Peptide-based probes for protein N-Methyltransferases

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Chapter 1

Introduction

1. Introduction to protein methyltransferases

Protein methyltransferases (PMTs) are responsible for the methylation of amino acid residues in a number of proteins including histones. PMTs that act on histones are in turn highly involved in the regulation of gene expression and transcription.[1] Protein methylation can occur on lysine, arginine, histidine, or glutamine side chains as well as on α-N-terminal residues.[2] All PMTs use a common mechanism of catalysis, in which the universal methyl donor S-adenosyl-L-methionine (AdoMet) and the target protein substrate bind the enzyme to form a ternary complex and, after transfer of the methyl group, the methylated protein substrate and the demethylated cofactor S-adenosyl-L-homocysteine (AdoHcy) are released from the active site. Methyltransferase enzymes share high homology in the active site residues that interact with the AdoMet cofactor, but can be distinguished by their ability to methylate specific amino acids of a small selection of protein substrates.

The methylation of lysine side chains and N-terminal amino acids result in mono-, di and trimethylated products,[3] while the arginine side chain can be monomethylated or symmetrically or asymmetrically dimethylated on its guanidyl group.[4] Histidine can be monomethylated on its imidazole nitrogen atom[5] and glutamine can be monomethylated to N^5-methylated glutamine (Figure 1).[6] Histone methylation has been extensively investigated since it was first recognized in the early 2000s and it has since been shown to be vital in mediating cell signalling and a range of cellular functions.[7-10] For example, histone H3 methyltransferases control the DNA methylation [11] and the lysine methyltransferase Dot1p is involved in telomere silencing.[12] Aberrant expression of PMTs and their concomitant deleterious effects can be observed in cancer[13], diabetes,[14] and neurological diseases.[15] Due to their role in a variety of key cellular functions, the discovery of selective inhibitors of PMTs has increasingly become an avenue of interest for therapeutic development[16, 17.] Inhibitors of protein lysine methyltransferases, such as UNC0642 (G9a/GLP) against breast cancer,[18] EPZ005687 (EZH2) against lymphoma cells,[19] and CPI-1205 (EZH2) against B-cell lymphoma[20] have been investigated in preclinical trials. In addition, protein arginine methyltransferases inhibitors, MS049 (PRMT4/6),[21] TP-064 (CARM1),[22] and EPZ020411 (PRMT6),[23] are highly selective chemical tools for inhibiting specific protein arginine methyltransferases (PRMTs). The different histone protein methylations regulate gene expression and transcription through a dynamic interplay of chromatin readers, writers, and erasers.[24, 25] To gain a deeper understanding of the mechanistic processes related to histone methylation, peptide-based probes are valuable tools for understanding the roles of specific enzymes in the complexity of epigenetics. In this thesis, the development of peptide-based probes specific for the PRMT family of methyltransferases is discussed. Inspired by naturally occurring PRMT substrates, the general

methodology to design the peptide probes is based on the substitution and modification of the guanidine moiety of the target arginine residue. Through this methodology, the peptide based probes retain the selectivity and specificity of the peptide substrate, while the guanidine side chain modification mimics the methyltransferase transition state, turning a substrate into an inhibitor. These tool compounds provide detailed insight into the interactions of peptide substrate and AdoMet cofactor in the active site of the targeted methyltransferase.

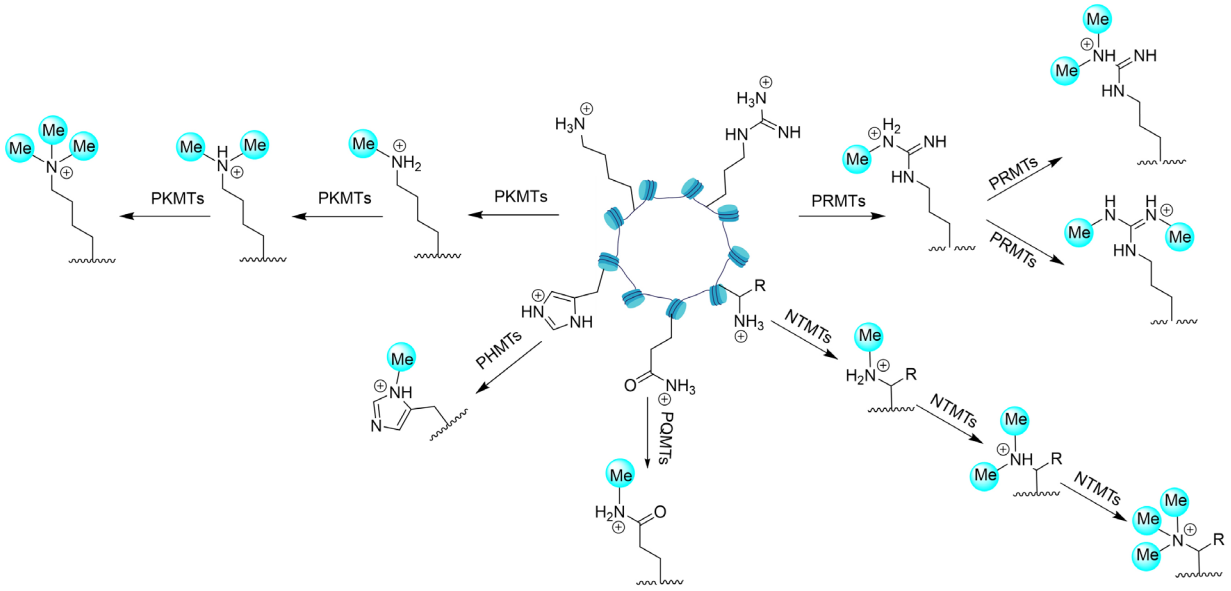

Figure 1. The different types of histone protein methyltransferases and their products. Protein lysine methyltransferases (PKMTs) form mono-, di- or tri-methylated lysine, Protein arginine methyltransferases (PRMTs) form mono-, asymmetrically or symmetrically dimethylated arginine; Protein histidine methyltransferases (PHMTs) form monomethylated histidine; Protein glutamine methyltransferase (PQMTs) form N^5-methylated glutamine; and Protein N-terminal methyltransferases (NTMTs) form mono-, di-, or tri-methylated N-terminal.

2. Peptide Based Inhibitors and Mechanistic Probes for Protein Arginine Methyltransferases

Protein arginine methyltransferases catalyse the transfer of the methyl group from cofactor AdoMet to the guanidine moiety in the sidechain of arginine residues of protein substrates. After binding of the substrate and the cofactor to the catalytic domain of the PRMT, the methyl group is transferred from AdoMet to the arginine side chain following an S_N2–like mechanism, to yield the products S-adenosyl-L-homocysteine (AdoHcy) and the methylated protein substrate (Figure 2). There are three distinct types of PRMTs found in mammalian cells,[4] classified by their methylated product resulting in either ω-N^G-monomethylarginine (MMA), symmetric ω-N^G,N'^G-dimethylarginine (sDMA), or asymmetric ω-N^G,N^G-dimethylarginine (aDMA).[26, 27] The type-I PRMTs catalyze the formation of MMA

and aDMA, and include PRMT1, PRMT3, PRMT4 (CARM1), PRMT6, or PRMT8[4]. The type II PRMT5 and PRMT9 form MMA and sDMA,[4, 27] while PRMT7 is the only type-III PRMT known to date that exclusively produces MMA. PRMT1 was the first mammalian PRMT discovered and is responsible for about 85% of total protein arginine methylation activity.[28] Type-I and type-II PRMTs are responsible for the majority of arginine methylation in humans and their aberrant expression has been linked to different cancers, such as prostate cancer[29] and leukemia[29, 30] as well as other pathologies including cardiovascular disease.[31, 32] Considering the impact of histone methylation on gene regulation and by extension epigenetic processes, PRMTs have been most heavily researched as potential therapeutical targets in a variety of cancers.[33-35]

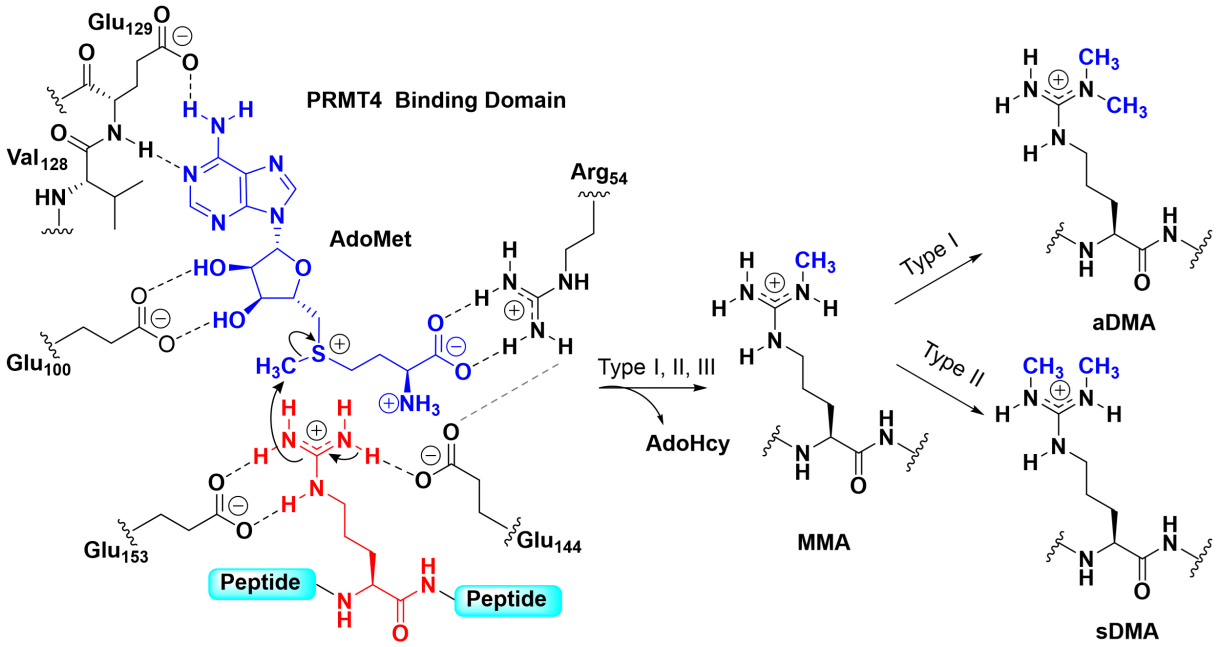

Figure 2. The overview of the PRMT binding site (residue numbering shown for PRMT4) bound to cofactor AdoMet in blue and a protein substrate in red indicating the interactions with the active site residues and the formation of monomethylated arginine (MMA) and subsequently asymmetrically or symmetrically dimethylated arginine (aDMA and sDMA) produced by the different types of PRMTs.

2.1 Peptide probes with substituted guanidino groups

The first peptide-based probes targeting PRMTs focussed on the substitution of the guanidine moiety in the arginine side chain in the context of a peptide fragment of a protein substrate. [36-38] Starting from the sequence of a known PRMT substrate to achieve selectivity, a variety of different substitution of the ω-nitrogen of the target arginine residue were explored to evaluate the effects on the methylating activity of the target PRMT. The first generation N^{η}-substituted arginyl peptides prepared in our group investigated the effects of substituent electronics and sterics on the second methylation step performed by PRMT1. The introduction of

ethyl, or mono-, di- and tri-fluoroethyl groups on one of the terminal nitrogens of the target arginine in the R1 peptide resulted in the inhibition of PRMT1 with IC_{50} values in the micromolar range (13-29 μM) (Figure 3A).[36] Peptides R1-1, -2, -3, and -4 demonstrated a 5- to 24-fold increase in potency compared to the products of the methylation process (i.e. the aDMA or sDMA-containing peptides). Notably, the potency of the probes against PRMT1 increased with an increasing number of fluorine atoms. In addition, comparable activity was observed against PRMT6, but only weak inhibition of CARM1 was found. Inspired by these results, a second generation of N^{η}-substituted PRMT probes were explored that expanded the range of substitutions on target arginine specifically examining steric, electronic, and pKa and effects (Figure 3B). PRMT1 demonstrated methylating activity towards R4, R5, R6, and R7, but only low levels of methylated product were found for R1 and R3 and no product was observed for R2 in which the arginine residue was modified with a strongly electon withdrawing nitro group. A similar approach was subsequently applied to another PRMT substrate, the HIV-Tat protein. A variety of modifications were introduced onto arginine residue 52 in the HIV-Tat$^{48\text{-}60}$ peptide sequence (Figure 3C) and their effects on enzyme activity analysed. These peptide probes were found to be substrate inhibitors of PRMT1 and PRMT6 and the results obtained demonstrated that a wide range of substitutions were accepted by the PRMTs.

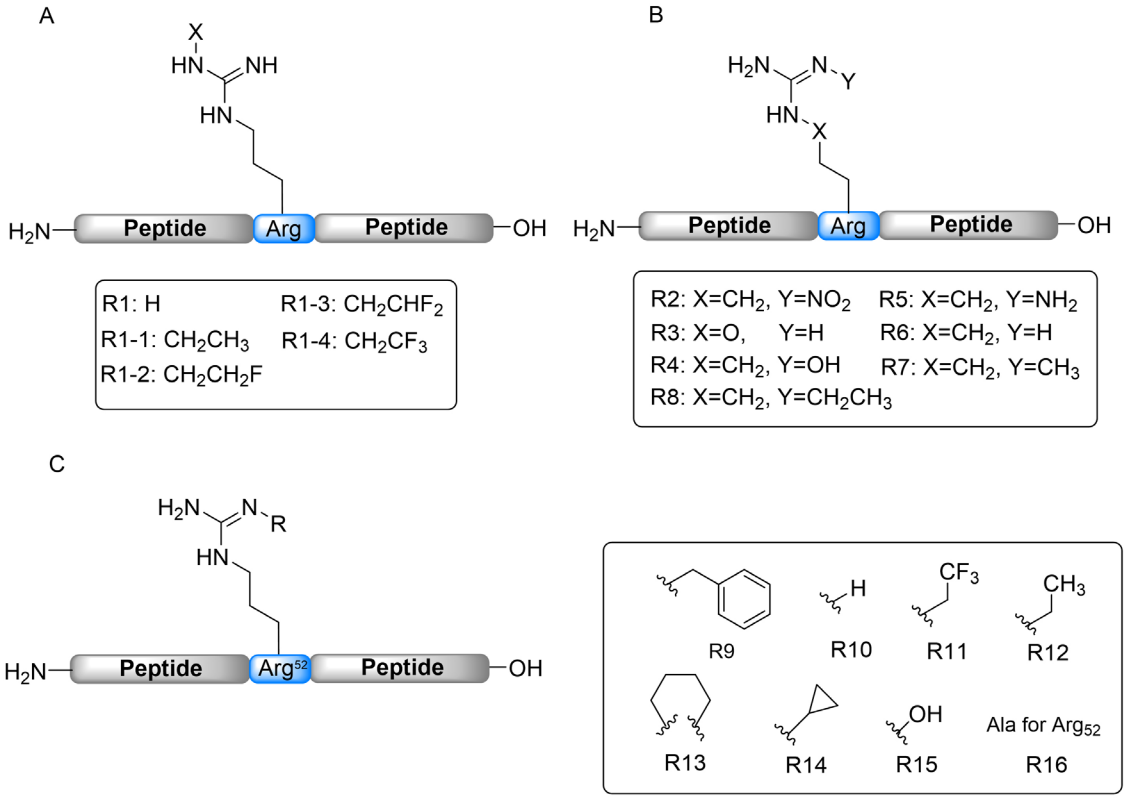

Figure 3. The peptide-based N^{η}-substituted arginine analogues. A and B showed the guanidino modified peptide inhibitors; C. HIV-Tat$^{48\text{-}60}$Arg^{52} guanidino modified peptide inhibitors.

2.2 Chloroacetylated arginines in peptide-based covalent inhibitors of PRMT1

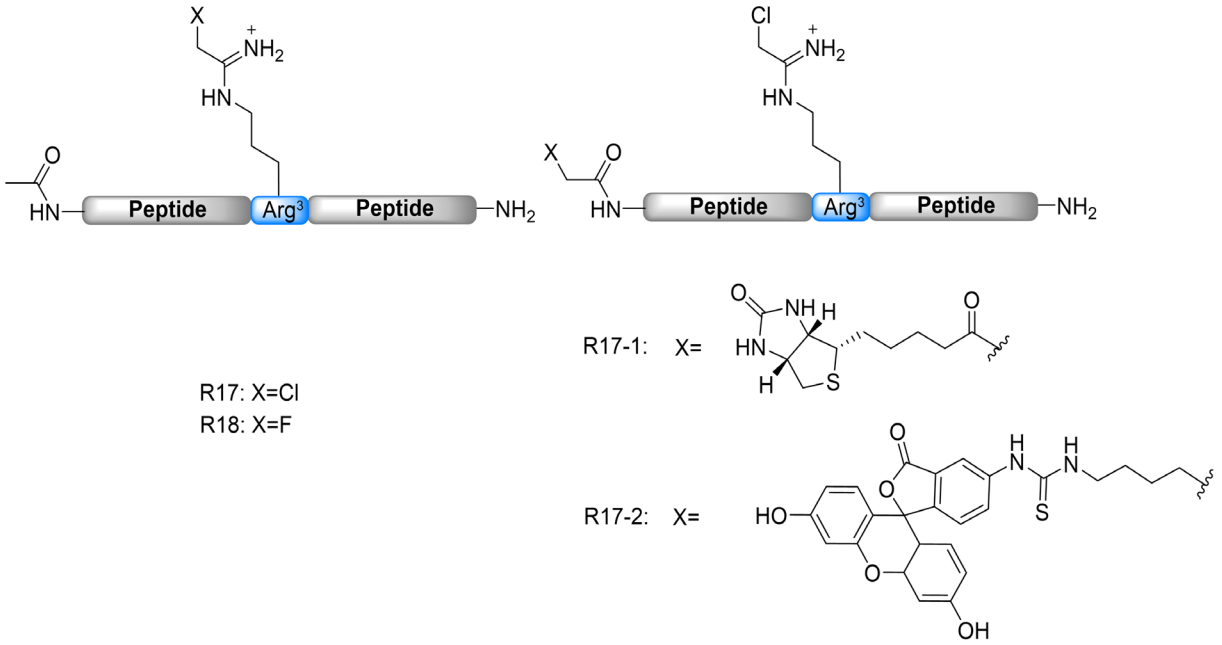

Figure 4. A. Structure of H4 peptide based covalent inhibitors R17 and R18. B. Structure of H4 activity-based probes (ABPs) R17-1 (biotin-conjugated R17) and R18-1 (fluoresce-in-conjugated R18).

Covalent inhibitors have been increasingly considered a viable option in clinic and more frequently enter the market.[39, 40] The covalent interaction with its target blocks the enzyme permanently and the enzyme's activity can be recovered only through de novo protein production. While this can be a downside when de novo production is fast or when prolonged effects are undesirable, covalent inhibitors do have the potential to alter disease pharmacology.[39] Recently, several covalent inhibitors of PRMT5 and PRMT6 have been described, showing promising activity outperforming the most potent competitive inhibitors.[41, 42] The crystal structure of the target enzyme plays a vital role in the design of the covalent inhibitors to select the correct spacers and warheads to evaluate. The work on covalent PRMT inhibitors was initiated by the group of Thompson who designed and synthesized two histone H4-based peptides consisting of the first 21 amino acids of the H4 tail and contained a chloroacetamide warhead on Arg^{17} or a fluoroacetamide on Arg^{18} (Figure 4). To investigate their potencies, the chloroacetamide peptides were incubated with PRMT1 showing that R17 (IC_{50} 1.8 ± 0.1 μM) is about 52-fold more potent than R18 (IC_{50} 94 ± 17 μM). Also of note, while R17 exhibited irreversible inactivation of PRMT1, the fluoroacetamide in R18 was found to be a competitive inhibitor.[43]

The covalent chloroacetamide-containing H4 peptide R17 was subsequently conjugated at the N-terminus to biotin (R17-1) or fluorescein (R17-2) turning the

peptides into PRMT1-targeting activity-based probes (ABPs) (Figure 4).[44] ABPs can be used as chemical tools for the investigation of novel functions, binding partners, expression levels or cellular localisation of enzymes as well as for the screening of inhibitors.[45-47] The results indicated that N-terminal labelling of R17 did not affect the compound's inhibition of PRMT1. Both ABPs showed good labelling of PRMT1 in MCF-7 cells and R17-2 was found efficient to enrich and isolate the PRMT1.[44]

2.3 Peptide-based probes mimicking the methyltransferase transition state

In an attempt to develop more potent PRMT inhibitors, our group reported a series of small bisubstrate molecules with a guanidine group attached to the adenosine unit of cofactor AdoMet with different linker lengths.[48] While this approach resulted in potent inhibitor with somewhat surprising selectivity, the bisubstrate inhibitors were subsequently optimized towards further enhancing their selectivity through the incorporation of a PRMT-specific peptide sequence.[49] This strategy was initially applied on PRMT4 (also known as coactivator-associated arginine methyltransferase 1 (CARM1)). The CARM1 transition state (TS) mimics were designed and prepared based on the peptide sequence of its known substrate poly(A)-binding protein 1(PABP1) (Figure 5). Different linkers between the adenosine and guanidine moieties were also evaluated revealing the three-carbon linker to most closely mimic the distance between the AdoMet adenosine moiety and the substrate arginine guanidino group. The PABP1$^{447-459}$ and PABP1$^{456-466}$ TS mimics showed a nanomolar range of inhibition against CARM1 with up to ~300-fold selectivity over PRMT1.[49]

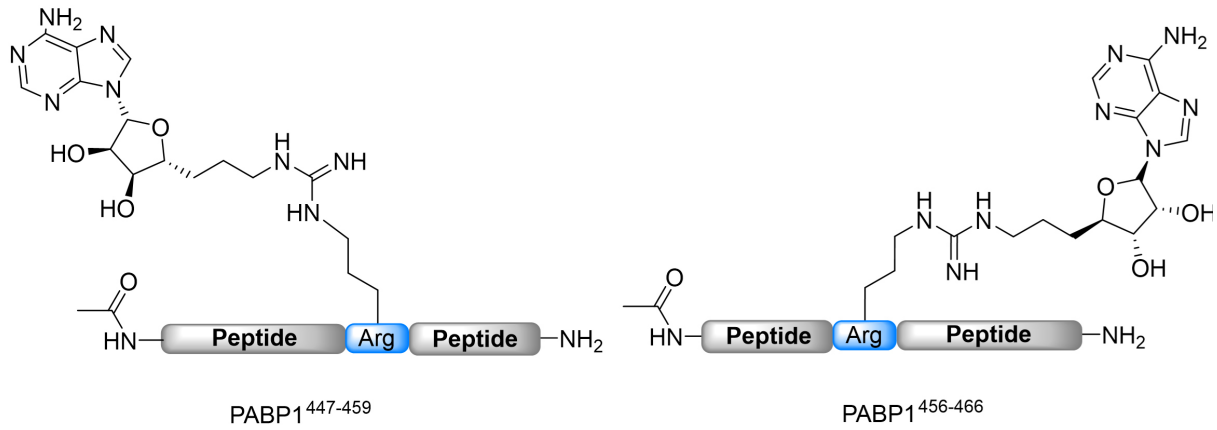

Figure 5. The structure of transition state mimic peptide inhibitors based on CARM1 substrate poly(A)-binding protein 1 (PABP1).

Co-crystal structures obtained with CARM1 and these PABP1 inspired adenosine-peptide conjugates (Figure 6) showed that they effectively mimic the TS of the first methylation step performed by PRMTs. [49] These structural results provided new insights into the binding interactions of the PRMTs with the specific peptide

substrate. This approach to generating PRMT-specific TS mimics is in principle also applicable to all PRMTs. In this regard, part of the work contained in this thesis was aimed at extending this approach to other PRMTs.

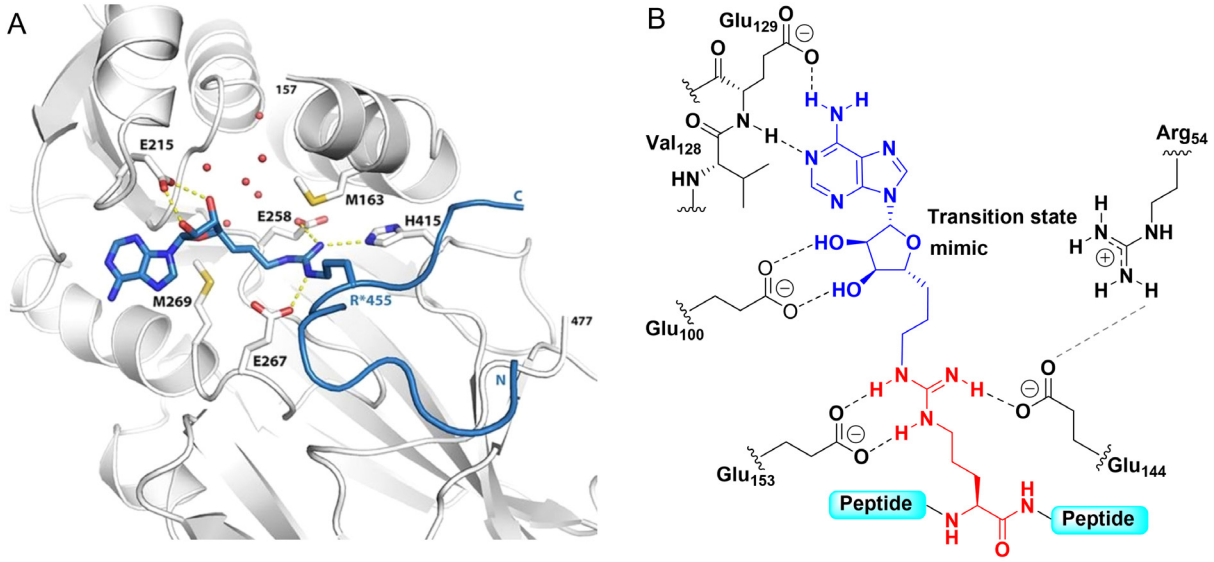

Figure 6. A.The crystal structure of CARM1 and PABP1 peptide substrates. B. Transition state mimic bind to CARM1 catalytic pocket.

3. Thesis Outline

In Chapter 2, the general applicability of the PRMT TS mimic methodology is reported. Specifically, TS mimicking adenosine-peptide conjugates were designed and synthesized to target PRMT1.[50] Based on the previous work targeting CARM1,[49] the three-carbon spacer was selected to covalently connect arginine side chains in histone $H4^{1-7}$ and $H4^{1-8}$ peptides to the adenosine moiety. Both sequences were evaluated with a free and an acetylated N-terminus and the ability of the corresponding TS mimics to inhibit PRMT1 compared to the asymmetrically dimethylated arginine (aDMA) product. In all cases the H4 transition state mimics showed inhibition of PRMT1 and PRMT6 with micromolar IC_{50} values. Interestingly, the extra C-terminal lysine present in $H4^{1-8}$ decreased the selectivity of the compounds towards PRMT1 over PRMT6.

Chapter 3 describes the development of a new series of CARM1 TS mimics based on the sequence of histone H3 around target arginine R^{17}. To examine the impact of neighbouring lysine acetylation in histone H3 on the recognition of arginine residues by CARM1, adenosine-linked peptides were synthesized with the neighbouring lysine residue K^{18} present as either the free amine or in its acetylated form. The potent inhibition observed for both $H3^{10-25}$ and $H3^{10-25}(K^{18}Ac)$ TS mimics, led to further evaluation of shorter peptidomimetics by sequentially omitting N- and C-terminal residues to generate the corresponding deca-, octa-

, hexa-, and tetra-peptide analogues 7–14 (See Chapter 3, Table 1). Each of these truncated peptidomimetics were prepared with and without acetylation of the neighboring Lys^{18} residue to probe the interplay between peptide sequence and lysine acetylation on recognition by and inhibition of CARM1. Inhibition studies subsequently showed the most potent inhibition for the hexapeptide with nanomolar IC_{50} values. Structural studies performed with the H3-based TS mimics provide insight into the effect of acetylation on CARM1-binding. The findings point to the intriguing possibility that crosstalk between lysine acetylation and arginine methylation may also serve to reinforce PRMT specificity beyond the primary sequence of the peptide substrate.

The development of a direct, specific, and convenient analytical method for measuring the activity of CARM1 is described in Chapter 4. The LC-MS based method we developed applies multiple reaction monitoring (MRM) for the detection and quantification of a methylated peptide substrate. The assay presents a significant simplification over existing ELISA and radiometric methods while benefitting from high sensitivity and convenient sample preparation. The application of the MRM LC-MS method has been demonstrated by assaying the inhibitory activity of a selection of known CARM1 inhibitors, showing good comparability with previously published data.

Chapter 5, reports the development of peptide-based inhibitors for a different class of methyltransferase, nicotinamide N-methyltransferase (NNMT). NNMT is an AdoMet-dependent small molecule methyltransferase responsible for the methylation of pyridinyl compounds including nicotinamide (vitamin b3) and is implicated as a driver of both metabolic disease and many cancers. Macrocyclic peptide-based NNMT inhibitors were identified using an mRNA-peptide display technique known as the random nonstandard peptides integrated discovery (RaPID) system. The most highly enriched cyclic peptides from both L-tyrosine and D-tyrosine initiating libraries were synthesized using Fmoc- solid phase peptide synthesis (SPPS) and subsequently evaluated for their inhibitory activity against NNMT. Interestingly, while good inhibition of NNMT was observed, none of the macrocyclic peptides identified in our study exhibit significant competition with the substrates of NNMT; AdoMet or nicotinamide. These findings indicate that rather than binding in the active site, the macrocyclic peptides may instead bind at an allosteric site on the enzyme. Furthermore, in cell-based assays, administration of our macrocyclic peptides was found to result in a significant reduction in the production of MNA by endothelial HAEC cells and A549 lung carcinoma cells.

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