central alanine with valine led to a new strategy for homolog specificity for CBX6 within the CBX family.

Kme reader proteins, the CBX7 chromodomain contains an aromatic cage that recognizes Kme3 predominantly through π-cation interactions [Kaustov et al., 2011]; however, the formation of the aromatic cage of CBX7 seemingly requires the binding of a methylated peptide to form a fourth antiparallel beta sheet, indicating the necessity of the surrounding sequence for Kme3 recognition [Kaustov et al., 2011; Stuckey et al., 2016]. Peptide ligands lend themselves to this induced-fit mode of binding as they are capable of making the critical backbone hydrogen bonds that are required to mediate CBX7 folding.

Pioneering CBX7 peptidic inhibitor exploration began in the Hof group utilizing a SETDB1 peptide shown to have higher in vitro potency for CBX7 than the native H3K27me3 peptide [Kaustov et al., 2011]. This work led to a set of 5-mer peptide inhibitors with sub-micromolar affinity for CBX7 [Simhadri et al., 2014]. The most potent and selective CBX7 inhibitor ($K_d = 200$ nM; 10-fold and 1.5-fold selective over CBX8 and CBX4, respectively) retained the Kme3, alanine, and phenylalanine residues of the SETDB1 peptide, yet key alterations at other positions allowed for a significant increase in potency (Compound 1, Fig. 2A). Substitution of a propanediol group for serine likely preserved key hydrogen bond donors that interact with a glutamate residue in CBX7, while introduction of a cyclopentylalanine residue highlighted a preference for more hydrophobic residues at the position occupied by an arginine in the histone sequence. Finally, installation of a para-(methoxycarbonyl)benzamide group at the N-terminus fared best out of many well tolerated para-substituted benzyl substituents [Simhadri et al., 2014].

Parallel efforts in our group similarly began with optimization of the SETDB1 peptide, but we instead focused initially on tertiary amine replacements for the Kme3 to simultaneously improve affinity and permeability [Stuckey et al., 2016]. Our efforts culminated in the discovery of UNC3866 (Fig. 2B), the first cellular chemical probe for CBX Polycomb chromodomains. While UNC3866 retains many of the structural features of compound 1, replacement of the Kme3 with a diethyl-lysine was crucial for cellular efficacy. Moreover, extensive SAR around the N-terminal benzamide position demonstrated that this region added dramatic potency enhancements through key hydrophobic contacts when substitutions were made at the para-position. A potential explanation for the significance of this position was posed by molecular dynamics simulations which suggested the N-terminus as the first point of contact between histone H3 and CBX7,which in turn initiates the induced-fit mode of binding. Surface plasmon resonance (SPR) studies further supported this hypothesis by demonstrating that the rigidity and complimentary hydrophobicity of the cap enhances association, as opposed to dissociation, rates. Thus, the

induced-fit binding hypothesis complimented ligand property optimization to facilitate the discovery of a cellularly efficacious peptidomimetic chemical probe.

Careful characterization of UNC3866's selectivity profile led to the discovery that, despite high selectivity outside of the chromodomain family and versus the HP1 CBX domains (CBX1, −3, and −5), UNC3866 did have modest affinity for all of the Polycomb chromodomains and off-target affinity for the CDY chromodomain family. Given that these chromodomains have all been shown to recognize H3K27me3, this result is unsurprising, but the nearly 10-fold selectivity for CBX4 and −7 over other Polycomb CBXs was interesting and suggests some differences in the interaction interface that may yield improved selectivity in second generation ligands. Importantly as tools for Polycomb biology, these ligands do not bind the other known H3K27me3 reader in this pathway, embryonic ectoderm development (EED), which is a WD40 domain Kme reader. By targeting the subtle differences at the CBX-ligand interface, selective peptidomimetic compounds have been developed for CBX6, a Polycomb chromodomain that does not yet have a well-defined function [Milosevich et al., 2016]. These efforts capitalized on the slightly larger alanine binding pocket of CBX6 versus CBX4 and −7 by substituting the alanine with a valine to deliver a peptidomimetic ligand that is 6- to 20-fold selective for CBX6 over the other Polycomb CBXs, and ∼90-fold selective over CBX1 (Compound 2, Fig. 2C). Unfortunately, this substitution does not improve CBX6 affinity and these ligands retain only around 1 μM affinity for CBX6, while differentially losing affinity for other Polycomb CBX domains tested. Additionally, CBX2 and −8 possess identical alanine binding pockets to CBX6 but experienced a loss of affinity similar to that of CBX4 and −7, suggesting other selectivity determinants outside of this pocket that are not yet known. The challenge moving forward is taking a non-selective peptidic scaffold and incorporating non-peptide functionalities to facilitate selective and potent chemical probe development within the Polycomb CBX family. The multiple co-crystal structures obtained during these initial efforts should prove invaluable to this process [Stuckey et al., 2016].

Although UNC3866 has been successfully applied as a cellular chemical probe and its initial mouse PK following intraperitoneal administration is promising, this compound has fallen short of finding utility in an in vivo context owing to poor cell permeability. The modest cellular potency of UNC3866 (EC_{50} ∼7 μM) which is permeability limited illustrates the remaining challenges in developing an in vivo chemical probe using this template [Stuckey et al., 2016]. Ongoing studies are aimed at further enhancing permeability, target affinity, and the utilization of in vivo appropriate delivery systems to overcome these issues.

MINING PEPTIDE CHEMICAL SPACE VIA COMBINATORIAL CHEMISTRY

The extensive effort required to investigate structural hypotheses for chromodomain inhibition is indicative of the challenges inherent in systematic peptide optimization. When carried out via solution phase synthesis, peptide SAR is costly, time-consuming, and often fails to discover synergistic modifications. Designing selective compounds from a modestly selective, large scaffold is not always straightforward and often comes at the expense of potency or permeability. Fortunately, the past three decades have witnessed the advent of impressive combinatorial technologies to address these very issues. Applying combinatorial chemistry to the optimization of peptidomimetic ligands affords a strategy for the rapid discovery and optimization of peptidic and peptidomimetic ligands while interrogating hundreds-to-thousands of key structural hypotheses with minimal effort and cost.

Combinatorial chemistry provides an economically efficient route to mine chemical space via the rapid creation and screening of libraries on the order of 10^4–10^{12} compounds. Unlike small molecules, peptides are well-suited to traditional combinatorial methodologies due to their efficient chemistries, modular assembly, and straightforward structural deconvolution via tandem mass spectrometry. Additionally, peptides are uniquely capable of harnessing the cellular machinery to develop vast phage and mRNA display-based libraries. In applying combinatorial peptide assembly, the advantages are by no means limited to hit discovery as combinatorial hit optimization can also be achieved via rapid exploration of targeted chemical space [Aina et al., 2007; Gao et al., 2015]. Mining targeted chemical space in turn informs an understanding of the structural requirements for binding and yields extensive SAR. Ultimately, a successful combinatorial platform for peptidic ligand optimization requires an informed library design and a screening infrastructure to reap the benefits of this technique without falling prey to its pitfalls.

Multiple groups have previously explored combinatorial peptide optimization focused on improving affinity. Where less advancement has occurred is in the application of combinatorial chemistry to improve compound properties and deliver biologically relevant and selective chemical probes. Rationally designed combinatorial libraries and appropriate screening strategies are likely to enable the identification of ligands with improved physicochemical properties. Furthermore, the

amenability of combinatorial libraries to multiple rounds of screening facilitates a target class screening effort to yield selectivity and affinity information prior to structural deconvolution [Barnash et al., 2016].

The successful use of a combinatorial strategy to facilitate peptide-to-peptidomimetic optimization can be seen in our development of ligands for the central Kme reader of Polycomb repressive complex 2 (PRC2), EED [Barnash et al., 2017]. PRC2, which contains three core components, enhancer of zeste homolog 1 or 2 (EZH1/2), EED, and suppressor of zeste 12 (SUZ12), coordinates the methylation of H3K27 via the enzymatic activity of EZH1 or −2. EED binds and interprets the product of PRC2 activity, H3K27me3, which in turn allosterically stimulates EZH2 methyltransferase activity mediating propagation of this PTM [Margueron et al., 2009]. Starting with a five-residue non-histone methylated peptide (K_d ~10 μM), the design, synthesis, and screening of two combinatorial libraries paired with traditional SBD resulted in a 10-fold more potent EED ligand with a reduced number of hydrogen bond donors and a lower charge state. Ultimately, the final ligand, UNC5115, has a molecular weight under 600 Da, has a charge state of +2, includes only three hydrogen bond donors, and contains only a single natural amino acid (Fig. 3A). While methylated proteins that bind to the EED reader pocket are known to stimulate PRC2 methyltransferase activity, our peptidomimetic ligands inhibit PRC2 methylation of an H3K27 peptide. Since these inhibitors were inactive against EED aromatic cage mutants and they were shown to compete with H3K27me3, we concluded that they likely function allosterically by disrupting the stabilization of a critical EZH2 α-helix that facilitates opening of the enzyme's substrate binding channel. Consequently, PRC2 retains basal activity but is not stimulated upon ligand binding. Disruption of the Kme reader function of EED is consequently a viable strategy for targeting PRC2, and interestingly, the effects are unlikely to be identical to those of previously published EZH2 inhibitors due to the retention of basal methyltransferase activity.

In addition to EED ligand development, we exploited the ability of on-bead libraries to be screened repeatedly to pursue a target class screening strategy, with the intention of repurposing UNC3866 to discover high affinity ligands versus the chromodomains of the CDY family. After the synthesis of a UNC3866 derivative library and a preliminary negative selection, we screened our biased library against the target chromodomain, CDYL2, and employed competitor exchange kinetics to selectively isolate higher affinity ligands than UNC3866. Subsequent homologous chromodomain negative selections against the CDYL2 binding beads removed non-selective ligands, leading to the final isolation of ligands with unique potency and selectivity profiles as compared to UNC3866. UNC4991 is a submicromolar ligand of the CDYL chromodomains that demonstrates the importance of the N-terminal residue in mediating chromodomain potency and selectivity [Barnash et al., 2016]. Moreover, the library hits demonstrate minimal tolerance to alteration at the alanine, lysine, and serine positions yielding important insights into the requirements for peptidomimetic binding across chromodomains. Although these ligands have not yet been exploited to discern novel CDYL2 biology, we anticipate continued optimization and expanded interest in CDYL proteins will greatly enhance the impact of this series of ligands [Liu et al., 2017]. Moreover, this combinatorial strategy is readily applicable to other chromodomain-containing proteins and can rapidly yield ligands of novel selectivity and affinity against new targets.

The dependence of hit deconvolution on sequencing efficiency in one-bead-one-compound libraries limits the utility of this approach for interrogating larger peptides, and screening libraries of more than a few million compounds is practically quite challenging for most academic settings. Consequently, alternative peptide library technologies have emerged over the past three decades to interrogate larger libraries [McCafferty et al., 1990; Hanes and Plückthun, 1997; Roberts and Szostak, 1997]. These display technologies (ribosomal, phage, and mRNA) all apply DNA sequencing, not peptide sequencing, for final hit deconvolution. Moreover, screening of the libraries can be conducted on a much smaller scale due to the homogenous nature of the selection platform which typically includes an affinity enrichment step for protein-bound peptide ligands. Suga and co-workers discovered a mechanism-based, isoform selective SIRT2 macrocyclic peptide inhibitor through mRNA display [Morimoto et al., 2012]. Their library incorporated three non-natural amino acids including an acetyl-lysine mimetic (trifluoroacetyl-lysine) that dramatically reduces the diacylation reaction rate and α-*N*-(2-chloroacetyl)-L-tyrosine or α-*N*-(2-chloroacetyl)-D-tyrosine to covalently react with nucleophilic residues in or near the catalytic site. Final ligands derived from these efforts exhibited affinity in the single digit nanomolar range, demonstrating the utility of using combinatorial libraries to rapidly develop potent and selective ligands against challenging targets. Ultimately, display techniques substantially expand the diversity achievable with large peptide libraries, but they are currently hindered by the limitations of genetic encoding; however, significant effort has been invested to address this issue and engineer technologies to incorporate PTM-mimetic amino acids [Guo et al., 2008;

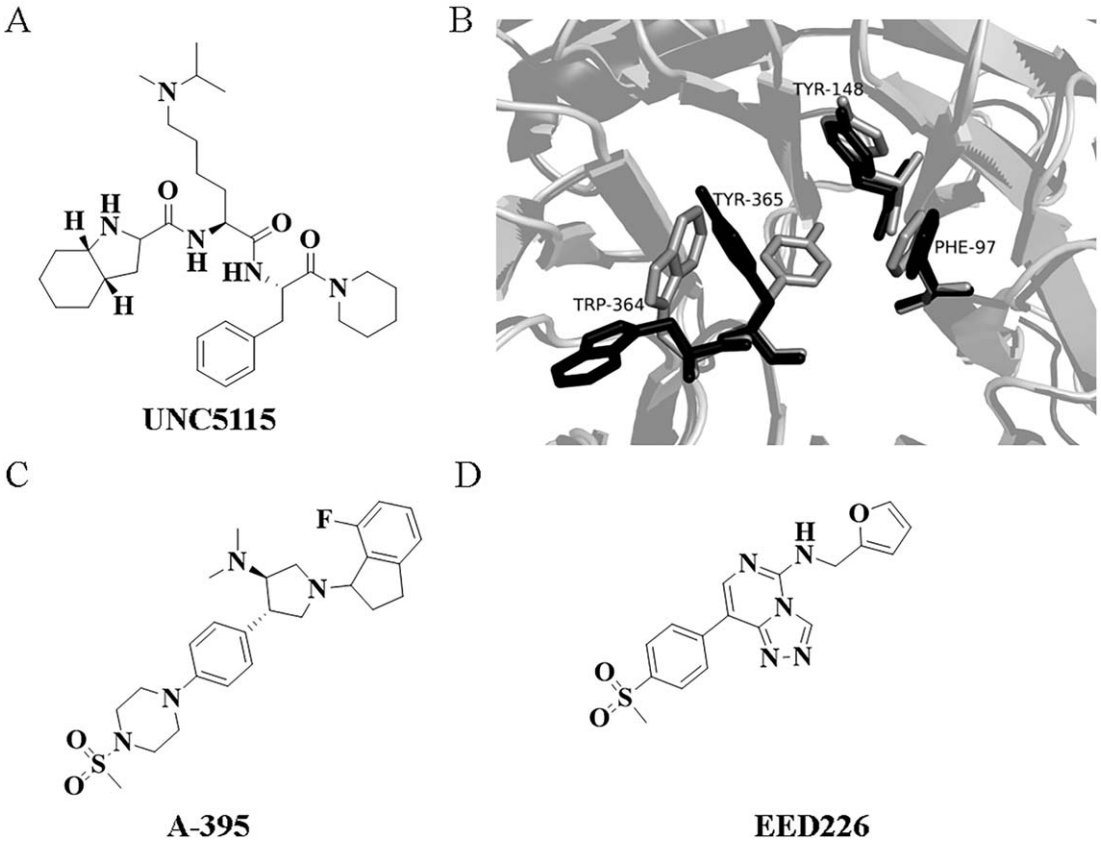

Fig. 3. EED inhibitors. (**A**) UNC5115 is a peptidomimetic ligand of EED that was optimized via paired combinatorial chemistry and SBD. (**B**) Small molecule inhibitors of EED restructure Trp364 and Tyr365 of EED's aromatic cage (black, PDB 5GSA) as compared to the binding mode of methylated peptides (PDB 5HYN). The small molecule inhibitors (**C**) A-395 and (**D**) EED226 are structurally divergent but induce similar binding modes in EED.

Morimoto et al., 2012; Kawakami et al., 2013; Knight and Cropp, 2015; Elsasser et al., 2016]. It is likely that the use of such display technologies will greatly increase as improvements are made to the available chemical diversity.

PEPTIDE AND SMALL MOLECULE COMPLEMENTARITY FOR LIGAND DEVELOPMENT

Our own efforts in peptidomimetic inhibitor development yielded significant advances in the understanding of PRC1 chromodomain function, chromodomain family selectivity, and allosteric regulation of PRC2. Pursuing such peptidomimetics is advantageous in an academic setting where diversity oriented synthesis of compound libraries would be both high risk and prohibitively expensive; yet, limited cell permeability and in vivo efficacy restrict the current use of these peptidomimetic ligands toward understanding chromatin regulation at an organismal level. Peptidomimetics are, however, advantageous as a window of insight into the ligandability of PPIs. Additionally, they are easily derivatized to facilitate new assay methodologies as our sights shift back to small molecule discovery.

Our own preliminary efforts at small molecule discovery against the malignant brain tumor (MBT) family of Kme readers were distinctly biased toward peptidic fragments which evolved into small molecule Kme mimetics off of an aromatic core [Herold et al., 2011; Herold et al., 2012]. These ligands were intended to bind analogously to the native Kme but with conformational constraints and improved contacts in and around the targeted aromatic cage. Cross-screening of these compounds within the Kme reader and methyltransferase target classes has led to some success, but has often resulted in modestly potent ligands [James et al., 2013; Ma et al., 2014; Perfetti et al., 2015; Robaa et al., 2016; Wagner et al., 2016]. Elaborating on the current peptidomimetic scaffolds that target chromatin regulatory proteins might spur innovative strategies to evolve peptidomimetics toward small molecule-like chemical

space. Known mimetics of peptide cores have also not been explored in this context, and future efforts may benefit from assessing the amenability of these peptides to such replacements [Lao et al., 2014; Bhardwaj et al., 2016; Craven et al., 2016; Watkins et al., 2017].

Recent efforts targeting the preformed aromatic cage of EED are suggestive of a new strategy for small molecule antagonism of Kme reader domains [He et al., 2017; Qi et al., 2017]. The reported EED chemical probes, published simultaneously by AbbVie and Novartis, completely restructure the "open" aromatic cage of EED resulting in analogous binding modes. In both structures, the tryptophan of the cage (Trp364) is flipped out toward the protein surface due to the movement of tyrosine 365 upon binding (Fig. 3B). This movement is facilitated by compensatory ligand cation-π interactions with the two tyrosines of the aromatic cage in the case of A-395 (Fig. 3C), whereas EED226 (Fig. 3D) appears to form a network of π stacking interactions through its electron deficient aromatic core. The binding mode of the latter compound suggests the possibility of targeting other Kme reader proteins with small molecules in a similar fashion. Unlike A-395, EED226 is completely void of chiral centers and its structure is almost fragment-like, yet its affinity for EED is low nanomolar. Moreover, EED226 is the first Kme reader chemical probe to lack a discernible Kme mimetic due to replacement of this moiety with an electron deficient aromatic ring system.

These small molecule EED modulators exemplify conversion of the "undruggable" into the druggable via the creation of an induced-fit pocket. The implications of these successes are that, given an appropriate small molecule library, motifs capable of restructuring aromatic cages could be reapplied in a target class screening effort to reassess the small molecule ligandability of Kme reader proteins. Accordingly, the challenge lies in developing and pursuing a more appropriate chemical space to induce rearrangement of aromatic cages. The success of EED226 suggests that electron deficient aromatic fragments represent a promising starting point for library design and inclusion in peptide frameworks for ligand discovery. Perhaps this motif will represent a privileged scaffold for Kme reader antagonists?

CONCLUSIONS

Modulation of chromatin compaction via PTM deposition and removal requires exquisitely controlled protein machinery. Multiprotein complexes, such as PRC1 and PRC2, directly regulate individual marks and indirectly regulate numerous PTMs, but the complexity of their biology prevents molecular and genetic techniques from elucidating a comprehensive understanding of their function. Moreover, teasing apart the individual contributions of specific domains remains an even more daunting task when solely relying on these traditional approaches.

The growing trend toward targeting chromatin-modifying proteins demonstrates the advantages of chemical probes for interrogating domain function. Kme readers are a fascinating target class that exemplifies the challenge of interrogating PPIs with chemical probes. The disruption of the surface groove binding of Kme readers to their cognate histone peptides via high-throughput small molecule hit discovery has been only modestly successful to date [He et al., 2017; Huang et al., 2017; Li et al., 2017; Lingel et al., 2017; Qi et al., 2017]. In contrast, optimized peptidomimetic inhibitors are a viable alternative for cellular chemical probe discovery with known challenges to be overcome. Peptides offer several advantages including a rational starting point for ligand optimization, simple chemistry, and complementary geometric and structural features to target large protein surfaces. In contrast, improving the potency, selectivity, and physicochemical properties of peptide ligands in order to achieve high-quality probes that engender a biologically relevant endpoint can be challenging. Strategies that enable the multi-parameter optimization of peptidic ligands hold the potential to drastically increase the rate of chemical probe discovery for chromatin-associated machinery, while merging on-bead peptide ligand optimization with the evolution of small molecules will likely also be advantageous.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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