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Reviews

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Protein Arginine Methylation and Citrullination in Epigenetic Regulation

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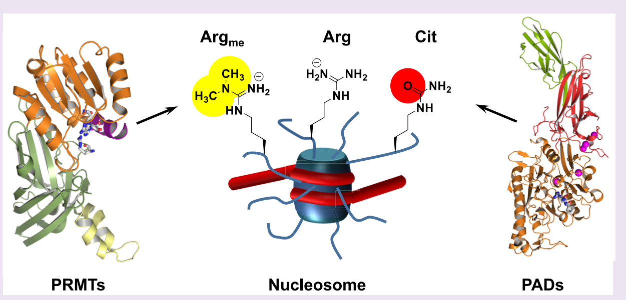
ABSTRACT: The post-translational modification of arginine residues represents a key mechanism for the epigenetic control of gene expression. Aberrant levels of histone arginine modifications have been linked to the development of several diseases including cancer. In recent years, great progress has been made in understanding the physiological role of individual arginine modifications and their effects on chromatin function. The present review aims to summarize the structural and functional aspects of histone arginine modifying enzymes and their impact on gene transcription. We will discuss the potential for targeting these proteins with small molecules in a variety of disease states.



Epigenetic regulation of gene expression is essential to eukaryotic life, and its dysregulation is involved in numerous human diseases. This regulatory mechanism is controlled, at least in part, by a diverse set of post-translational modifications (PTMs) of histone proteins.[1] Histone proteins are small, basic proteins that constitute the building blocks of nucleosomal particles. These proteins form octamers around which the genomic DNA is spooled. Projecting out of this nucleosomal core are unstructured lysine/arginine-rich N-terminal tails.[2] Notably, the N-terminal tails of each histone harbor the majority of known PTMs that are critical for the epigenetic control of gene expression. Since arginine residues are important for DNA binding and protein−protein interactions, it is not surprising that they are subject to extensive modification. Currently, there are four known types of enzymatic arginine modifications, i.e., methylation, citrullination, phosphorylation, and ADP-ribosylation,[3,4] and all four have been shown to occur on histone arginine residues.[4] The best characterized modifications, however, are arginine methylation and citrullination. In this review, we discuss the chemical biology of protein arginine modifications in the epigenetic control of gene transcription, focusing on the enzymes that catalyze protein citrullination and arginine methylation as well as their regulatory effects on the core histone tails and chromatin function. Additionally, we highlight the recent progress in targeting these proteins using small molecule inhibitors.

The Epigenetic Role of Arginine Modifications. *The Biological Effects of Histone Arginine Methylation.* Protein arginine methylation is a common post-translational modification, with many cytoplasmic and nuclear proteins being methylated on arginines.[5−7] In fact, arginine methylation impacts numerous cellular pathways, and, when dysregulated, human disease, particularly the development and progression of cancer.[8] This modification is mediated by a family of nine protein arginine methyltransferases (PRMTs) that can be grouped into three types based on their arginine methylation products, i.e., monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA; for a detailed description, see below). Histone proteins are well-established PRMT substrates for all types of PRMTs.[7] The main sites of histone arginine methylation include H2AR3 and R11, H2BR29, R31 and R33, H3R2, R8, R17 and R26, H4R3, R17, R19, and R23 (Figure 1). In addition, there is evidence that arginine methylation affects not only the histone tails but also the histone core, such as in H3R42$_{me2a}$, where it is implicated in transcriptional activation by weakening the histone−DNA interactions.[9] Typically, asymmetric dimethylation of histones has been associated with transcriptional activation while symmetric dimethylation is linked to transcriptional repression.[10] Here, we provide a brief overview about individual PRMT members and their influence on histone methylation.

PRMT1 is an essential gene product and is responsible for the majority of ADMA modifications in mammalian cells.[11] The PRMT1 deposited methylation mark (H4R3$_{me2a}$) is associated with transcriptional activation of nuclear receptor regulated genes.[12] This coactivator activity is facilitated by the subsequent

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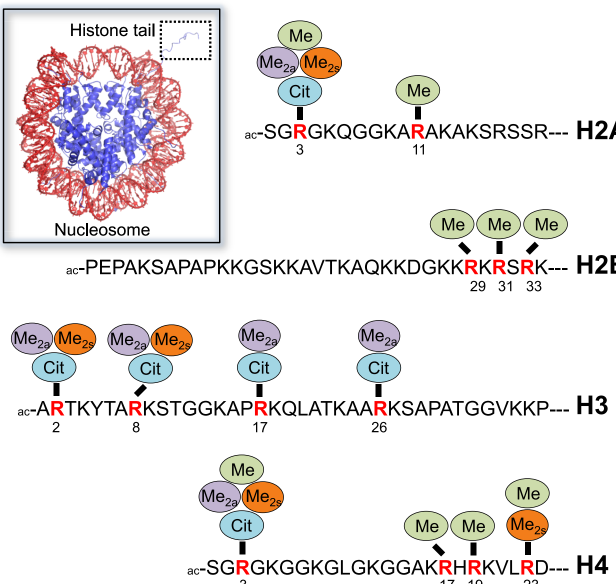

Figure 1. Sites and types of histone arginine modifications. Arginine methylation and citrullination sites of individual histone N-terminal tails. Abbreviations: Me, monomethylation; Me_{2a}, asymmetric dimethylation; Me_{2s}, symmetric dimethylation; Cit, citrullination. The inset on the left depicts the nucleosome core particle (PDB code: 1AOI); DNA is colored in red, and the histone octamer is highlighted in blue, including a protruding H3-derived histone tail that is otherwise barely defined for the other histone proteins in the crystal structure.

acetylation of the H4 tails by the histone lysine acetyltransferase p300.[12] Notably, the previous acetylation of H4 by p300 prevents the methylation by PRMT1,[12] most likely by reducing the positive charges in the remote sequences that are required for efficient PRMT binding (see below). In addition, PRMT1 functions synergistically with CARM1 and p300 as transcriptional coactivators of the tumor suppressor p53.[13] Blythe and colleagues showed that during embryonic development, β-catenin recruits PRMT2 to distinct promoters, where it asymmetrically dimethylates H3R8, thereby priming a genetic program for dorsal development.[14] The PRMT4/CARM1 enzyme was shown to be responsible for transcriptional activation by the asymmetric dimethylation of H3R17 and H3R26 and to be required for the maintenance of cellular pluripotency[15] and also muscle cell differentiation.[16] Moreover, upon growth stimulation, PRMT4 is recruited to the Cyclin E1 encoding gene promoter where it methylates histone H3 at R17 and R26 and thereby functions as a transcriptional coactivator and likely accelerates tumor progression.[17] In contrast to the ADMA mark deposited by type I PRMTs, the symmetric dimethylation of H4R3 by PRMT5 represents a repressive mark that is required for the formation of DNMT3A-mediated transcriptionally repressive DNA methylation.[7,18,19] PRMT5 methylates histones H2A and H4 at R3, respectively, as well as H3 at R2 and R8. Interestingly, the cooperator of PRMT5, COPR5, binds to the amino terminus of histone H4 and thereby recruits PRMT5 to preferentially methylate histone H4 at R3.[20] A similar recruitment of PRMT5 to histones is also mediated by the protein MEP50, which interacts with the histone fold of the H3−H4 tetramer and thus promotes the proper positioning of the substrate arginine to the catalytic site.[21] Notably, Alinari and colleagues reported that B cells transformed with Epstein−Barr virus (EBV) show high levels of nuclear PRMT5 and a concomitant increase in PRMT5-mediated $H4R3_{me2s}$ and $H4R8_{me2s}$ symmetric dimethylation marks and a decrease of the type I PRMT-dependent asymmetric dimethylation of $H4R3_{me2a}$.[22]

Similar to PRMT1, PRMT6 was shown to deposit ADMA marks on H2AR3 and H4R3, and these modifications have been shown to be linked to transcriptional activation.[23] However, PRMT6 can also asymmetrically methylate H3R2, and this modification is associated with transcriptional repression by blocking the recruitment of transcriptional activators to trimethylated H3K4.[24] PRMT7 mediates the monomethylation of H2AR3 and H4R3 that are both associated with DNA damage repair.[25] The presence of these monomethylation marks blocks the transcription of DNA polymerase encoding genes.[25]

Furthermore, epigenetic regulation of gene expression by arginine methylation goes beyond histone methylation and can also directly impact the activity of diverse transcription factors such as CBP,[26] ERα,[27] p53,[28] and BRCA1,[29] as well as RNA polymerase II.[30] Although, there is only limited information available regarding the readers of the histone methylarginine marks, it is now well established that several members of the Tudor protein family specifically recognize methylarginine.[31] For instance, the Tudor domain containing protein TDRD3 recognizes ADMA modified H3R317 and H4R3 and acts as a transcriptional coactivator.[32] It remains to be shown whether other proteins specifically bind to methylarginines or whether competition with other histone modifications is the primary mode of action of these marks.

The Biological Effects of Histone Citrullination. Protein citrullination is mediated by a family of five enzymes called protein arginine deiminases (PADs), which hydrolyze the arginine guanidinium into a urea group. Based on electrostatic considerations, histone arginine citrullination best compares to histone lysine acetylation. In both cases, the positively charged functionalities (guanidinium group in arginine and amino group in lysine) are converted into neutral forms (urea in citrulline and acetamide in acetyllysine). Since histone lysine acetylation is usually associated with an open chromatin structure that can be accessed by RNA polymerases as well as transcription factors and thus typically correlates with gene activation,[1] a similar trend was expected for histone arginine citrullination. Indeed, it was recently shown that PAD4 induced citrullination of the linker histone H1 at R54 leads to extensive chromatin decondensation in pluripotent stem cells.[33] The loosened chromatin structure allows for the enhanced expression of genes involved in stem cell development and maintenance such as *Klf2*, *Tcl1*, *Tcfap2c*, *Kit*, and *Nanog*.[33] It was proposed that the observed overexpression of *PADs* in several cancers might induce a similar chromatin decondensation and thus promote a stem-cell-like state.[34]

However, more detailed analyses regarding the functional effects of histone citrullination reveals that this mark is associated with both transcriptional repression and activation.[35−37] It was suggested that its distinct roles on gene expression can be mediated either by preventing activating arginine-methylation events or by the recruitment of further histone modifying enzymes.[36,38] PAD4 was shown to citrullinate histone H3 on arginines 2, 17, and 26, as well as histones H2A and H4 on arginine 3, respectively (Figure 1).[35,36,39] Specifically, the citrullination at H3R17 represses the expression of estrogen receptor regulated genes.[36] Moreover,

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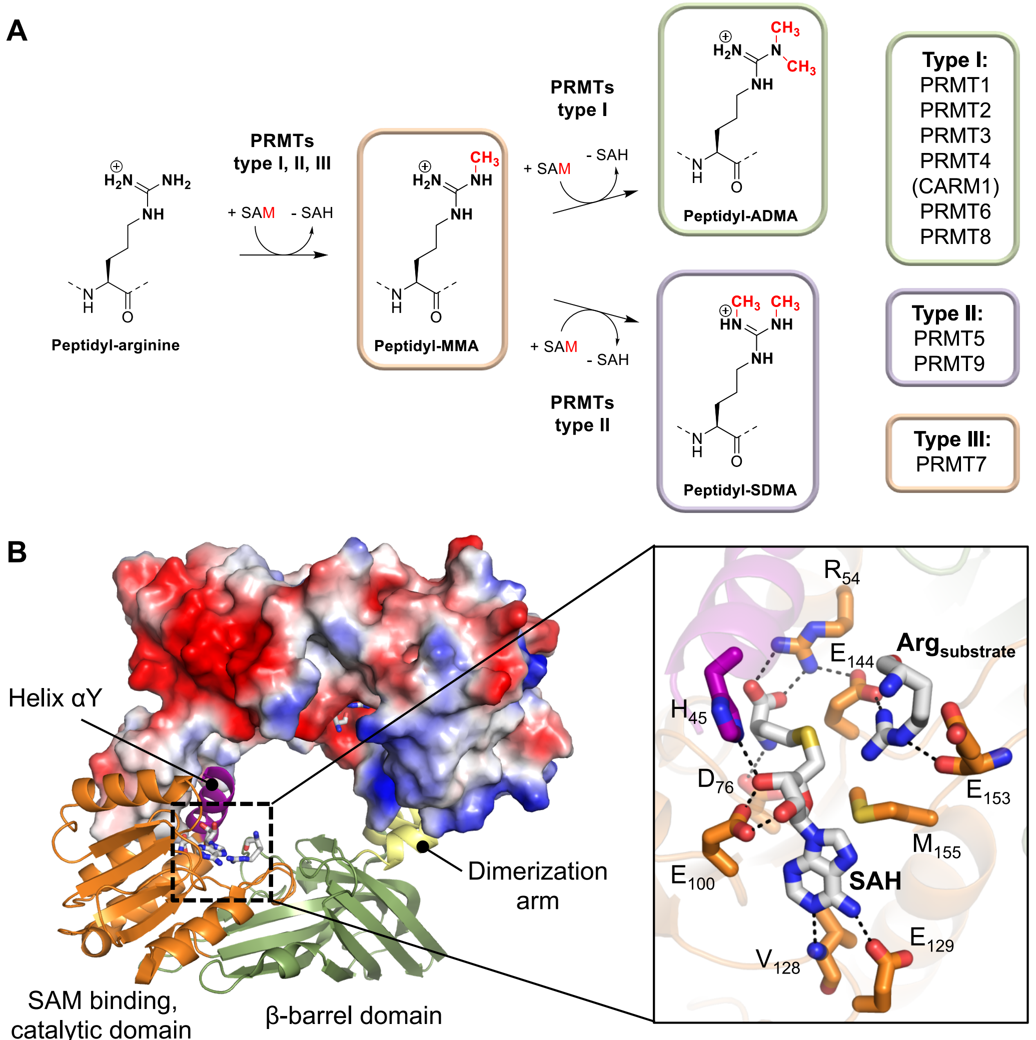

Figure 2. Structure and mechanism of PRMTs. (A) Schematic representation of the PRMT catalyzed arginine methylation reactions including the different types of PRMTs mediating these enzymatic reactions. The classification of individual PRMT members is shown on the right side. (B) The crystal structure of dimeric PRMT1 bound to SAH and arginine (PDB code: 1OR8). The protomer on top is shown as surface representation colored according to its electrostatic potential (negative electrostatic potentials are highlighted in red, whereas positive electrostatic potentials are illustrated in blue). The inset on the right depicts a close up view of the PRMT1 active site residues implicated in substrate and cofactor binding as well as catalysis.

PAD4 seems to act as a p53 corepressor by H3 citrullination at the p21 promoter site, thereby blocking downstream gene transcription.[40] Histone H3 citrullination at the promoter region of the pro-apoptotic tumor suppressor gene *OKL38* was shown to associate with transcriptional repression as well.[41] Estrogen-induced stimulation of PAD4 induces citrullination of H3R8 that is linked to transcriptional activation at ERα-dependent promoters by interfering with the H3K9me3 directed binding of HP1α.[42] There, it was shown that the citrullination of H3R8 in peripheral blood mononuclear cells is involved in the increased expression of cytokines *TNFα* and *IL8*; the overexpression of these cytokines is associated with an uncontrolled immune response and T-cell activation in multiple sclerosis.[42] Besides the direct effect of histone citrullination on transcriptional regulation, the citrullination of the histone acetyltransferase p300 was shown to enhance its coactivator ability to stimulate gene transcription indicating a role for nonhistone mediated epigenetic functions of protein citrullination.[43]

Based on cellular localization studies employing overexpressed PAD enzymes, PAD4 is the only isozyme located in the nucleus and thus has been suggested to be solely responsible for histone H2A, H3, and H4 citrullination.[44] Moreover, sequence analysis revealed that only PAD4 contains a canonical nuclear localization signal.[45] However, several recent studies revealed that PAD2 can also reside in the nucleus, where it citrullinates histone H3 at arginines R2, R8, R17, and R26.[37,46,47] The PAD2 catalyzed citrullination of histone H3 in EGF stimulated mammary epithelial cells has been suggested to modulate the expression of lactation related genes during the estrous cycle.[46] In addition, stimulation of estrogen receptor α (ERα)-positive cells with 17 β-estradiol (E2) induced PAD2 dependent citrullination of H3R26 at ERα target genes.[37] This modification leads to local chromatin decondensation, thereby increasing the accessibility for ERα to its target sites and consequently transcriptional activation of ERα regulated genes.[37,47] Guertin and colleagues further proposed that PAD2 mediated citrullination at H3R26 might be a potential

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prognostic marker for estrogen receptor positive (ER+) tumor development.[47]

Apart from the epigenetic consequences of histone citrullination, PAD4-mediated hypercitrullination of histones is critical for the innate immune system and the development of inflammatory diseases such as rheumatoid arthritis (RA) and lupus. Specifically, it was shown that PAD4 is essential for neutrophil extracellular trap (NET) formation,[48] also termed NETosis, a specialized pro-inflammatory form of cell death that is involved in the defense against bacterial infection.[49] During NETosis, histone hypercitrullination promotes chromatin unraveling on such a massive scale that the chromatin complex is extruded from the cell to form a web-like structure that captures pathogens. These large extracellular structures of decondensed chromatin include hypercitrullinated histone H3, which is a key marker of this form of cell death.[48,50] Notably, aberrantly increased NETosis has been recognized as a central player in the pathogenesis of several systemic autoimmune diseases, including lupus and RA, as well as Alzheimer's disease.[51−54] Interestingly, citrullination of H4R3 was also shown to be associated with apoptosis in osteosarcoma cells and suggested to promote apoptotic fragmentation by increasing the accessibility of genomic DNA for DNase attack.[55]

Structure and Function of Arginine Modifying Enzymes. *The Structure and Function of PRMTs.* PRMTs catalyze the transfer of a methyl group from a donor molecule, S-adenosylmethionine (SAM), to the terminal guanidino nitrogens of arginine residues. As mentioned above, there are currently nine known PRMTs that can be further classified into three distinct types according to their regiospecificity, i.e., the generation of (i) ADMA, performed by type I enzymes, (ii) SDMA, mediated by type II PRMTs, and (iii) MMA, which is catalyzed by type III enzymes (Figure 2A).[4,7] Notably, the mono- or dimethylation of arginine residues does not alter the overall positive charge on the arginine guanidinium group; however, it affects the hydrogen bonding capabilities of this residue.[4] The availability of numerous crystal structures of type I and type II PRMTs reveals a conserved architecture wherein two monomers form a head-to-tail homodimer. The dimer interface is stabilized by interactions between the catalytic domain and the helix-turn-helix dimerization arm that protrudes from the C-terminal β-barrel domain (Figure 2B).[56−60] Notably, the known structures of all the dimethylation specific PRMTs show a central hole and two opposing active sites that are separated by ~3 nm. By contrast, the only type III enzyme, PRMT7, lacks the central cavity and consists of a monomer constituting two consecutive PRMT modules that fold into a homodimer-like structure.[61,62] The PRMT active site contains a SAM binding pocket that consists of a series of highly conserved sequence motifs that are critical for SAM binding and the structural organization of the active site. In addition, the arginine binding pocket is characterized by two invariant glutamate residues (E144 and E153 in PRMT1), which are located on the double E-loop and are thought to properly align and orient the substrate guanidinium group for nucleophilic attack.[57,63]

The structural determinants that dictate product selectivity and thereby steer the formation of ADMA, SDMA, or MMA remain unclear. However, recent biochemical studies and the availability of high resolution crystal structures of several PRMT members reveals striking signature features specific for each PRMT type.[56,57,59−61,64] For instance, a conserved methionine residue (M48 in PRMT1), present in all type I and type III enzymes, is replaced by a phenylalanine in the type II enzyme PRMT5 (F327). It was shown that swapping this residue from a methionine to a phenylalanine in PRMT1 led to the slight formation of SDMA.[65] The complementary experiment, i.e., replacing the phenylalanine in PRMT5 to a methionine residue, results in the generation of both ADMA and SDMA.[64] These experiments highlight the important role that this residue plays in specifying the regiospecificity of the PRMTs. However, the very recent demonstration that PRMT9 acts as a type II enzyme[66,67] questions the general importance of this site for product specificity, as this enzyme possesses a methionine at this position.[4] More recently, it was proposed that subtle steric constraints, among different PRMT types, may be important for conferring the observed product selectivity.[4,61,68] In this respect, two major determinants have been suggested. The first one consists of differences in the THW loop-motif. This motif is only present in type I and type III enzymes, and the critical histidine residue is thought to narrow the substrate arginine binding pocket. In the case of type II enzymes, the histidine is replaced with a serine (in PRMT5) or cysteine (in PRMT9) residue that increases the available volume to fit a methylated nitrogen atom while placing the other nonmethylated guanidine nitrogen close to the SAM methyl transfer site.[4] This orientation is compatible with the formation of symmetrically dimethylated arginine residues. The second critical determinant comprises the conserved YF/YXXY motif in the αY helix that is only found in type I PRMTs. There, the two invariant tyrosine hydroxyl groups hydrogen bond to one glutamate residue of the double E-loop, thereby forming a small pocket that allows the accommodation of a methyl group on the attacking nitrogen atom for asymmetric dimethylation.[68]

Apart from the generation of different methylation products, individual PRMTs also have distinct substrate specificities. Typically, PRMTs prefer to methylate glycine and arginine-rich (GAR) sequences as encountered in numerous RNA binding proteins and the histones H2A and H4.[4,6,69,70] A plausible reason for the requirement of glycine residues is their enhanced conformational flexibility and the ability to form β-turn-like structures that are critical for the enzyme−substrate interaction as shown in the crystal structure of PRMT5 bound to a histone H4 peptide.[60,71] However, the substrate specificity of PRMT5 is not only restricted to GAR sequences as it can also accommodate arginines within a wider spectrum of sequence contexts.[72] Notably, PRMT4 (CARM1) mainly methylates arginine residues present within proline-, glycine-, and methionine-rich (PGM) sequence motifs as found in several splicing factors and histone H3.[73] The type III enzyme, PRMT7, specifically monomethylates arginines within an RXR motif encountered in H2B and H4 (Figure 1).[74] In addition, substrate recognition in most PRMTs is drastically enhanced by remote sequences that are typically more than 10 residues apart from the arginine methylation site.[75−77] The recognition of distal substrate elements is mainly mediated by electrostatic interactions. In this respect, it is interesting to note that all PRMTs have acidic isoeletric points (p*I*), with typical p*I* values between 5.0 and 5.3. The only exceptions are PRMT5 (p*I*, 5.9), PRMT4 (p*I*, 6.3), and PRMT8 (p*I*, 6.5), which possess slightly higher p*I*'s. Notably, PRMTs contain large areas of negative electrostatic potentials located on the β-barrel domain and the catalytic domain (Figure 2B) that are proposed to bind the positively charged residues of the remote substrate re-

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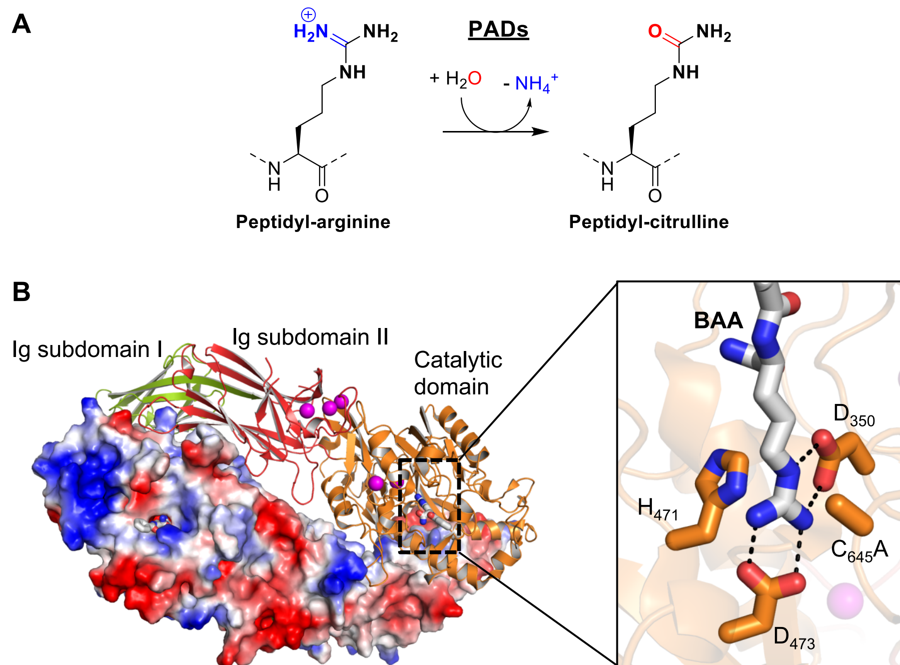

Figure 3. Structure and mechanism of PADs. (A) Schematic representation of the PAD catalyzed citrullination reaction. (B) The crystal structure of the PAD4 C645A dimer bound to the arginine mimicking substrate BAA (PDB code: 1WDA). The structure on top is colored according to domain organization. The C-terminal catalytic domain (orange) contains the bound substrate (BAA, gray). The bound calcium ions are illustrated as purple spheres. The inset on the right shows the PAD4 active site residues implicated in catalysis. The protomer on the bottom is shown as a surface representation colored according to its electrostatic potential.

gions.[56,57,75,78] This effect is further enhanced by the presence of dimeric PRMTs that can act as a negatively charged "sponge" to tether the positively charged substrates close to the active site cavity. This in turn facilitates the processive dimethylation of arginine residues, where the remote sequence elements anchor the substrate, while the arginine methylation site can swing from one active site into the other thereby promoting efficient dimethylation reactions. Evidence for such a mechanism stems from kinetic studies that show a partially or semiprocessive mechanism of dimethylation for PRMT1 and PRMT6.[75,79,80] Interestingly, the extent of processivity is influenced by the substrate employed, and thus different patterns of methylation can be obtained by the same PRMT enzyme in a substrate-dependent manner.[80] Conversely, the type II enzyme PRMT5 uses a distributive mechanism for the symmetric dimethylation of histone H4.[81] This might be a consequence of much slower reaction kinetics of symmetric methylation compared to asymmetric dimethylation reactions and a weaker interaction between the monomethylated substrate and PRMT5.[21]

The Structure and Function of PADs. As mentioned above, citrullination is the conversion of arginine into citrulline via the hydrolysis of the guanidinium group to form the neutral urea. In essence, an imine is replaced by a carbonyl; therefore this is termed a deimination reaction. Protein citrullination is mediated by the PAD enzymes (Figure 3A). There are five human PAD isozymes that show distinct tissue distributions and cellular localizations.[4,45,82] Four of these enzymes (PADs 1−4) are catalytically active, whereas PAD6 appears to be a pseudoenzyme with no detectable enzymatic activity.[83] Interestingly, PAD activity is strictly dependent on the availability of high concentrations of calcium ($K_{0.5,Ca}$ = 130−710 μM) and the enzymes bind to five (PADs 1, 3, and 4) or six (PAD2) calcium ions at distinct sites.[84−86] Although, calcium is not directly involved in catalysis, the recent crystal structures of apo and calcium-bound PAD2 show that calcium induces a series of structural rearrangements that are essential for the formation of a catalytically competent active site.[86] In particular, the movement of the catalytically important cysteine, C645 in PAD4 and C647 in PAD2, into the active site is triggered by calcium. Interestingly, calcium binding itself occurs in an ordered fashion, and residues involved in calcium interactions are conserved across the PAD family.[86]

On the basis of sequence analyses and structural comparisons, PADs belong to the pentein superfamily that is characterized by the pentameric arrangement of five subdomains around a central hollow, forming an α/β propeller.[87,88] This central cavity accommodates the active site within the catalytic domain. Besides the C-terminal catalytic domain, PADs also contain an N-terminal domain that is further composed of two immunoglobulin-like (Ig) subdomains (Figure 3B). Like the PRMTs, PADs exist as homodimeric proteins, with the individual monomers arranged in a head-to-tail fashion such that both active site pockets are located on the same side of the dimer.[88] The active site cavity of an individual monomer harbors all the critical residues for catalysis (Figure 3B). These residues include a strictly conserved cysteine (C645 in PAD4) that is important for nucleophilic attack onto the central guanidinium carbon atom of the arginine substrate, as well as two invariant aspartate residues (D350 and D473 in PAD4), which are thought to attract and properly position the arginine guanidinium group via electrostatic interactions. In addition, the active site contains a histidine (H471 in PAD4) that is important for the protonation of the ammonia leaving group and the subsequent activation of an incoming water

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Table 1. General Methyltransferase Inhibitors

Structure and name	IC_{50}	Structure and name	K_i
1, SAH	PRMT1 = 6.2 μM PRMT4 = 0.67 μM PRMT6 = 0.2 μM	2, Sinefungin	K_i PRMT1 = 0.2 μM PRMT4 = 0.1 μM PRMT5 = 0.51 μM PRMT8 = 0.31 μM

Table 2. Inhibitors of PRMTs

Structure and name	IC_{50}	Structure and name	IC_{50}
3, Stilbamidine (NSC35605)	PRMT1 = 56.9 μM, (15.2 μM) PRMT4 = ~400 μM PRMT5 = 44.1 μM PRMT6 = 173 μM	9, SGC707	PRMT1 = > 20 μM PRMT3 = 0.031 μM PRMT4 = > 20 μM PRMT5 = > 20 μM PRMT6 = > 20 μM PRMT7 = > 20 μM PRMT8 = > 20 μM
4, allantodapsone	PRMT1 = 1.7 μM	10, CMPD2	PRMT1 = > 10 μM PRMT3 = > 10 μM PRMT4 = 0.027 μM
5, Furamidine, DB75	PRMT1 = 9.4 μM PRMT4 = > 400 μM PRMT5 = 166 μM PRMT6 = 283 μM	11, CMPD1	PRMT1 = > 10 μM PRMT3 = > 10 μM PRMT4 = 0.03 μM
6	PRMT1 = 1.3 μM PRMT4 = 0.56 μM PRMT6 = 0.72 μM	12, CMP5	PRMT1 = > 100 μM PRMT4 = > 100 μM PRMT5 = < 50 μM PRMT7 = > 100 μM
7, C21 (GKGGKGLGKGGAKRHRKV−OH)	PRMT1 = 1.8 μM PRMT3 = > 500 μM PRMT4 = > 500 μM PRMT6 = 8.8 μM	13, EPZ015666	PRMT1 = > 50 μM PRMT2 = > 50 μM PRMT3 = > 50 μM PRMT4 = > 50 μM PRMT5 = 0.022 μM PRMT6 = > 50 μM PRMT7 = > 50 μM PRMT8 = > 50 μM
8, E-84	PRMT1 = 3.4 μM PRMT4 = 21.5 μM PRMT5 = 35.4 μM PRMT6 = 84.9 μM	14, EPZ020411	PRMT1 = 0.119 μM PRMT3 = > 20 μM PRMT4 = > 20 μM PRMT5 = > 20 μM PRMT6 = 0.01 μM PRMT7 = > 20 μM PRMT8 = 0.223 μM

molecule that ultimately cleaves the thiouronium reaction intermediate.[89]

The proposed reaction mechanism, including the covalent intermediate, is shared by other members of the guanidino-group modifying pentein superfamily, such as dimethylarginine dimethylaminohydrolase (DDAH) and arginine deiminases (ADI).[90,91] However, in contrast to these proteins, PADs modify peptidyl-arginine residues.[84] As a result, PADs have evolved a more accessible active site and contain several residues that specifically recognize the substrate peptide backbone.[92] For example, residue R374 is critical for the formation of a bidendate hydrogen bond with two substrate peptide carbonyls. The structures of PAD4 bound to histone peptides reveal that substrate recognition is mainly mediated via interactions with the substrate peptide backbone.[92] As such, there is limited sequence specificity regarding PAD4 substrate

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Table 3. Inhibitors of PADs

Structure and name	IC_{50}	Structure and name	k_{inact}/K_I (M^{-1} min^{-1})
15, Chlortetracycline	PAD4 = 100 μM	**21**, TDFA	PAD1 = 1,700 PAD2 = 500 PAD3 = 400 PAD4 = 26,000 (**IC_{50}**: PAD4 = 2.3 μM)
16	PAD4 = 36% inhibition at 10 μM	**22**, YW3-56	**IC_{50}**: PAD4 = 1–5 μM
17	PAD4 = 0.1 μM	**23**, BB-Cl-amidine	PAD1 = 16,000 PAD2 = 5,000 PAD3 = 6,000 PAD4 = 14,000 (**IC_{50}**: PAD4 = 8 μM)
18, GSK 184	**K_{is}** PAD1 = 108 μM PAD2 = 107 μM PAD3 = 2090 μM PAD4 = 6.8 μM (**IC_{50}**: PAD4 = 250 nM)	**24**, biphenyl tetrazole *tert*-butyl-Cl-amidine	PAD1 = 2,775 PAD2 = 10,900 PAD3 = 4,940 PAD4 = 6,090
19, Cl-amidine	**k_{inact}/K_I (M^{-1} min^{-1})** PAD1 = 37,000 PAD2 = 1,200 PAD3 = 2,000 PAD4 = 13,000 (**IC_{50}**: PAD4 = 5 uM)	**25**, Streptonigrin	PAD1 = 3,700 PAD2 = 12,000 PAD3 = 3,500 PAD4 = 440,000 (**IC_{50}**: PAD4 = 1.8 μM)
20, *o*-Cl-amidine	**k_{inact}/K_I (M^{-1} min^{-1})** PAD1 = 106,000 PAD2 = 14,100 PAD3 = 10,400 PAD4 = 38,000 (IC_{50}: PAD4 = 2.2 μM)	**26**	PAD1 = 360 PAD2 = 1,110 PAD3 = 15,600 PAD4 = 1,460 (**IC_{50}**: PAD3 = 4.5 μM)

selection. In addition, and in contrast to PRMTs, substrate recognition by the PADs does not depend on long-range interactions originating from remote sequences in the substrate.[84] Despite limited sequence specificity, it was shown that PAD4 bound peptide substrates adopt a β-turn-like conformation, similar to PRMT bound substrates.[92] Therefore, the propensity of peptide substrates to adopt such kinked conformations might dictate substrate selection in PADs. Moreover, apart from structural constraints, PAD substrate specificity may be regulated by the accessibility of arginine residues in chromatin structures, through interaction of a PAD with other proteins and by cross-talk with distinct PTMs, such as arginine methylation. In this regard, it was claimed that PADs can also act on methylated arginine residues.[35] However, several lines of evidence questioned the physiological relevance of this activity and even indicated that arginine methylation prevents arginines from being PAD substrates.[36,84,93,94] Thus, citrullination and arginine methylation are now considered to be antagonistic modifications.[4,95]

Inhibitors of Arginine Modifying Enzymes. *PRMT Inhibitors.* In recent years, a diverse set of PRMT inhibitors have been discovered. The most common and general PRMT inhibitors are *S*-adenosyl-*L*-homocysteine (SAH, **1**) and sinefungin (**2**). These molecules are structurally related to SAM, and numerous biochemical and crystallographic data confirm that they are SAM-competitive inhibitors that block PRMT activity in the low micro- to nanomolar range (Table 1).[96] SAH is the reaction product of SAM-dependent methyl transfer reactions. Normally, it is rapidly degraded by SAH hydrolase; however, its activity can be blocked by adenosine dialdehyde to artificially increase the endogenous level of SAH.[97] This approach is frequently employed to study the global effects of inhibiting cellular methyltransferases including the PRMTs. The natural product sinefungin was originally

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isolated from Streptomyces species[98] and was shown to act as a pan-methyltransferase inhibitor similar to SAH.[99]

One of the first high-throughput screens to identify small molecule inhibitors of PRMT1 was performed by the Bedford group.[100] These screening efforts led to the identification of several compounds termed arginine methyltransferase inhibitor (AMI). One of these small molecules, AMI-1, was shown to be cell-permeable and to inhibit cellular PRMT1 in a concentration dependent manner.[100] However, most of these small molecules were nonspecific and also inhibited protein lysine methyltransferases. In addition, subsequent studies revealed that AMI-1 actually does not bind to PRMTs but rather interacts with the histone substrates via electrostatic interactions, thereby preventing substrate access.[101]

To obtain PRMT specific inhibitors, Spannhoff *et al.* employed a target-based virtual screening approach.[102] The identified compounds include the diamidine stilbamidine (**3**; Table 2) and the dapsone derivative allantodapsone (**4**) that both act as competitive inhibitors of the protein substrate. Moreover, these compounds were shown to block PRMT1 methylation of H4R3 in cellular assays while having minimal inhibitory effects on lysine methylation of H3K4.[102] However, they show limited potency and PRMT isozyme specificity. Recently, an improved diamidine compound, furamidine (**5**), was described.[103] This small molecule is selective for PRMT1 with a ∼18-fold lower IC_{50} for PRMT1 compared to PRMT5. Based on molecular modeling studies, the positively charged amidinium in **5** was proposed to bind to the substrate binding site, occupying the position of the substrate guanidinium group. In addition, furamidine was shown to be cell permeable, resulting in inhibition of cellular PRMT1 and a decrease in cell proliferation in leukemia cell lines.[103] However, care has to be taken regarding the utilization of diamidine derivatives as PRMT-specific inhibitors because they are also reported to bind DNA with high affinity;[104] as such we do not recommend their use as PRMT inhibitors.

There are several studies on PRMT inhibitor development strategies aimed at substrate, cofactor, and in particular partial bisubstrate analogs.[105−110] Although these compounds showed decent inhibition, they are usually nonspecific with regard to different PRMT isozymes. For example, one of the most potent compounds (**6**), consisting of the SAM adenosine moiety linked to a guanidinium group, shows an IC_{50} of 560 nM for PRMT4 but also effectively blocks the activity of PRMT1 and PRMT5.[110] An exception is the peptide-based inhibitor C21 (**7**) that is composed of the first 21 residues of histone H4 and contains a chloroacetamidine warhead instead of the substrate arginine guanidinium group.[106] This compound acts as an irreversible inhibitor wherein the chloroacetamidine group reacts with a hyperreactive cysteine, C101 present in the PRMT1 active site, to form a stable thioether bond.[111] Notably, the peptide inhibitor C21 is >100-fold more potent than the chloroacetamidine warhead containing compound Cl-amidine (**19**, Table 3, discussed below).[106] These data further highlight the requirement of remote sequences, distal from the arginine substrate site for efficient inhibitor/substrate peptide binding. C21 shows high preference for PRMT1 over PRMT3 and PRMT4 with an IC_{50} of 1.8 μM for PRMT1; however, it can also block the activity of PRMT6 (IC_{50}, 8.8 μM). In addition, C21 was adapted as a chemical probe by incorporating either fluorescein or biotin reporter tags to monitor and isolate active PRMT1.[112] On the basis of a previously identified cyanine scaffold,[113] Hu and colleagues developed a PRMT1 inhibitor (**8**), denoted as E-84.[114] This small molecule inhibitor blocks PRMT1 with an IC_{50} of 3.4 μM and shows 6-fold selectivity over PRMT4 and over 10- and 25-fold selectivity over PRMT5 and PRMT8. On the basis of molecular docking studies, it was proposed that E-84 binds to the SAM binding pocket as well as the arginine substrate binding site. In addition, this compound slightly reduced the level of asymmetrically dimethylated arginine in leukemia cells and displays cytotoxic activity toward these cells.[114] However, experimental evidence of target engagement is still lacking, and it thus remains to be shown if this compound directly interacts with PRMTs.

In recent years, several highly potent isozyme-specific and more drug-like PRMT inhibitors have been developed. One of these compounds, **9**, represents an optimized hit derived from a HTS approach that selectively targets PRMT3.[115,116] This compound is an allosteric inhibitor that does not bind the active site pocket. Structural analysis revealed that **9** binds at the PRMT3 homodimerization interface and prevents the proper orientation of helix αY for catalysis.[116] In addition, it was shown that **9** is active in cellular assays and efficiently blocks the PRMT3-dependent dimethylation of H4R3 with nanomolar efficacy.[116] Sack and colleagues described the identification of two PRMT4 (CARM1) selective inhibitors, the pyrazole derivative **10** and the indole derivative **11**.[117] Both of these compounds were derived after optimization of compounds identified from initial HTS screens.[118] Detailed structural investigations showed that these small molecule inhibitors bind to the arginine-substrate binding cavity of PRMT4 and require bound SAH.[117] In addition to PRMT3 and PRMT4, specific inhibitors of PRMT5 have also been identified. The carbazole ring containing CMP5 (**12**) was shown to block PRMT5 activity but did not inhibit PRMT1, PRMT4, or PRMT7.[22] This compound was predicted to occupy the SAM binding pocket and form $\pi-\pi$ stacking interactions with the signature phenylalanine (F327) residue implicated in product selectivity of PRMT5. Cellular studies indicate that treatment of transformed B-cells, expressing high levels of PRMT5, with CMP5 blocks Epstein−Barr virus driven B-lymphocyte transformation while leaving normal B cells unaffected.[22] Another highly selective and even more potent PRMT5 inhibitor was recently developed by Chan-Penebre *et al.*[119] This small molecule, EPZ015666 (**13**), is an optimized version of a compound derived from a library of 370 000 compounds. Inhibition studies with PRMT5 revealed that EPZ015666 is competitive with the peptide substrate, and this was further confirmed by structural analyses.[119] Similar to PRMT4 inhibitors, it was shown that the binding affinity of EPZ015666 for PRMT5 was greatly increased by SAM binding. Interestingly, EPZ015666 also engages in $\pi-\pi$ stacking interaction, via its critical tetrahydroisoquinoline moiety, with F327 of PRMT5 as predicted for compound CMP5. These data highlight the potential for developing selective PRMT inhibitors by harnessing isozyme specific differences in the active site such as the characteristic phenylalanine residue, F327, of PRMT5. On the basis of functional studies, EPZ015666 reduces the level of global symmetric dimethylation in the mantle cell lymphoma (MCL) cell line Z-138. Moreover, EPZ015666 exerts antiproliferative effects in numerous MCL cell lines at nanomolar concentrations, and oral administration of this compound induces antitumor activity in different MCL xenograft mouse models.[119] Very recently, a PRMT6 specific inhibitor, EPZ020411 (**14**), has been reported.[120] This compound shows high potency, IC50 = 10

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nM, for PRMT6, and good selectivity over PRMT1 (12-fold) and PRMT8 (22-fold) and excellent selectivity (>100-fold) compared to PRMT3, PRMT4, PRMT5, and PRMT7. The crystal structure of EPZ020411 bound to a ternary PRMT6-SAH complex revealed that the inhibitor binds into the arginine substrate site via its diamine side chain and the pyrazole core structure.[120]

PAD Inhibitors. Since dysregulated citrullination levels have been implicated in numerous diseases including rheumatoid arthritis, several autoimmune diseases, as well as cancer, PADs represent a promising target for pharmaceutical intervention.[121] Thus, several small molecules have been developed that block the activity of this enzyme class. Because of the involvement of PADs in the development of RA, a small panel of disease modifying antirheumatic drugs were tested for the presence of potential PAD inhibitors.[122] Interestingly, some of these compounds showed modest PAD inhibition in the low milli- to micromolar range. One of the most potent inhibitors was the tetracycline derivative minocycline. Further investigations, using different tetracycline derivatives, revealed chlortetracycline (**15**) as a modest PAD4 inhibitor with an IC_{50} of ~100 μM (Table 3).[122] However, due to the weak inhibitory activity, it is unlikely that these compounds exert inhibition of cellular PAD4. Since the arginine guanidinium moiety is the major contributor for efficient PAD−substrate interactions, several guanidinium group containing compounds have also been evaluated. In this respect, the derivatized guanidine compound **16** displays 36% inhibition of PAD4 activity at 10 μM.[123] Moreover, the acylguanidine derivative **17** was shown to block PAD3 with an IC_{50} of 100 nM.[124] However, given that acylguanidines have a low pK_a value, typically several orders of magnitude lower than that of guanidines, and are expected to be poorly suited as arginine substrate mimicking inhibitors, the strong inhibitory activity of **17** needs further validation. Although it was hypothesized that these compounds act as competitive inhibitors, their detailed mode of inhibition has not been studied. The limited potency for these reversible PAD inhibitors likely relates to the small active site pocket that only accommodates the side chain of an arginine residue. Therefore, the recent discovery of a distinct binding site occupied by compounds GSK 199 and its more potent derivative GSK 484 (**18**) opens a promising approach to develop high affinity reversible PAD inhibitors.[125] Specifically, structural studies revealed that these compounds bind to the solvent exchange channel in PADs and induce large conformational changes around the active site.[125] In addition, compound **18** is a PAD4 specific inhibitor that displays at least 35-fold selectivity for PAD4 over the other PAD isozymes. Interestingly, **18** preferentially binds a calcium-deficient form of PAD4 that lacks calcium in the Ca2 binding site and has an IC_{50} value in the nanomolar range in the absence of calcium, whereas calcium binding decreases its potency by at least 5-fold.[125] These data further highlight the dynamic nature of the PAD4 active site that fluctuates between different conformations in a calcium dependent manner. Moreover, it further indicates that these conformational states (calcium-deficient, inactive and calcium-bound, active) can be targeted by distinct inhibitors and pave the way for developing conformer-specific PAD inhibitors. Inhibitors targeting the resting apoenzyme are of particular interest as they stabilize the inactive conformation that likely represents the major form inside the cell. By shifting the equilibrium toward the inactive conformation, these inhibitors do not directly compete with endogenous substrates and might be better suited to prevent burst activation of PADs such as during NETosis, triggered by massive calcium influx. In this respect, pretreatment of stimulated mouse neutrophils with 10 μM GSK484 markedly diminished hypercitrullination of histone H3 and NET formation, thus highlighting the biological activity of this compound.[125] Apart from these reversible inhibitors, substantial progress has been made in developing irreversible PAD inhibitors. One of the most widely used and best characterized irreversible PAD inhibitors is Cl-amidine (**19**), which blocks PAD4 activity with an IC_{50} of 5 μM.[126] This compound contains a reactive haloacetamidine warhead, as in the PRMT inhibitor C21 (**7**). The positively charged electrophilic group acts as a mimic of the substrate guanidinium group and covalently attaches to the active site cysteine residue (C645 in PAD4) forming a stable thioether bond.[126,127] By varying the side chain length in a Cl-amidine derivative, it was confirmed that a three-carbon linker between the chloroacetamidine moiety and the amino acid backbone is most effective.[128] Notably, and in contrast to GSK484, haloacetamidine inhibitors require the high-calcium bound form of PAD4, thus confirming their substrate mimicking mode of inhibition and preference for the calcium primed conformation of PAD4.[126] Cl-amidine has been used successfully in several preclinical models of RA,[129] lupus,[130] colitis,[131] and even breast cancer,[132] by effectively reducing aberrant hypercitrullination levels. To improve the potency and specificity of Cl-amidine, several derivatives were developed. For instance, the *ortho*-carboxylate containing Cl-amidine derivative **20** has a more than 2-fold higher inhibitory activity compared to **19** but a similar PAD isozyme selectivity profile.[133] On the basis of a peptide library approach, the fluoroacetamidine containing compound TDFA (**21**) has been identified.[134] The tripeptide TDFA shows high selectivity for PAD4 compared to the other PADs with more than 15-fold preference for PAD4 inhibition over PAD1. Interestingly, both, **20** and **21**, contain a negatively charged carboxylate group that occupies a similar position in PAD4-inhibitor crystal structures and is involved in direct or water mediated interactions with the side chain amide of Q346 that might explain the increased potency of these compounds over Cl-amidine.[4,133,134] Since Cl-amidine is a polar and highly water-soluble compound and thus exhibits poor bioavailability, several attempts to increase its hydrophobicity have been undertaken. For example, Wang *et al.* attached a diverse set of hydrophobic groups to the amide backbone of Cl-amidine.[135] One of the most potent compounds of this series, YW3−56 (**22**), contains an N-terminal dimethyl-naphthylamine and C-terminal methylbenzene moiety. Yw3−56 shows similar rates of inhibition compared to Cl-amidine, but its antiproliferative activity toward mouse sarcoma cells was increased by a factor of 50.[135] The same trend of increased cellular activity, bioavailability, and *in vivo* half-life was observed with BB-Cl-amidine (**23**) that possesses an N-terminal biphenyl and a C-terminal benzimidazole group.[136] Notably, using the PAD4 expressing U2OS osteosarcoma cell line, the EC_{50} value of BB-Cl-amidine has been demonstrated to be 8.8 μM compared to >200 μM for Cl-amidine.[136] Biphenyl-tetrazole-*tert*-butyl-Cl-amidine (**24**), a further apolar derivative of Cl-amidine, preferentially inhibits PAD2.[137] This compound harbors a C-terminal *tert*-butyl-tetrazole group that was shown to increase the specificity toward PAD2 over other PAD isozymes. Employing a fluorophore labeled Cl-amidine derivative in a fluorescence polarization HTS assay, Knuckley *et al.* described the identification of streptonigrin (**25**) as a potent and very

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selective PAD4 inhibitor.[138] Streptonigrin acts as an irreversible inactivator of PAD4 and was shown to block PAD4 activity in cellular studies; however, it also binds to several off targets thereby limiting its physiological utility.[138,139] Recently, Jamali and colleagues employed a substrate-fragment discovery approach to identify a PAD3 isozyme selective inhibitor (**26**) that shows >10 selectivity for PAD3 over other PAD enzymes.[140] This compound contains a chloroacetamidine warhead for reactivity as well as a biphenyl-hydantoin group for selectivity.

CONCLUSIONS AND PERSPECTIVE

In the past decade, there has been tremendous progress in our understanding of the epigenetic influences of histone arginine methylation and citrullination; however, much remains to be learned about the chemistry and biology of these fascinating modifications. In future studies, it will be interesting to test whether protein arginine methylation and citrullination are reversible modifications. Although it was proposed that the Jmjd6 protein acts as an arginine demethylase,[141] subsequent studies showed that it does not remove the methyl mark from methylated arginine residues and actually acts as a lysine hydroxylase.[142,143] Nonetheless, the dynamic appearance and disappearance of citrullination and arginine methylation marks on histones hints at the existence of enzymes that might reverse these modifications.[36,144] In this respect, it will also be of great importance to identify proteins that act as "readers" to recognize the modified arginine residues such as the tudor domain-containing proteins that were shown to bind methylated arginine residues.[31] Moreover, it will be interesting to evaluate the scope and impact of other enzymatic and nonenzymatic arginine modifications such as phosphorylation,[145−147] ADPribosylation,[148,149] carbonylation,[150] and the formation of arginine derived advanced glycation end products[151] on epigenetic regulation. The goal is to combine this information with other histone PTMs to generate a map of individual histone modifications and to delineate the underlying crosstalk to ultimately decipher the "language" of histone PTMs.

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The manuscript was written through contributions of both authors. Both authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): The authors declare competing financial interests. P.R.T. is a cofounder and consultant to Padlock Therapeutics.

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KEYWORDS

Epigenetics: describes the heritable alterations in states of gene expression apart and beyond alterations in the genomic DNA sequence. It comprises DNA and/or histone modifications

Histones: small, highly basic proteins that combine into octamers. These histone octamers tightly bind to genomic DNA forming nucleosome particles that represent the building blocks of higher chromatin structures

Posttranslational modification (PTM): protein alterations that affect the mature protein after translation. Typically, these modifications include but are not limited to the covalent addition of chemical groups to the amino acid side chains as well as cleavage of existing bonds

Protein arginine methylation: a PTM in which one or two methyl groups are added to an arginine residue on the nitrogen(s) of its guanidine side chain. There are three types of arginine methylation: monomethylation, symmetric dimethylation, and asymmetric dimethylation

Protein arginine methyltransferases (PRMTs): enzymes that methylate arginine residues. They can be subdivided into three types: type I, yielding asymmetrically dimethylated arginine (ADMA); type II, forming symmetrically dimethylated arginine (SDMA); and type III enzymes, i.e., monomethyltransferases, generating monomethyl arginine (MMA)

S-adenosyl-L-methioline (SAM): a cofactor that serves as a methyl donor for PRMT catalyzed methylation reactions

Protein citrullination: also known as arginine deimination; defines the post-translational conversion of arginine to citrulline. Specifically, it is characterized by the replacement of an imine by a carbonyl group, thereby forming a neutral urea

Protein arginine deiminases (PADs): an enzyme family composed of five members in humans that specifically deiminates peptidyl arginine residues yielding citrulline

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