Histone Methyltransferase Inhibitors: Novel Epigenetic Agents for Cancer Treatment

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Abstract: Epigenetics is defined as heritable changes in gene activity and expression that occur without alteration in DNA sequence. The gene transcription is strictly correlated to chromatin structure, which could undergo covalent modifications of histones involving acetylation, methylation, phosphorylation and ubiquitination. Alterations in histones are implicated in many diseases, including cancer, by leading to tumor suppressor silencing or pro-apoptotic proteins downregulation. Although post-translational addition of methyl groups to the histone lysine has been discovered three decades ago, the importance of this epigenetic modification has emerged only in the last few years. Thenceforward histone methyltransferase inhibitors have been developed as potential therapeutic cancer agents. It should not be long before some selective inhibitors make their way into clinical trials. This review is mainly focused on the evolution in the development of new epigenetic modifier molecules modulating histone marks.

INTRODUCTION

Over the past few years many evidences indicated that the expression of genes is not only regulated by DNA sequence and transcription factors but is also under epigenetic controls. Epigenetic is defined as heritable change in gene expression that is not correlated to changes in DNA sequence [1] and represents the critical mediator for turning 'ON' or 'OFF' expression of particular genes. It is known that epigenetic cell memory is particularly important in multicellular organisms, whereas cells with identical genomes have distinct functional identities [2]. Epigenetic gene control has now been well recognized to play an important role in many biological processes involving stem and progenitor cell differentiation and maintenance, and cell cycle control. Aberrant epigenetic expression has been associated with several diseases such as cancer, neuropsychiatric conditions, neurogenerative syndrome, inflammation and metabolic disorders [3]. Epigenetic acts at chromatin levels changing the conformation of the chromatin and regulating gene expression mechanisms. DNA methylation and histone modification are the main epigenetic factors, by which gene expression could be regulated.

The repeating unit of chromatin is the nucleosome, composed of two copies of each four core histone proteins H2A, H2B, H3 and H4 around which 147 bp of DNA are wrapped [4]. Histones are subject to a wide variety of post-translational covalent modifications, mainly occurring on their N-terminal tails rich of positively charged lysine and arginine residues, and potentially confer a large information capacity on each nucleosome. These most common modifications of histones include lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, lysine ubiquitylation, arginine and glutamate ADP ribosylation, lysine sumoylation, arginine deimination and proline isomerization Fig. (**1**) [5, 6].

In 2001, Jenuwein and Allis proposed the existence of 'histone code' that may considerably extend the information potential of the genetic (DNA) code. The histone code hypothesis predicts that the modification marks on the histone tails should provide binding sites for effector proteins and alter chromatin structure, thereby leading to inherited differences in transcriptional 'ON-OFF' states [7]. Several enzymes are involved in this process: 'writers', which introduce post-translational modifications into a given protein

(e.g., histone methyltransferase or histone acetyltransferase), 'erasers', which remove posttranslational modifications from a protein (e.g., a histone demethylase or histone deacetylase) and 'readers', proteins or complexes that bind specifically to a post-translationally modified protein that recognize specific marks [8]. Precise combinations of histone modifications regulate distinct chromatin structures and thereby result in individual transcriptional outputs. Perturbations of chromatin structure can cause inappropriate gene expression and genomic instability, resulting in cellular transformation and malignant outgrowth. Proteins controlling chromatin organization therefore constitute key players in cancer pathogenesis [9].

Histone methylation was discovered several decades ago, but its functional role in the regulation of gene expression is emerging only in the last years. This review will focus on this particular histone modification, on the enzymes and proteins involved in the process, and on the new histone methyltransferase inhibitors discovered.

DNA METHYLATION AND DNA METHYLTRANSFERASES INHIBITORS

Several studies have indicated that DNA methylation and histone methylation at certain positions are connected. Earlier it was suggested that histone modification was secondary to DNA methylation, but recent studies on fungus revealed that histone modification can on its own commence the process of DNA methylation [10]. DNA methylation is a covalent chemical modification, resulting in the addition of a methyl group at the carbon 5 of the cytosine ring, in the sequence context 5′CG3′ (also called the CpG dinucleotide) located in CpG islands, catalyzed by the C5-DNA methyltransferases (DNMTs) [11]. Most CpG islands are found in the proximal promoter regions of almost half of the genes in the mammalian genome and are, generally, unmethylated in normal cells. In cancer, however, the hypermethylation of these promoter regions is the most well-categorized epigenetic change to occur in tumors [12]. It is found in every type of human neoplasm and is associated with the inappropriate transcriptional silencing of tumor suppressor genes (TSG). Since DNA methylation is reversible, the use of specific inhibitors of DNMT (DNMTi) might reactivate TSG and induce the reprogramming of cancer cells, leading to their proliferation arrest and ultimately to their death. Many DNMT inhibitors have been described and are divided into two families: the nucleoside analogs and the non-nucleoside inhibitors. 5-Azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine) are two cytidine analogs in which the carbon atom in position 5 is replaced by a nitrogen atom and linked to a ribose or a deoxyribose, respectively [13]. They are metabolized by kinases that convert the nu-

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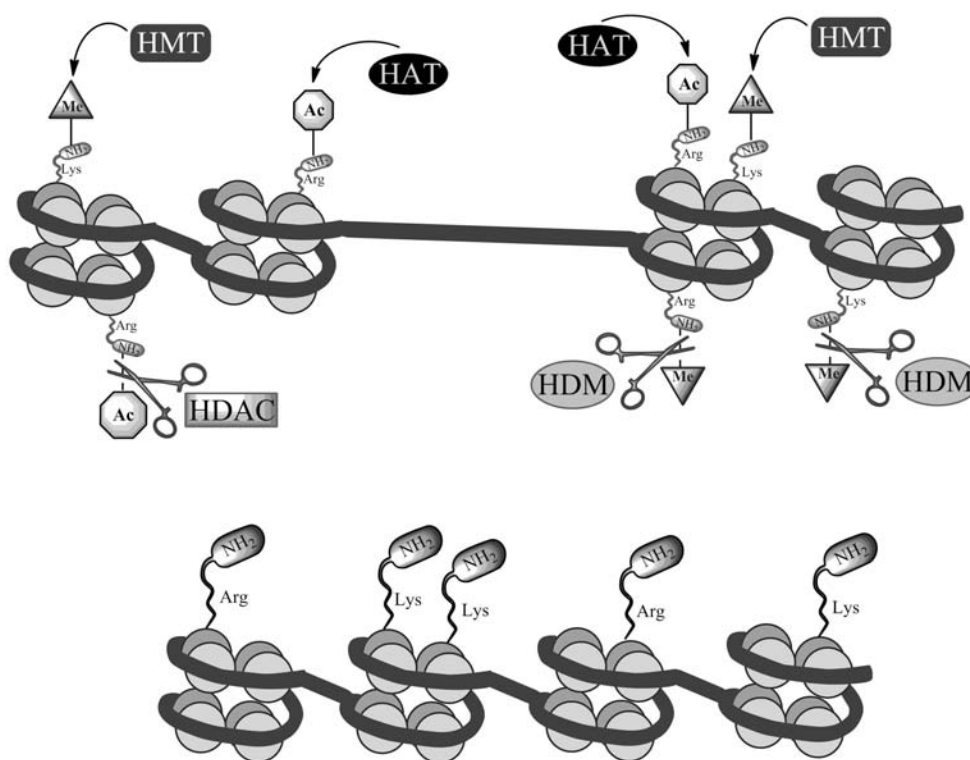
Fig. (1). Representative of histone modifications.

cleosides into nucleotides for incorporation into DNA and/or RNA. Once into the DNA, the cytosine analogs are recognized by the DNMTs and undergo the same reaction as normal cytosines with the formation of the covalent intermediate between the catalytic cysteine of the enzyme and 6-position of cytosine analogs. However, unlike with cytosine, the β-elimination reaction can no longer occur because of the presence of the nitrogen atom in 5-position, resulting in a covalent irreversible complex Fig. (**2**) [14]. As a result, cellular DNA methyltransferase is rapidly depleted, and concomitantly genomic DNA is demethylated as a consequence of continued DNA replication. Azacitidine and decitabine exhibit greater cytotoxicity and have been approved by the Food and Drug Administration (FDA) in 2004 and 2006, respectively, for the treatment of Myeloid Dysplasic Syndrome (MDS) and Acute Myeloid Leukemia (AML) [15].

Unlike cytidine analogs, non-nucleoside DNMTi do not require incorporation into DNA, and thus might exhibit less cytotoxicity. Some of the compounds assessed for their potential to induce hypomethylation in solid tumors are hydralazine and procainamide. These compounds, however, have limited DNA hypomethylation activity in living cells.

HISTONE METHYLATION: PcG AND trxG ROLE

Lysine methylation is currently one of the most studied process because it can be an activator or repressor of gene expression, depending upon the position of the modified residue. The Polycomb group (PcG) proteins are mainly responsible for maintaining the inactive expression state of numerous genes [16]. PcG are histone methyltransferase (HTMase) proteins discovered in *Drosophila melanogaster* as the product of genes that are required to prevent inappropriate expression of homeotic (Hox) genes [17, 18]. In contrast, a group of proteins called trithorax (trxG) neutralizes PcG repression, activating the transcription [19, 20]. Polycomb and trithorax proteins have been demonstrated to have intrinsic HMTase activity toward H3-K27 (i.e. histone 3 at lysine 27) suggesting the

possibility that they participate in cellular memory through methylating core histones [21-23].

To date, three different major polycomb repressive complexes have been identified in *Drosophila melanogaster*: polycomb repressive complex 1 (PRC1) [24], polycomb repressive complex 2 (PRC2), and pleiohomeotic-repressive complex (PhoRC) [25]. PRC1, is less conserved than PRC2. ESC-E(z)-mediated H3-K27 methylation recruits PRC1 complex by facilitating PC binding, a core component of the PRC1 complex. Recruitment of PRC1 prevents the access of nucleosome remodeling factors leading to the formation of a repressive chromatin state [26]. Finally, PhoRC includes the DNA-binding PcG protein Pleiohomeotic (Pho), which binds to sites within Polycomb Response Elements (PREs) that serve as docking platforms for PcG proteins. Pho directly interacts with components of both PRC1 and PRC2, and is required for recruitment of both complexes [27]. The second PcG complex, PRCter2 consists of four core subunits in Drosophila: enhancer of zeste [E(z) or EZH2 in human]; extra sex combs [ESC, E(z) catalytic activity enhancer]; suppressor of zeste (SUZ12); Nurf55 (a protein implicated in histone binding). PRC2 is known to have methyltransferase activity targeted towards H3-K27 [22, 28, 29]. The trimethylated form of H3-K27 (H3-K27me3) is currently considered as the predominant form that conveys biological function *in vivo*. Trithorax group is also involved in histone methylation, as demonstrated by Ash-1 and Trx/Mll, to be HMTases for activating marks, primarily H3-K4 methylation. H3-K4 recruits complexes that enhance the accessibility of transcriptional factors to the chromatin.

Thus, histone lysine methylation has been correlated with both gene activation and silencing. The most studied lysine methylation residues involve K4, K9, K27, K36, and K79 of histone H3, and K20 of histone H4. In general, methylation at H3-K9, H3-K27, and H4-K20 is correlated with transcriptional repression, while methylation at H3-K4, H3-K36, and H3-K79 is related to gene transcription [23, 30]. Therefore, the action of these enzymes is crucial in controlling gene regulation, and biochemical and biological data
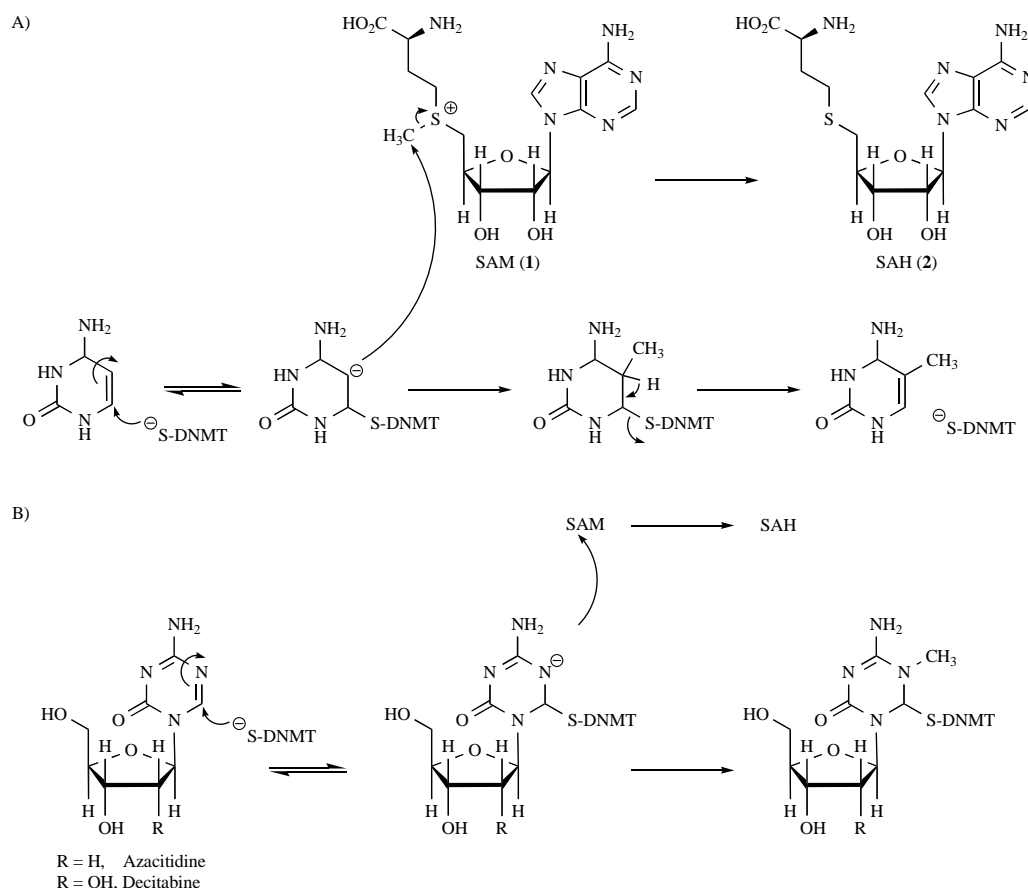
Fig. (2). A) Catalytic mechanism of C5-DNA methylation. **B**) Mechanism of the 5-azacytidine and 5-aza-2′-deoxycytidine.

suggest that these proteins have pathogenic roles in cancer and in many other diseases [1, 31].

THE ACTIVE SITE OF HISTONE METHYLTRANS-FERASES (HMTs)

In contrast to other histone modifications, histone methylation does not determine changes in the steric interactions or charge of the histone tail, but rather modifies its basicity, hydrophobicity and the affinity of other proteins such as transcription factor toward DNA. Histone methylation can occur on lysine or arginine amino-acidic residues and is carried out by two enzymes: lysine methyl-transferases (HKMTs or PKMTs) and arginine methyltransferases (HRMTs or PRMTs), respectively. Lysine residue can be mono-, di- and trimethylated, whilst arginine can be only mono- or dimethylated [32].

To date, more than 90 HMTs are known in the proteome. They can be divided into three classes: SET-domain lysine methyltransferases which contain a 130 amino acids catalytic domain - discovered for the first time in Drosophila suppressor of variegation [Su(var)3-9], enhancer of zeste and trithorax: hence the name SET - [33], non SET-domain lysine methyltransferases (DOT1L) [34], and arginine methyltransferases [35]. Except for DOT1L, all HMTs contain a conserved SET-domain, responsible for the enzymatic activity, with a unique structural fold and different from other classes of protein methyltransferases that use S-adenosyl-L-methionine (**1**, SAM also known as AdoMet) as the methyl donor cofactor. The SET-domain contains a series of β-strands folding into three discrete sheets that surround a knot-like structure. The structures in some mammalian SET-domain proteins have been solved [36]. Lysine and arginine methylation probably follow the same mechanism Fig. (**3**).

The S_N2 reaction starts when the lone pair electrons of the nitrogen atom (from lysine or arginine) attacks the electrophilic methylsulphonium cation of SAM at 180° angle to the leaving group, to form a penta-coordinate carbon transition state. The transition state structure collapses, with methyl group relocation to the nitrogen atom of the lysine or arginine side chain and formation of S-adenosyl-L-homocysteine (**2**, SAH also known as AdoHcy) as a product. The methyl transfer can occur only if the ε-amine of the lysine substrate is deprotonated [17, 31, 37, 38]. It is supposed that the lysine substrate might be deprotonated upon binding, since SET-domain methyltransferases possesses an unusually high pH optimum of ~10 [39].

The catalytic active site of HKMTs and HRMTs consists of a SAM-binding pocket and a hydrophobic narrow acceptor channel that extends to the opposite face of the protein surface [40]. The target lysine is inserted into the channel so that the target nitrogen is located in proximity to the methyl donor SAM at the opposite end of the channel. The methylene group of the target lysine chain forms van der Waals interactions with the aromatic residues that form the channel, while the terminal ε-amino group of the lysine hydrogen binds the hydroxyl group of the catalytic Tyr of SET-domain at the bottom of the channel. Even though there is no difference between HKMTs and HRMTs regarding the cofactor binding, the configuration adopted to bind SAM is the hallmark. The SET-domain of HKMTs, that has been co-crystallized with SAM or SAH, showed that the cofactor adopts a 'U-shaped' configuration within the active site, a region rich in aspartate or glutamate residues able to form hydrogen bonds with the two ribose hydroxyls of SAM. The methylsulfonium cation is sited at the base of the hydrophobic channel in which the lysine substrate binds, while the lysine forms a salt bridge with the carboxylate of SAM [37, 41].
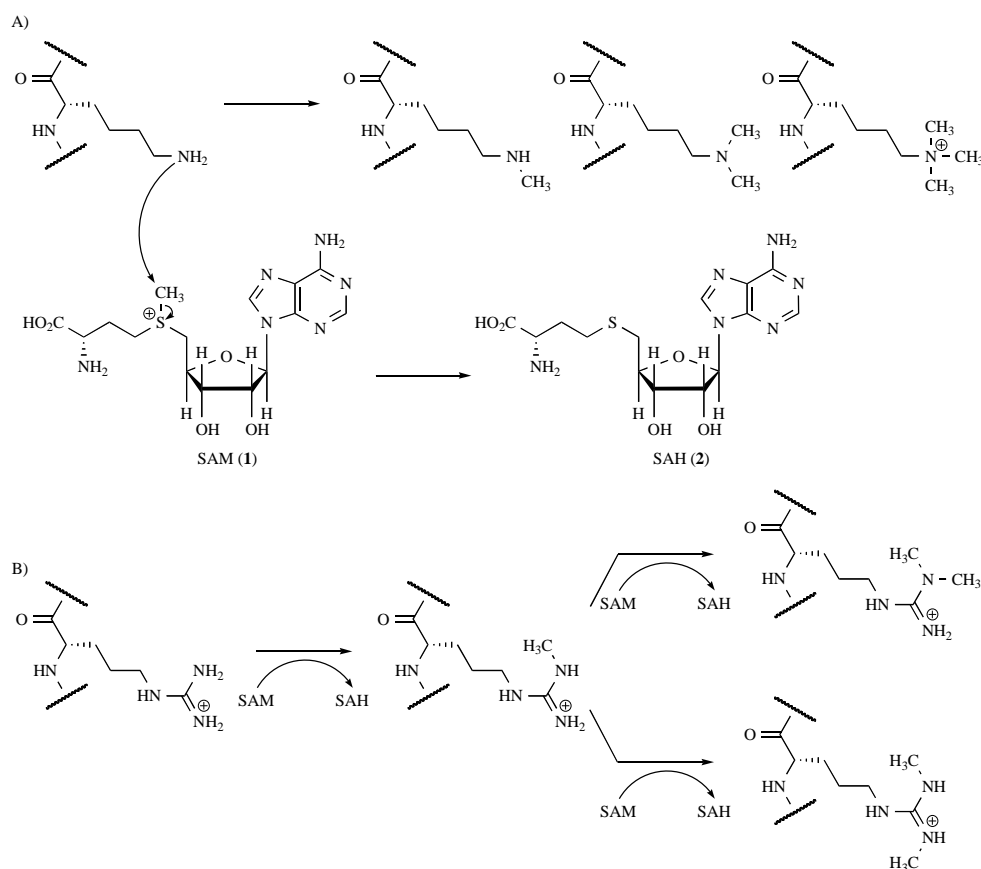
Fig. (3). Lysine and arginine methylation by SAM.

In contrast, the SET-domain of HRMTs, co-crystallized with SAM or SAH, has shown that arginine methyltransferases active site adopts an extended configuration when is bound to SAM, with the methylsulphonium group aligned with the base of the acceptor binding channel.

Despite DOT1L is a lysine methyltransferase enzyme, it seems to be more similar to the HRMT group. The co-crystal structures of human DOT1L with SAM has shown that the cofactor is bound in the extended configuration, similar to that observed in the HRMTs, with some differences in specific torsion angles [42].

In the last years the development of selective inhibitors for the HMTs was based on the difference of the static structure and the structural dynamics of the active site of the enzyme [38].

HKMT: CLASSIFICATION AND FUNCTION

Several efforts have been made to group the HKMTs on the basis of sequence homology and substrate [31, 43]. The specificity of HKMTs is not correlated with the SET-domain, common to all methyltransferase enzymes, but is rather defined by protein domain or homologous sequence next to the target lysine in the substrate. Thus, HKMTs have been classified in eight distinct subfamilies on the basis of these sequence differences (Table **1**) [44].

HKMT1 proteins include SUV39, G9a and GPL enzymes as family members [36]. SU(VAR)3-9 was the first SET-domain protein lysine methyltransferase discovered, initially identified in *Drosophila melanogaster*, and successively discovered in human homologous as SUV39H1 [45]. This subfamily is responsible for the H3-K9 specific methylation and, in addition to the SET-domain, contains two adjacent cysteine-rich regions (preSET and postSET) that are required for its enzymatic activity [46]. Methylated H3-K9 constitutes a binding site for the recruitment of heterochromatin

protein 1 (HP1) that, through the chromodomain located at its C-terminus, can oligomerize to form the heterochromatin [47]. SUV39H1 is an important epigenetic regulator, suggesting that pericentric H3-K9 methylation has a potential tumor suppressing activity by protecting the stable segregation of chromosomes [48, 49]. SUV39H1 is involved in the functions of the retinoblastoma (Rb) protein cooperating with Rb to repress the cyclin E promoter. G9a (also known as EHMT2) is responsible for mono- and dimethylation of H3-K9 [50]. G9a has shown *in vitro* 10- to 20-fold stronger HMTase activity toward H3-K9 compared to SUV39H1. Moreover G9a can methylate H3-K27 on non-heterochromatic loci, correlated with transcriptionally active genes. Localization of G9a in nuclei has suggested that it could target sites at transcriptionally active euchromatin rather than repressive pericentric heterochromatin [51]. GLP (also known as EHMT1) is a H3-K9 HMT that shares 80% of the primary sequence with G9a in their respectively SET-domains. The N-terminal region of G9a and GLP contains six centrally located ankyrin repeats able to bind to N-terminal histone H3-K9me or H3-K9me2 (mono- and dimethylated) suggesting that G9a and GLP are involved in both making and reading the histone code [52]. Although G9a and GLP can independently methylate H3-K9 *in vitro*, G9a/GLP heteromeric complex formation seems to be essential for exerting their function as a euchromatic H3-K9 methyltransferase. Both G9a and GLP are components of several transcriptional repression complexes and have been found to methylate also non histone proteins, e.g. p53 at K373, correlated with inactivation of p53 [53]. Furthermore, generation of GLP-deficient mice results in embryonic death [54].

HKMT2 family includes *Drosophila Trithorax* homolog mixed lineage leukemia (MLL) proteins. MLL1 is a major regulator of HOX gene expression, a transcription factors that participate in the development of multiple tissues, including the hematopoietic and

Table 1.   Lysine Methyltransferase Enzymes (HKMTs)

Classes	Members	Function
HKMT1	SUV39, G9a and GPL	SUV39 expression is increased in colorectal tumors, associated with transcriptional repression. G9a is overexpressed in various human cancers including leukemia, prostate carcinoma, epatocellular carcinoma and lung cancer. GPL is overexpressed in gland tumors.
HKMT2	MLL1	Translocation MLL1 is found in myeloid and lymphoblastic leukemia
HKMT3	SET 2 and NSD1	Translocation fuses NSD1 to nucleoporin 98 in human acute myeloid leukaemia
HKMT4	DOT1L	Recruited by MLL fusion partners, mediates MLL-rearranged leukaemias
HKMT5	PR-Set7 (Set8)	Methylates p53 leading to repression of p53 target genes in the absence of DNA damage
HKMT6	EZH1 and EZH2	Overexpressed in various human cancers including leukemia, prostate carcinoma, hepatocellular carcinoma and lung cancer
HKMT7	Set7/9	Modulates p53 activity in human cancer cells; regulates NF-κB-dependent inflammatory genes
HKMT8	RIZ1	Loss of RIZ1 expression was also commonly found in hepatoma tissues, neuroblastoma and breast and lung cancer

embryonic systems. MLL is involved in a large number of chromosomal rearrangements that lead to myeloid and lymphoblastic leukemia (ALL, AML) [55]. This family efforts a H3-K4 methylation and share a different SET-domain with an essential post-SET region at the C-terminus [43].

HKMT3 family methylates H3-K36 and H4-K20 through SET 2 and NSD1 activities, respectively [56, 57].

HKTM4 group comprises the first and only non SET-domain protein DOT1L, found in 1998 in yeast as disruptor of telomeric silencing 1 [58]. Despite DOT1L protein is structurally more similar to arginine methyltransferases, it catalyzes histone lysine methylation rather than arginine methylation [34]. Crystallographic studies have shown similarity between DOT1L and HRMTs in comparing the conformation of the substrate (SAM) and product (SAH) ligands bound to SET-domain. DOT1L binds SAM and SAH in an extended conformation, different from the 'U-shaped' conformation exhibited in SET-domain HKMTs but highly overlapping with the ligand conformation seen for the HRMTs [59]. DOT1L is the only enzyme responsible for H3-K79 mono-, di-, and trimethylation, that is correlated with active transcription [60]. In contrast to the other lysine methyltransferase, DOT1L target is not located in the tail region of the histone but in the globular core of the histone itself. It is clear that the main function of H3-K79 methylation is to mark open chromatin domain since around 90% of the chromatin is methylated by DOT1L [61]. Mammalian DOT1L is an essential gene required for embryogenesis, hematopoiesis, and cardiac function [62]. Furthermore, DOT1L mediates the interaction between MLL and AF10 and AF9, MLL fusion partners in human leukemia, through its HMTase activity [63, 64].

HKMT5 family includes PR-Set7 (also known as Set8). PR-Set7 specifically methylates nucleosomal H4-K20. It is the sole H4-K20 mono-methyltransferase whereas other enzymes catalyze the transition from H4-K20me1 to H4-K20me2/3 [65].

HKMT6 comprises EZH1 and EZH2 enzymes that act on H3-K27. They functions as the catalytic subunit of polycomb repressive complex 2 (PRC2). In particular, EZH1 is part of a non canonical PRC2 complex that catalyzes addition of methyl groups on H3-K27 and prevents the depression of PRC2 target genes [66]. EZH2 is directly required for establishment and maintenance of embryonic stem cells pluripotency and plasticity. Both enzymes contribute in maintaining the transcriptional repressive state of chromatin and are upregulated in several human cancers [67]. Overexpression of the SET-domain HKMT EZH2 and suppressor of zeste 12 homologue (SUZ12) has been associated with prostate, breast, bladder, colon, skin, liver, endometrial, lung, gastric, lymphoid, and myeloid cancers. The mechanism by which loss of the H3-K27 methylation system leads to cancer is still poorly characterized [63].

HKMT7 contains only one protein, Set7/9, which monomethylates histone at H3-K4 [68]. Set7/9 methylates also non-histone proteins, including p53, DNA methyltransferase 1 (DNMT1), nuclear factor kappa B (NFkB) and nuclear hormone estrogen receptor alpha (ERα) [59].

HKMT8 includes only one member, RIZ1. It was identified as retinoblastoma (RB) protein-interacting zinc-finger protein and is responsible of H3-K9 methylation [69]. Mutation or silencing of RIZ1 was observed in many human cancers [70].

HKMTs constitute an important, novel drug target class for the development of small-molecule drug therapies against a number of serious human diseases [38].

HKMT SET-DOMAIN INHIBITORS

Histone lysine methylation plays an important role in the chromatin organization and the regulation of gene expression [71]. HMTs have more than 50 different SET-domains and differ in their specificity for a target lysine residue. Many HMTs such as SUV391H, EZH2, MLL, Nsd1, RIZ, and others are implicated in tumor development. Therefore, in the last ten years most epigenetics research has focused in the research of histone methyltransferase inhibitors (HMTi). The initial HMTi reported were analogs of SAM, such as SAH and sinefungin (**3**), a natural inhibitor isolated from Streptomyces spp. cultures Fig. (**4**). SAM exhibits higher affinity for HKMTs than the product SAH, while for the HRMTs both substrate and product have similar activity. Unfortunately, no selectivity was observed for these compounds. The antifungal compound **3** (C**3**), with a pendant amino functional group replacing thiol cation of SAM, has been reported to be 3-10 fold more active than SAH [72].

In 2005, Greyner *et al.*, using a standard radioactive filter-binding assay, screened a small library of 2976 compounds to test their ability to inhibit the activity of recombinant *Drosophila melanogaster* SU(VAR)3-9 protein. They identified a specific lysine histone methyltransferase inhibitor, (-)-chaetocin (**4**) Fig. (**4**), a fungal micotoxin that belongs to the 3,6-epidithio-diketopiperazines class and acts as a competitive inhibitor for SAM. Biological studies have confirmed strong inhibitory potential of **4** for SU(VAR)3-9 ($IC_{50} = 0.6$ μM) and SUV39H1 ($IC_{50} = 0.8$ μM), but its poor selectivity constitutes a problem since **4** has also shown activity toward other HKMTs such as G9a and DIM5 at 2.5 and 3.0 μM, respectively, and cell toxicity has been observed. Although **4** lack in subclasses member specificity, it was selective over non-H3-K9 HKMTs such as SET7/9 and E(z) in respected of which it showed to be active at concentration below 90 μM [73]. Furthermore, total synthesis of (+)-chaetocin has been accomplished. Interestingly, (-)-**4** and (+)-**4** inhibits G9a equally ($IC_{50} = 2.4$ and 1.7 μM, respectively). In contrast, sulfur-deficient analogues ($IC_{50} > 50$ μM) are inactive [74].

A selective G9a inhibitor, BIX-01294 (**5**), has been identified by virtual and high-throughput screening of Boehringer Ingelheim's
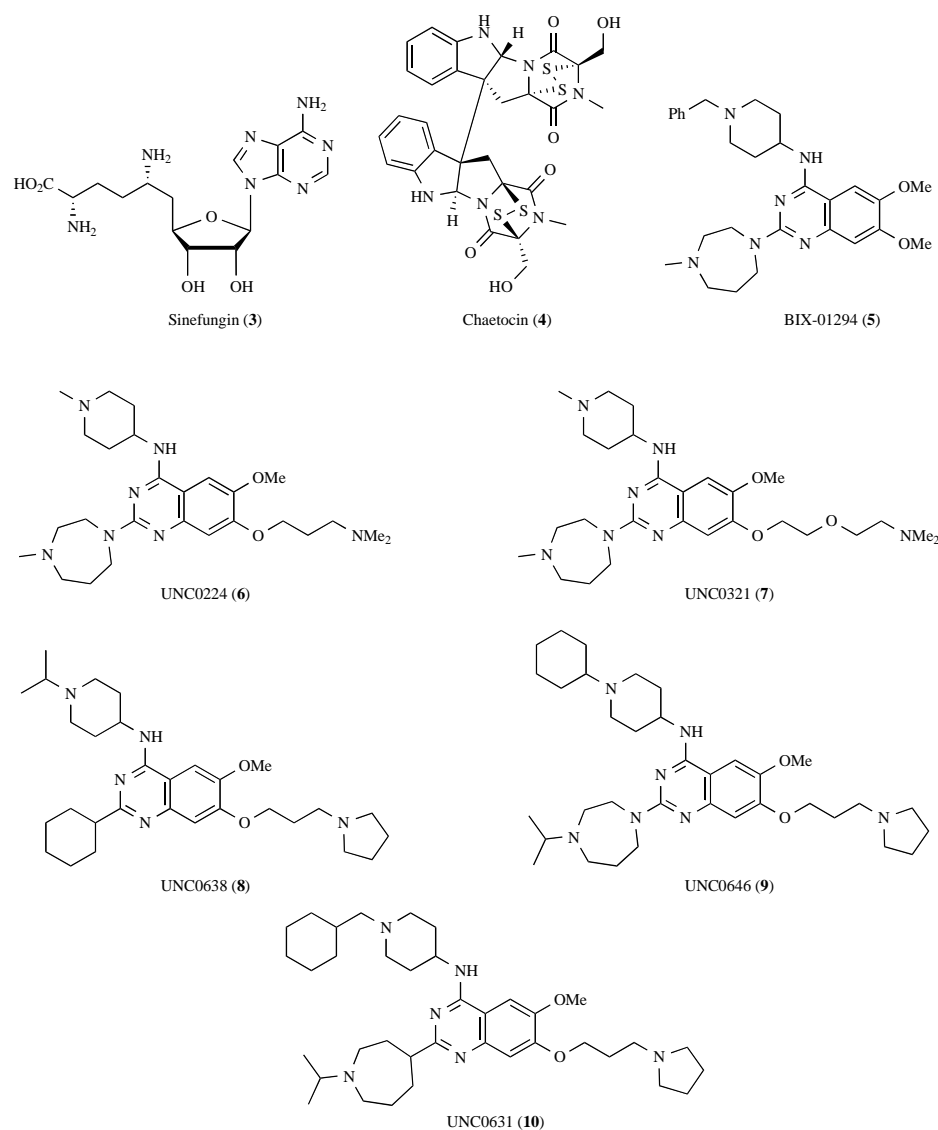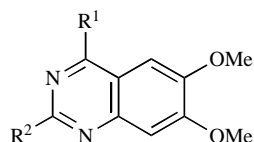
Fig. (4). Representative SET-domain lysine methyltransferase inhibitors.

compounds collection for a total of 125000 natural compounds evaluated. C**5** displayed G9a selectivity, with IC_{50} of 1.7 µM, over SUV39H1 and PRMT1 which showed no inhibition at the tested concentration range (up to 45 µM) [23]. G9a and GPL structure similarity suggested to investigate whether **5** is active toward GPL enzyme. Linear condition assay showed that **5** inhibits GPL at concentration of 0.7 µM. Chemically, **5** consists of a central quinazoline ring linked to a seven membered diazepane one and a benzylated six-membered piperidine. X-ray crystal structure of **5** and GLP in the presence of SAH showed that **5**, in contrast to **4**, does not bind the enzyme in the SAM-binding site but occupies the histone peptide binding pocket. The contacts between the enzyme and **5** are mostly concentrated on the quinazoline ring, its associated dimethoxy moieties and the diazepane ring. Quinazoline ring binds the acidic surface of the SET-domain of human GLP in the peptide binding groove, interacting with four aspartates and preventing the binding of peptide substrate [75]. In order to obtain more potent HMTi, structure activity relationship (SAR) studies have been developed. The first to be investigated was the 4-amino moiety. Accordingly to X-ray crystal structure data, the substitution of the 1-benzylpiperidin-4-ylamino groups with 1-methylpiperidin-4-ylamino resulted in no potency loss, as proved by G9a AlphaScreen assay for the detection of methylated histone peptides [76]. Replac-

ing the 1-methylpiperidin-4-ylamino with piperidin-4-ylamino, tetrahydropyran-4-ylamino, or cyclohexylamino results in substantial potency loss, demonstrating that alkylated nitrogen is important for inhibitory activity (Table **2**). Analogues containing a smaller amino group such as cyclopropylamino and isopropylamino have displayed less activity. Also, modifications to quinazoline 2-amino region are, in general, well tolerated. The methylhomopiperazine group could be replaced with the methylpiperazine or piperidine one without significant activity loss. Analogues containing morpholine, diethylamine or dimethylamine group had moderate potency.

As the methoxy group of **5** does not interact whit the narrow lysine binding channel, the addition of a 7-aminoalkoxy side chain was proposed to engage the lysine cavity and hence increase the inhibitory activity. This new potent HMTi, UNC0224 (**6**), resulted seven times more potent than **5** with an IC_{50} for G9a and GLP of 0.7 and 1.9 nM, respectively, and selectivity more than 1000-fold higher for G9a over SET7/9 and SET8. A high resolution X-ray crystal structure of the G9a-**6** complex revealed that the 7-dimethylaminopropoxy chain occupies the lysine binding channel of G9a interacting with Tyr1154 and Leu1086 [77]. However, the 7-alkoxy side chain does not completely occupy the lysine binding channel, and space remains to accommodate a longer side chain.

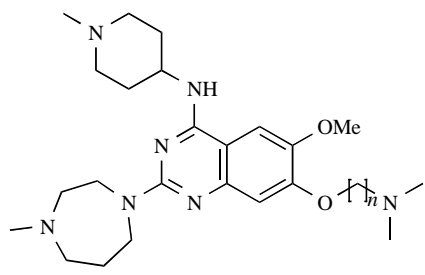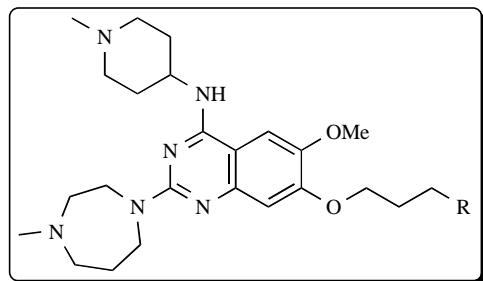**Table 2.** **SAR of 4-amino Moieties of the Quinazoline Scaffold**

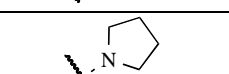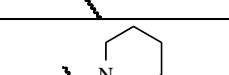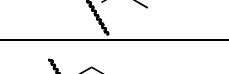| R^1	R^2	G9a IC$_{50}$ (μM)
		0.29
		0.23
		> 10
		> 5
		0.20
		0.51
		6.5
		6.0
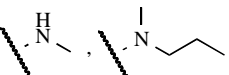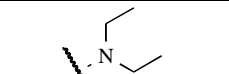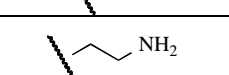
Further SAR studies have been conducted on the 7-aminoalkoxy region of **6** to optimize the potency of this series (Table **3**). Carbon chain elongation up to 5-carbons does not reduce the activity while analogue with 6-carbon chain significantly reduces the potency. Substitution of N,N-dimethylamino group with N-methylamino, N,N-diethylamino, N-methyl-N-propylamino or with the primary amino group, does not result in a significant potency loss. Analogues with cyclic amino groups such as the pyrrolidine and piperidine are more potent. In particular, the pyrrolidine is about 5-fold more potent than **6** with an IC$_{50}$ of 11 nM in chemiluminescence-based oxygen (CLOT) assay, used to detects monomethylation of histone H3 peptide, while piperidine showed higher cellular potency in the In-Cell Western (ICW) assay [78].
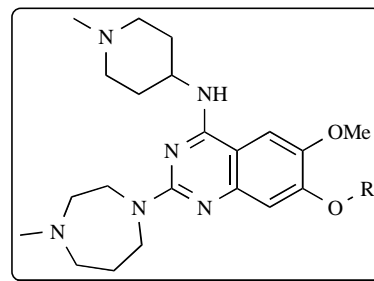
These results, together, suggest that modifications to the substituents on the basic nitrogen are well tolerated and a larger amino capping group can be accommodated. The basic nitrogen in the aminoalkoxy side chain was found to be essential for retaining high potency for G9a, since its substitution with methine, methoxy or the N-Boc protected amino group completely abolishes G9a inhibitory activity (Table **3**). Replacing the 5-carbon chain in compound **6** with a 2-(N-ethyl-N-methylamino) ethyl chain or increasing the size of the amino capping group with pyrrolidine leads to potency decrease. In contrast, substitution of the 5-carbon chain with an ethoxyethyl chain resulted in the discovery of a potent G9 inhibitor, UNC0321 (**7**) (IC$_{50}$ = 6 nM, Alpha Screen). Morrison Ki's were determined showing that C7 is 40-fold more potent than **6** and 250
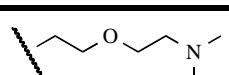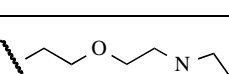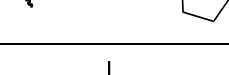
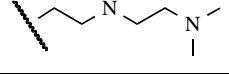**Table 3.**   **SAR of 7-aminoalkoxy Region of 6**

n	CLOT IC$_{50}$ (nM)
2	120
3	57
4	110
5	49
6	3200

R	CLOT IC$_{50}$ (nM)	ICW IC$_{50}$ (nM)
	45	–
	65	–
NH$_2$	110	–
	11	2.2
	20	0.91
	5200	–
NHBoc	> 10000	–

R	CLOT IC$_{50}$ (nM)
	6
	110
	173
	5100

fold more potent than **5** (Ki's = 63 pM, 2.6 nM and 16 nM, respectively). In addition, **7** is more selective for G9a over GLP (IC$_{50}$ = 23 nM) and inactive against other HKMTs, SET7/9 and SETD8, as well as HRMT3 (IC$_{50}$ > 40 μM) [79].

However, C**6** and C**7** resulted less potent than **5** in cellular assays even though they are more potent than **5** in biochemical assays. The low cellular potency shown by these compounds is probably due to the poor cell membrane permeability and low lipophilicity. In order to improve the lipophilicity, maintaining high *in vitro* potency, varies modifications to C**6** have been effectuated. An isopropyl group was added to the piperidine nitrogen, the diazepam ring was replaced with a cyclohexyl group and the methoxy at C5 was substituted with a propyl-pyrrolidin group. These efforts led to

the discovery of UNC0638 (**8**), a more potent and less toxic inhibitor as showed by cellular assay. C**8** has been found to have excellent *in vitro* potency with Ki for G9a of 3.7 nM, IC$_{50}$ < 15 nM against G9a and IC$_{50}$ = 19 nM against GLP. Moreover, **8** is highly selective for G9a substrate and GLP, being totally inactive against H3-K9 (SUV39), H3-K27 (EZH2), H3-K79 (DOT1L), H3-K4 (SETD7) and H4-K20 (SETD8) lysine methyltransferases as well as arginine methyltransferases HRMT1 and HRDM3. Treatment of several cell lines with **8** resulted in good cell penetration and great potency in the reduction of H3-K9me2 levels (with IC$_{50}$ ranging from 28 to 238 nM). Furthermore, X-ray crystal structure of the G9a-**8**-SAH complex (2.56 Å resolution) revealed that **8** is competitive with the peptide substrate and non-competitive with the cofactor SAM [78]. Several derivatives have been synthesized aiming

to improve the *in vivo* activity. In particular, further investigations have been conducted to efficiently explore the 7-aminoalkoxy region. Based on the findings that poor activity is correlated to low lipophilicity, the 7-aminoalkoxy portion of compound **8** was replaced with a piperidin-1-ylpropoxy group and the methyl in 4 of the diazepan ring was substituted with an isopropyl group. Both of these modifications are well tolerated. Furthermore, the substitution of the piperidine nitrogen with a cyclohexyl or methylene-cyclohexyl groups led to the formation of two potent compounds, UNC0646 (**9**) and UNC0631 (**10**) respectively. C**9** and C**10** show high *in vitro* potency versus G9a (IC$_{50}$ = 6 and 4 nM, respectively) and in reducing H3-K9me2 levels, also displaying low toxicity [80].

HKMT NON SET-DOMAIN INHIBITORS

To date, DOT1L is the only non SET-domain HMT known. This enzyme catalyzes an S$_N$2 reaction of the H3-K79 ε-NH$_2$ of the substrate nucleosome with the methyl group of SAM that is converted in SAH. Compound SAH is known to be a nonselective inhibitor of many methyltransferases, including DOT1 (Ki = 160 nM) [81]. However, SAH is quickly degraded by SAH hydrolase in adenosine and homocysteine and this limits *in vivo* use. The correlation between DOT1L and MLL protein has prompted the research of DOT1L inhibitors. Thus, DOT1L is considerate a potential therapeutic target in mixed lineage leukemia. MLL1, a gene that encodes for histone methyltransferase MLL, is mutated in most MLL leukemias. This mutation is responsible for MLL chromosomal translocations and its fusion to other translocation fusion partners, including AF4, AF5, AF9, ENL, and AF10 [82]. This protein complex recruits DOT1L resulting in RNA Polimerase II elongation. In particular, AF10 was identified as a DOT1L-interacting protein. This interaction is critical for leukemogenesis induced by MLL-AF10 since HMT activity of DOT1L is necessary for the transformation of hematopoietic progenitors [63].

The first SAM-competitive selective DOT1L inhibitor reported was EPZ004777 (**11**, Fig. **5**) [83]. C**11** is a SAM analog that includes a urea replacement for the methionine α-amino acid moiety

and a basic amine mimetic of the positively charged sulfonium group. This compound showed high activity (Ki = 0.3 nM) and great selectivity (>1,000-fold) for DOT1L compared to the other HMTs tested [84]. Treatment of MLL cells with **11** resulted in selective killing of those cells bearing the MLL gene translocation, with little effect on non-MLL-translocated cells. Moreover, administration of the drug in a mouse MLL xenograft model led to extension of survival [85]. However, the compound has poor pharmacokinetic properties due to the presence of six hydrogen bond donors that results in a high polar surface area and impairs passive membrane permeability [84].

In order to design selective DOT1L inhibitors, DOT1L-SAM complex has been investigated by crystallographic studies. This investigation reveled that SAM 6-amino group forms only one H-bond with DOT1L with a large hydrophobic cavity nearby. Consequently, the importance of NH$_2$ groups has been evaluated. Yao and co-workers designed and synthesized a series of molecules in which the nitrogen in 6 was differently substituted. SAR studies have shown that introduction of a CH$_3$ group, such as in **12**, provides good insertion into the hydrophobic cavity giving rise to a molecule with an excellent selectivity. Moreover, 6-NH methyl orientation allows the formation of a H-bond with Asp222 that is important for the binding of the adenine ring while all of the others 10 H-bonds, as well as the interactions between the ligand and the protein, remain essentially intact. The strong DOT1L selectivity is the reason of 6-NH substituent that in SET-domain HKMTs would be rise to intolerable steric repulsion with the protein, preventing these compounds from binding. C**12** showed a Ki value of 290 nM and only weak activities against two HRMTs, coactivator-associated arginine methyltransferase 1 (CARM1) and HRMT1, and two HKMTs, G9a and SUV39H1, with Ki values of 22.7 to > 100 μM. By replacing the S with the N, bringing an iodo-methyl substituent, C**13** was obtained. It was found to exhibit IC$_{50}$ = 15.7 μM, probably because C-N bonds (~1.47 Å each) in **13** are considerably shorter than the C-S ones (~1.82 Å). Elongation of the carboxylic chain from 4 to 5 carbon atoms, gave rise to C**14**. It displays extremely high DOT1L inhibition activity (IC$_{50}$ = 38 nM) and selectivity compared to the
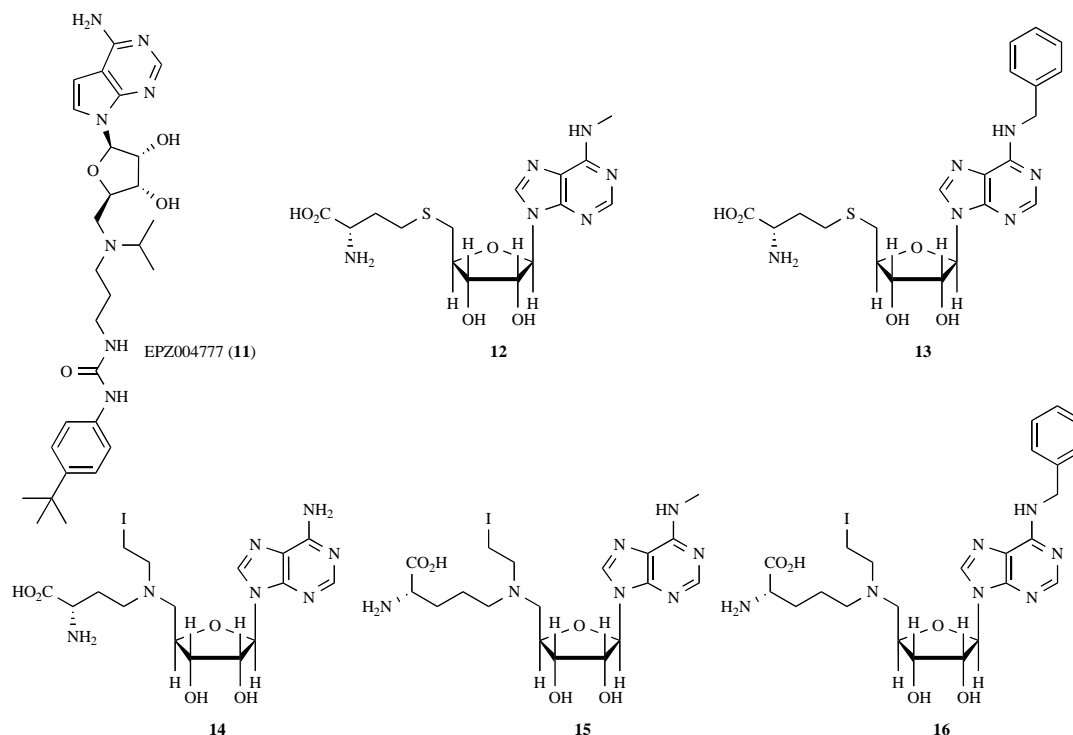

Fig. (5). Representative DOT1L lysine methyltransferase inhibitors.

other HMTs (IC_{50} of 1.1 to >100 μM). The reason of the selectivity of C14 is the locally more hydrophobic environment at the binding site of the putative aziridinium intermediate in DOT1L, which could protect the highly reactive aziridinium cation from nonspecific hydrolysis. To improve the selectivity, N6-substituted analogues of 14 have been synthesized (15 and 16). These two compounds exhibit strong activity against DOT1L, with IC_{50} values of 120 and 110 nM, respectively. As expected, 15 and 16 are inactive against other methyltransferases. The good activity and selectivity exhibited suggests that C15 and C16 could have wide applications as tools to study the biological functions of DOT1L [81].

HRMT: CLASSIFICATION AND FUNCTION

To date, about nine human arginine methyltransferases (HRMT 1-9) have been characterized and are classified in four different typology, depending on the type of methylation they accomplish. HRMT type I (HRMT1, HRMT2, HRMT3, HRMT4/CARM1, HRMT6 and HRMT8) catalyze an asymmetric reaction transferring two methyl groups from SAM to one guanidine nitrogen giving the asymmetric N,N′-dimethylarginine. Type II enzymes (HRMT5, HRMT 7, HRMT9) catalyze the formation of symmetric dimethylated arginine through transferring a methyl from SAM to guanidine nitrogen. [85]. Although histone tails are the main targets for this family of enzymes, HRMT7 catalyze the formation of ω-N^{G}-monomethylarginine in peptides. Thus, is common to consider HRMT7 as type III enzymatic activity [86]. Finally, type IV enzyme activity catalyzes the monomethylation of the internal guanidine nitrogen atom, and this type of activity has been described only in yeast [87]. HRMTs, as well as HKMTs, are implicated in the positive and negative control of the transcription. HRMTs are also involved in a number of basic cellular actions, including RNA processing, transcriptional regulation, signal transduction, and DNA repair [88]. At least two methyltransferases, HRMT1 and HRMT4, are linked to transcriptional activation, whereas HRMT5 negatively regulates the expression of some tumor suppressor genes [37]. All HRMTs contain a highly conserved catalytic core region of about 310 amino acids and a unique region comprised between N-terminal and the core [89].

The type I enzymes, HRMT1, HRMT3, and HRMT6, generally recognize glycine- and arginine-rich (GAR) motif containing substrates [87], except for CARM1 and HRMT5 that can also methylate the N-terminal region, rich in proline, glycine, methionine, and arginine termed PGM motif [90].

HRMTs form ring-like dimer that is essential for the catalytic activity. The active site is highly conserved and contains a 12-residue 'double-E loop', two invariant glutamate and one His-Asp proton-relay system [91]. HRMTs enzymes catalyze an ordered sequential bi-bi kinetic mechanism in which SAM binds initially to the arginine substrate. Conserved glutamate and arginine residues (Glu100 and Arg54 in HRMT1) interact with the two ribose hydroxyls and carboxylate groups of SAM, respectively. The methylsulfonium group of SAM is positioned at the base of a channel in which the arginine substrate binds. Two conserved glutamate residues (Glu144 and Glu153 in HRMT1) form hydrogen bond with the guanidinium side chain of the arginine substrate localizing the positive charge to one terminal nitrogen of the guanidinium side chain and leaving a lone pair on the other nitrogen to attack the methylsulfonium of SAM [37].

HRMT1 was the first mammalian protein arginine methyltransferase identified [92]. It is the main arginine methyltransferase, responsible for at least 85% of all the HRMT activity in mouse [93]. HRMT1 is a key regulator of protein function. HRMT1-knockout mice die shortly after implantation [94]. This enzyme catalyzes the transfer of methyl group to H4-R3, activating the transcription, but it can also methylate non-histone proteins (Table 4) [95].

HRMT2 was discovered because of its 50% sequence similarity to the conserved catalytic core of HRMT1. HRMT2 methylates the N-terminal tail of histone H4 [96] and the heterogeneous the nuclear ribonucleoprotein E1B-AP5 [97], and acts as a co-activator of both the androgen and the estrogen receptors [98, 99]. In addition, HRMT2 can promote apoptosis and inhibits NF-kB transcription by blocking IkB-a nuclear export [100]. HRMT2-null mice have been shown to be viable and completely normal [101].

HRMT3, located in the cytosol, has a zinc-finger domain at its N-terminus, which is responsible for its substrate recognition specificity [102]. HRMT3 catalyzes the methylation of ribosomal protein 40S S2 (rpS2) [103], inhibiting its ubiquitination [104], and regulating ribosome biosynthesis at a stage beyond pre-rRNA processing [105].

HRMT4/CARM1: the CARM1 was identified in a yeast two-hybrid screen using as bait the carboxy terminal domain of GRIP1, a member of the p160 family of coactivators [106]. CARM1 activates the transcription by methylation of H3 at Arg17 and nucleosomal histones. In the nucleosomal methylation activator complex (NUMAC), which plays an important role in the regulation of nuclear-receptor signaling pathway, HRMT4 acquires the ability to methylate nucleosomal histones [107]. CARM1 also binds to the carboxyl-terminal region of p160 coactivators, enhancing transcriptional activation by nuclear receptors. Furthermore, CARM1 is a transcriptional coactivator of NF-kB and functions as a promoter-specific regulator of NF-kB recruitment to chromatin [108]. Gene

Table 4.   Arginine Methyltransferase Enzymes (HRMTs)

Classes	Function
HRMT1	Coactivator of hormone receptor function; aberrant expression observed in several tumors including breast, colon and prostate cancers. Essential component of MLL oncogenic transcriptional complex
HRMT2	Coactivator of both androgen and estrogen receptor
HRMT3	Expressed in breast carcinoma
HRMT4 (CARM1)	Steroid receptor coactivator; overexpressed in both human breast tumors and prostate cancer; overexpressed in breast tumors and associated with transcriptional activation
HRMT5	Expression increased in lymphoid, leukemia cancer cells, gastric carcinoma and lung cancer; mediates p53 methylation, which promotes cell arrest rather than cell death
HRMT6	Transcriptional repressor
HRMT7	Potential target for the sensitization of tumor cells to camptothecins
HRMT8	Expressed in brain; overexpressed in germ cell tumor, glioma and primitive neuroectodermal tumor of the CNS
HRMT9/FBXO11	Unknown

ablation experiments suggested CARM1 is vital for existence, as well as HRMT1; indeed, CARM1 null mice die at birth [109]. Furthermore increased expression of CARM1 correlates with androgen independence in human prostate carcinoma [110] and CARM1 is overexpressed in breast tumors [87].

HRMT5 was initially identified as a human homolog of the *Schizosaccharomyces pombe* Shk1 kinase-binding protein 1 (SKB1Hs) and was later cloned as Jak2-binding protein. It showed to be able to methylate histones H2A, H3 and H4 [111]. HRMT5 seems to function in several types of complexes in both the cytoplasm, in a 20S complex called methylosome, and the nucleus [112]. In the cytoplasm, HRMT5 complex, implicated in methylation and storage of spliceosomal sphingomyelin (Sm) proteins, interacts with the spinal muscular atrophy (SMN) complex and enhances its activity [113]. HRMT5 can also interact with flag-tagged BRG1- and hBRM-based hSWI/SNF chromatin remodelers and both complexes can specifically methylate histones H3 and H4 N-terminal tails. These findings suggest that the BRG1- and hBRM-associated HRMT5 regulate cell growth and proliferation by controlling expression of genes involved in tumor suppression [114]. HRMT5 has also been associated with the regulation of interleukin 2 (IL-2) gene expression. Inhibition of methylation in Jurkat T lymphocytes with methylase inhibitors or HRMT5 siRNA impaired IL-2 gene expression [115].

HRMT6 is located only in the nucleus and is currently the only automethylating enzyme in the HRMT family exhibiting automethylation activity [116]. HRMT6 has been identified as the major cellular H3-R2 dimethyltransferase repressing the transcription by counteracting H3-K4 trimethylation. HRMT6 also methylates the N-terminal tails of histones H4 and H2A at R3. HRMT6 exploits this function by antagonizing chromatin recruitment of WDR5 as well as MLL1 of the K4 methyltransferase complex, and by inhibition of the catalytic activity of MLL1 [117]. Furthermore, HRMT6 interacts with DNA polymerase b (Pol b) by methylation of R83 and R152, stimulating its polymerase activity through enhancing its DNA binding. [118]. Thus, HRMT6 plays a role in regulating DNA base excision repair. Finally, HRMT6 mediated methylation acts in defense against HIV1 by regulating the HIV1 gene expression [119].

HRMT7 has type III activity toward arginine-containing peptides [86] or type II activity toward peptide and protein substrates [120]. It can methylate histones and non histone proteins such as myelin basic protein, GAR motifs and spliceosomal protein SmB [121]. HRMT7 also plays a role in imprinting control region (ICR) DNA methylation through its interaction with the transcriptional repressor CTCFL, a paralog of CTCF that is an 11-zinc finger factor involved in gene regulation [122]. HRMT7 might be also involved in embryonic stem cell (ESC) pluripotency [123] and is also a potential target for the sensitization of tumor cells to camptothecins [124].

HRMT8 is the only arginine methyltransferase known to be membrane associated. HRMT8 N-terminus contains a myristoylation motif that facilitates its association with the plasma membrane [125]. Its expression is mainly restricted to the brain [126]. It also has an adapter role for nuclear proteins at the cell membrane in addition to its methyltransferase activity [121].

HRMT9/FBXO11 is one of the most recently histone arginine methyltransferase discovered and its sequence does not resemble those of other members of the family. It is also called FBXO11 or 'F-box only protein 11' because it can mediate the formation of F-box protein complex. HRMT9 was identified from a 'forced' alignment of its amino acid sequence to the HRMT family. An *in silico* screening has shown that this methyltransferase is present in four isoforms that are different in length, but all of them have the methyltransferase domains SAM-dependent I, post I, II, and III. HRMT9 has a zinc finger domain at the carboxy terminus end of

isoform 1. This enzyme may regulate protein degradation in the ubiquitination complex. The shortest isoform of HRMT9 (isoform 4) lacks the F-box domain so it may not be associated with the ubiquitination complex [127]. The protein HRMT10 remains to be characterized.

HRMT INHIBITORS

The first arginine methyltransferase inhibitor was discovered in 2004 by Bedford and co-workers by random screening approach [128]. By using protein arginine methyltransferase, HMT1 (heterogeneous nuclear ribonucleoprotein methyltransferase), in the yeast *Saccharomyces cerevisiae,* as model, they identified nine potential HRMT inhibitor molecules (AMI 1-9) Fig. (**6**) [129].

Test conducted *in vitro,* supported by the ability to inhibit the methylation of the RNA binding protein, Npl3p, upon Hmt1p arginine methyltransferase in the yeast, confirmed the activity of these compounds at low micromolar concentrations (ranging from 0.17 to 7 μM). In particular, AMI-1 (**17**), a symmetrical sulfonated urea, was identified as lead compound showing activity against HRMTs but not against HKMTs as well as AMI-6. Activity studies in HeLa cells showed that AMI-1 is able to inhibit hHRMT1 with IC_{50} of 9 μM, decreasing methylation levels of the GFP-Npl3 fusion. Compound **17** inhibits arginine methylation by inserting into the arginine-binding pocket, which can explain its high degree of HRMT specificity. AMI-1 and -9 are structurally symmetrical, and this may reflect the fact that HRMT1 and Hmt1p are dimers and the active sites are symmetrical. Thus, a single symmetrical compound could inhibit both HRMT molecules within the active dimer [128]. The structural similarity of the binaphthylurea **17** with some sirtuin inhibitors, like suramin, has suggested to measure its ability to inhibit SIRT1. As expected, **17** has shown activity toward SIRT1 (IC_{50} = 32 μM versus IC_{50} = 300 nM of suramin) [130].

In 2007, by *in silico* studies, Ragno *et al.* designed and synthesized a series of new inhibitors against HRMTs using **17** and AMI-5 (**18**) as lead compounds [131]. They reported structure-based (SB) and ligand-based (LB) molecular modeling studies to investigate the binding mode of several small molecule AMIs in the catalytic domain of the HRMT1 fungal homologue RmtA [132]. Among the number of the analogues synthesized, basing on **18** core, the highest activity was shown by C**21** where bromination of the xanthenic moiety in positions 2, 4, 5, and 7 greatly enhanced inhibitory potency (IC_{50} for HRMT1 of 4.4 μM versus 1.4 μM of **18**). On the contrary, opening or debrominating the xanthenic moiety determines significantly decrease of inhibition capability. The addition of an amino group in position 5 of the phenyl ring of **18** was unfavorable for the activity unless thwarted by the effects of bromine substituents or N-dichlorotriazino moiety, like in AMI-6 (**19**). SAR studies on **17** structure have been also reported. The replacement of one of the two hydroxynaphthalene sulfonic substituents with the shorter and nonpolar phenyl group resulted in a significantly decrease of the inhibitory activity. Substitution of the oxygen of the urea moiety with a sulfur atom determined an additional decreasing of the inhibitory potency [131]. Substitution of the sulfonic group in **17** with its carboxylic isoster has given only a slight decrease in inhibiting activity. On the other hand, replacement of the carboxylic group with an ester or an amide function diminished the activity against HRMT1 [133]. The major outcomes of the docking and binding mode analysis were the positioning of **17** between the SAM and arginine binding sites without fully occupying them, whereas the non-arginine selective derivative **18** was positioned in the SAM binding site. In this screening, **17** resulted the first specific HRMT inhibitor, and C**18** one of the most potent, though less selective [131]. Using the chemical structure of **18** as a template Mai *et al.* identified a new series of analogues, 1,5-diphenyl-1,4-pentadien-3-ones, with anti-methyltransferase activity, inserting a hydrophobic spacer between the two *o*-bromo- or *o,o*-
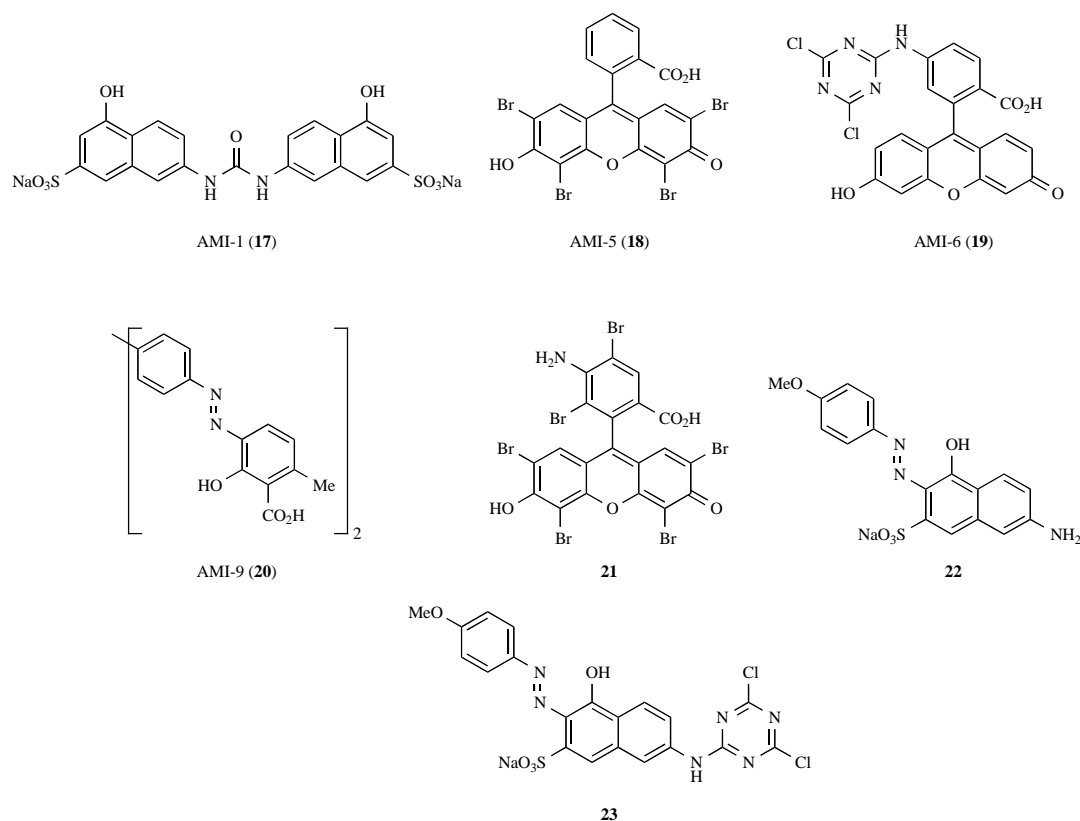
Fig. (6). Representative arginine methyltransferase inhibitors.

dibromophenol moieties (Table **5**). Compounds bearing different substituents, such as bromine atoms at C2 and C3-benzene, hydroxyl groups at C4 and C3, fluorine, nitro and methyl groups at C5-benzene, have been synthesized. All the compounds have been tested for methylation at H4-R3 against HRMT1. The highest activity was associated to the substitution on benzene ring with bromine atoms whiles substitution at C3-benzene with fluorine, nitro, or methyl group was tolerated. **C18d** was the most potent HRMT1 inhibitors with an IC_{50} value of 3.0 μM, being threefold more active than the lead **C18**, followed by **18e** with an IC_{50} of 9.9 μM, poorly active against SET7 (Table **5**). The introduction of further bromine atoms at the 5 or 5,5' position, replacing of the hydroxyl with a 4-methoxy group or removing of bromine substituents decreased the activity [134].

In an effort to develop a more potent selective inhibitor, Bonham and co-worker melted the characteristics of **17** (good selectivity) and AMI-9 (**20**, high activity). They synthesized two potent non-symmetric inhibitors bearing an azo moiety of **20** in one side, **C22**, and a dichlorotriazine group of **19** to the other side, **C23**, while the aminonaphthol sulfonate core of **17** was retained in both. **C23** is the most potent, with an IC_{50} of 4.15 μM for HRMT1 and 2.65 μM for HRMT4, similar to the IC_{50} value of 8.8 μM of the parent **17** for human HRMT1. In addition, **23** showed selectivity for HRMTs over HKMTs, SET7/9, in methylation assay [135]. Since HRMT1 activity is involved in T helper cell function the inhibitory effect of compound **23** towards T helper cell cytokine production was characterized [136, 137]. Biological assays reveled its ability to enhance T-helper cell proliferation. Finally, treatment with compound **23** reduced the production of IFN- levels of type 1 T-helper cells and IL-4 levels of type 2 T-helper cells [135].

Purandare and co-workers in 2008, with a high throughput screening, identified a new HRMTs inhibitor, the pyrazole amide derivative **24** Fig. (**7**). This compound exhibits a high selectivity toward CARM1 with an IC_{50} of 1.8 μM. Thus, hits to lead effort

had beginning to improve the *in vitro* potency of this derivative. SAR studies indicates that truncation of the biphenyl moiety is allowed whereas small alkyl amides are not tolerated. Modifications at the glycinamide portion or addition of substituent to the α-carbon do not result in less activity. Modifications to the amide portion linked to the pyrazole are not tolerated. Definitively, introduction of a methyl group to the α-carbon and simultaneously substitution of the biphenyl function with a phenyl group led to the discovery of **C25**, a potent CARM1 inhibitor with an IC_{50} of 80 nM and significant selectivity over HRMT1 and 3 with an $IC_{50} > 25$ μM [136].

Basing on the success of pyrazole derivatives as selective inhibitors of CARM1, other molecular modeling studies and high-throughput screening have been developed. Results obtained suggest a replacement of the central core phenyl ring with various heterocycle rings. Replacing the aromatic ring linked to the pyrazane with thiophene gave rise **C26**. It is the most potent CARM1 inhibitor with an IC_{50} of 60 nM and does not show activity against HRMT1 and SET7/9. Substitutions with other heterocycles like thiazole or pyridine result in decrease of the activity, while the isoindoline and benzofuran based analogues are devoid of activity (Table **6**). Despite the excellent potency toward CARM1 enzyme, these compounds does not show measurable cellular activities, probably due to the poor cell permeability [137].

In order to reduce hydrogen bond donor and to improve the permeability, Huynh *et al.* explored amine bioisosteres of **C25** at the east end of the molecules. Thus, they synthesized 1,3,4-oxadizole derivatives differently substituted at the position 5 of the heterocycle. Biological results showed higher activity of aryl groups with *o*-substituent compared with analogues *m*- or *p*-substituted. Introduction of nitrogen atom in the ring was not tolerated (Table **7**) The most potent analog in this series, **C27**, was obtained substituting the phenyl ring with a benzo[d]tiazol group displaying an IC_{50} of 40 nM. Although the improvement in the cell permeability, this compound lacks cellular activity [138].

Table 5.   SAR Studies Starting from C18

18a-f
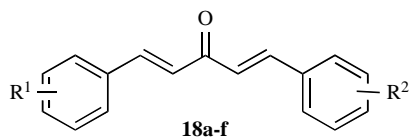
Cpd	R^1	R^2	PRMT1 IC$_{50}$ (µM)
AMI-5 (**18**)			9.8
18a	3-Br, 4-OH	3-Br, 4-OH	11.8
18b	3-NO$_2$, 4-OH	3-NO$_2$, 4-OH	614.3
18c	2,6-Br$_2$, 4-OH	2,6-Br$_2$, 4-OH	3.4
18d	3-Br, 4-OH	3,5-Br$_2$, 4-OH	3.0
18e	3,5-Br$_2$, 4-OH	3,5-Br$_2$, 4-OH	9.9
18f	3,5-Br$_2$, 4-OMe	3,5-Br$_2$, 4-OMe	433.9
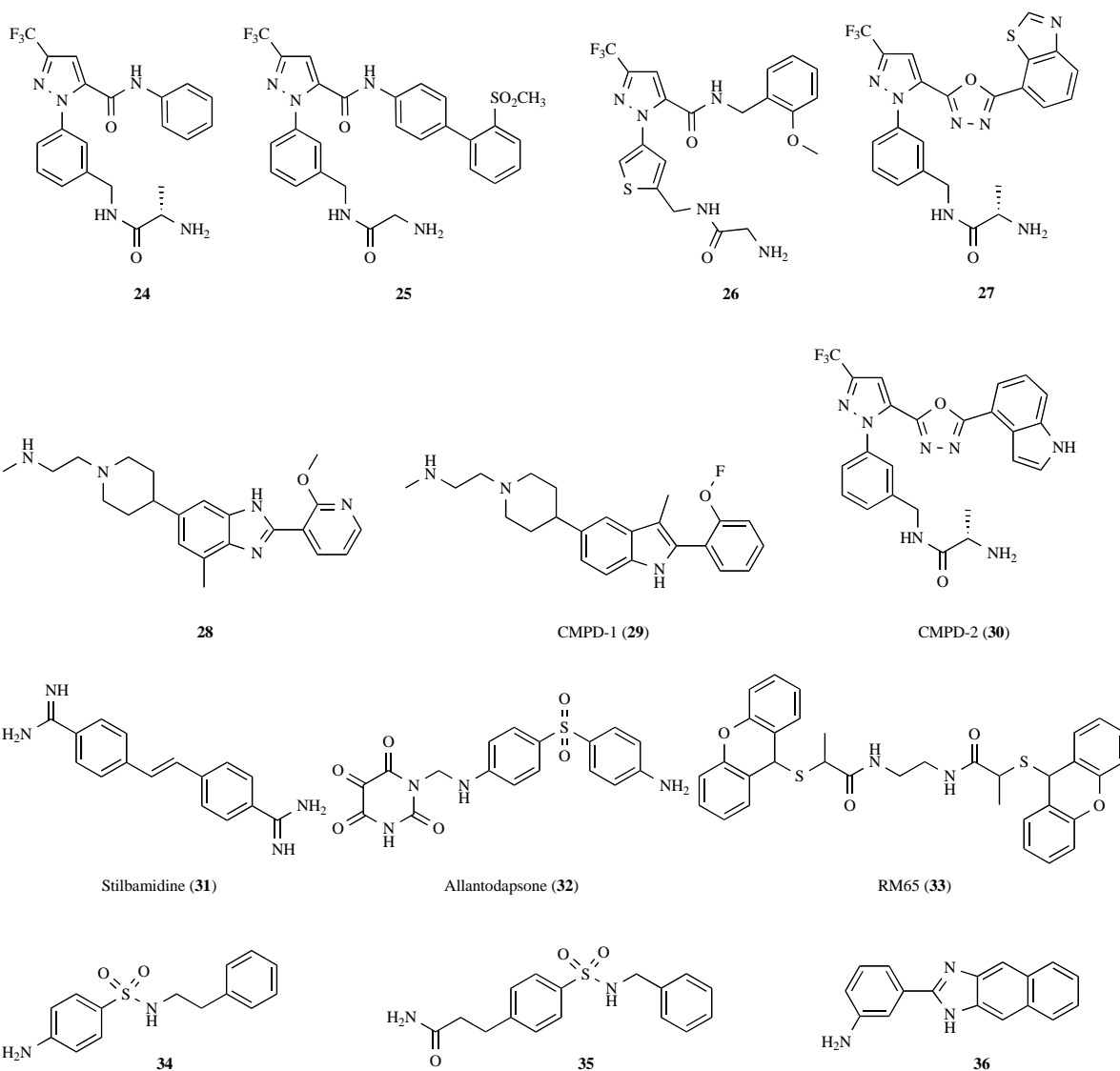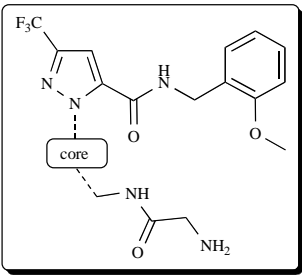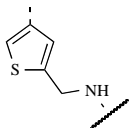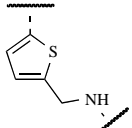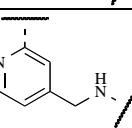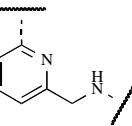
Fig. (7). Representative arginine methyltransferase inhibitors.
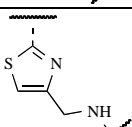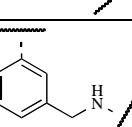
Table 6.   SAR Studies from C26
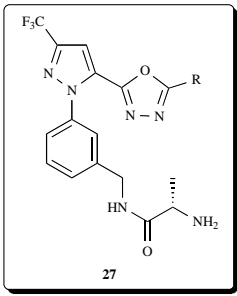
Core	CARM1 IC$_{50}$ (μM)
	0.06
	0.3
	2.20
	0.06
	4.20
	3.05

Table 7.   SAR Studies from C25

Cpd	R	CARM1 IC$_{50}$ (μM)
27		0.04
27a		0.06
27b		0.31
27c		1.54
27d		0.05
27e		0.16
27f		0.05
27g		0.05

Despite the optimal role of the (*S*)-alanine benzylamide in the enzymatic activity, Therrien *et al.* synthesized a series of pyrazole analogues substituting the amide function with several amino functions, ascribing the poor pK value observed to the presence of the amide group. However, substitution of this function with an ethyldiamino group led to a compound less potent (CARM1 IC$_{50}$ = 200 μM) devoid of cellular activity [139].

In 2009, a new screening of the corporate compound collection benzo[d]imidazole was conducted by Wan and co-workers. They identified, as hit compound, **28**, a moderate inhibitor of CARM1 with an IC$_{50}$ of 840 nM. In order to improve the *in vitro* potency of **28**, several modifications have been applied (Table **8**). Introducing an α-methyl group at the phenyl group, removal of internal nitrogen atom or abolishing basicity of nitrogen led to a significant loss of potency. Changing in the core, as introduction of methyl at the nitrogen of the benzo[d]imidazole, resulted in further loss of activity, while introduction of a nitrogen atom in the benzo- ring was well tolerated. Additional SAR on benzoimidazole substituents showed that almost all of the substituted phenyl and pyridyl groups were tolerated except the unsubstituted phenyl group leading to a decrease of the activity. The most potent inhibitor generated was **28b**, a 2,6-dimethoxyphenyl analogue of **28**. It displayed excellent activity, with CARM1 IC$_{50}$ of 70 nM, and a good selectivity over HRMT1 and HRMT3 (IC$_{50}$ > 25) [140].

Further modification at the indole and pyrazole inhibitor derivatives led to the discovery of two potent CARM1 inhibitors, CMPD-1 (**29**) and CMPD-2 (**30**) with IC$_{50}$ of 30 and 27 nM, respectively. Thus, in order to understand the basis for selectivity and to provide a starting point for structure-based design, crystal structures of the CARM1 catalytic domain in complex with cofactors and representative inhibitors of two different classes has been determined. The outcome of the study showed inhibitors **29** and **30** exploit the arginine-binding cavity to establish polar interactions using different regions of the pocket. C**29** interacts inside the cavity with Glu258, Met260 and His415, whereas C**30** forms similar polar interactions plus an additional one with Glu267. C**29** follows the mouth of the cavity wrapping over Glu267 and extending towards Ser146, whereas **30** follows the mouth of the cavity on the opposite side, packing against His415 and extending towards the N-terminus of the helix αZ. Is possible to conclude that **30** exploits better the region of the pocket between the arginine-binding cavity and the N-terminus of the helix αZ, whereas **29** exploits better the region between the arginine-binding cavity and Ser146. Presumably the arginine-binding cavity requires a specific shape; however, outside of the arginine-binding cavity the tolerance for variation increases. Noteworthy, has been identified a difference amino acidic sequence in the pocket of CARM1 compared with HRMT1 and HRMT3. The amino acid Phe153 in the CARM1 motif I corresponds to serine residue in both HRMT1 and HRMT3 (Ser38 and Ser220, respectively). This difference affects the shape of the pocket and the interactions with the molecules: Phe153 is directed into the pocket where makes hydrophobic interactions with the inhibitors while serine is responsible for hydrophilic binds [141].

In 2007, Spannhoff and co-worker applied a strategy combining virtual and biological assays using RmtA enzyme. The selection process was performed aiming at the potential ligands for the arginine and not for the SAM-cofactor binding site. Only 37 compounds of 140000 submitted to the National Cancer Institute have been tested in an *in vitro* assay for their ability to inhibit RmtA. Only the compounds that had showed favorable docking results had been chosen for *in vivo* test. The screening resulted in seven compounds with an IC$_{50}$ below 35 μM. Two competitive compounds for the protein substrate, stilbamidine (**31**) and allantodapsone (**32**), displayed a strong hypomethylating effect in HepG2 cells. C**31** and C**32** present a basic amine that interacts, through hydrogen bond, with the active site Glu152. In biological analyses, **31** and **32** have

Table 8.   SAR Studies from C28
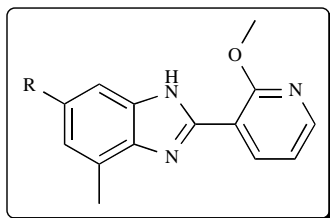
R	CARM1 IC$_{50}$ (µM)
	17
	> 30
	> 30
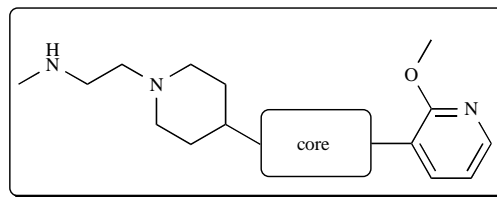
core	CARM1 IC$_{50}$ (µM)
	1.2
	2.9
	0.7

28
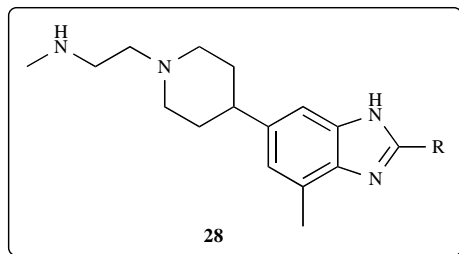
Cmp	R	CARM1 IC$_{50}$ (µM)
28a		0.41
28b		0.07
28c		0.26
28d		0.12
28e		4.6

shown strong inhibitory effect on the activation of the estrogen receptor E2, when tested in a stable transfection assay on MCF7a cells, while only **32** exhibited an inhibitory effect towards SET7/9 [142, 143].

A virtual screening for novel fragment-like leads (molecular weight below 200g/mol) led to the identification of an α-methylthioglycolic amide RM65 (**33**). It showed inhibition activity for RmtA/HRMT1 (IC$_{50}$ = 46 and 54, respectively) and no inhibitory effect on SET7/9. C**33** interacts with residues of the SAM binding pocket. In particular, all stereoisomers of C**33** demonstrated

favorable van der Waals interactions with several aromatic residues of the SAM and substrate binding pocket as well as hydrogen bonds with Tyr47, Tyr156, and Glu161. C**33** exhibited cell activity since HepG2 cells treated with it developed hypomethylation on histone H4-R3. Despite its cell permeability and activity in cancer cells, **33** lacked chemical stability and drug-like structure [144].

Founded on the good cell permeability of **32**, a new screening was conducted using a structure-based pharmacophore model on the basis of the HRMT1-allantodapsone interaction model. 328000 molecules of the Chembridge database were further selected by

Fig. (8). Representative arginine methyltransferase inhibitors.

docking program and tested in a biochemical HRMT1 assay. The aim was to discover compounds able to bind to the peptide binding site, and carrying basic or polar functional groups that can interact with key residues Glu152 and/or Tyr156. This research resulted in the finding of 9 compounds with activity below 35 μM. In particular, three of them, compounds **34-36**, were mainly active for HRMT1, displaying IC_{50} of 12.7, 15.3 and 14.3, respectively. All of these compounds have an aro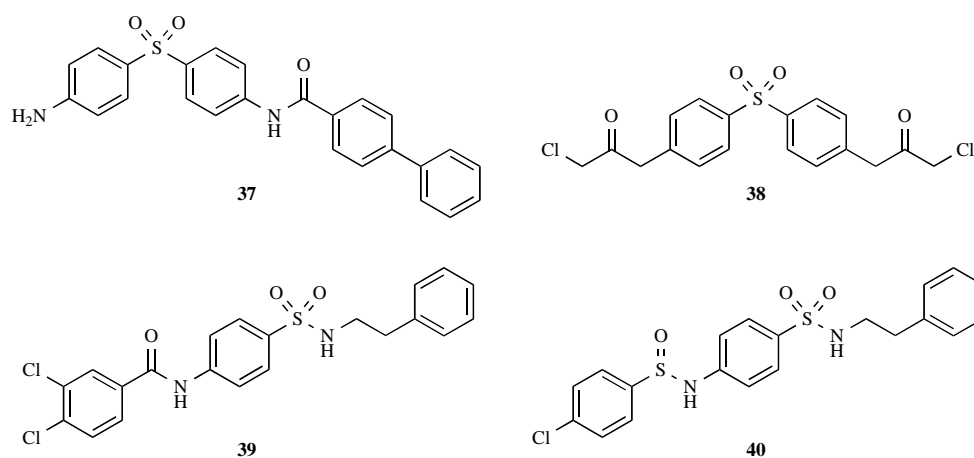matic group able to interact with tyrosine and tryptophan residues in the substrate binding pocket. The most potent, **36**, has a single amino group in the meta position of the aromatic ring that forms hydrogen bond to the side chain and backbone CO group of Glu152 as observed for **32**. C**36** also shows van der Waals interactions with the two aromatic residues Tyr147 and Tyr160 as detected for other potent inhibitors. Both of them seem to be a prerequisite for high inhibitory activity [145].

In order to improve the stability of **32**, acylated derivatives of *p*-aminobenzenesulfonamides have been synthesized. These compounds showed potent antiproliferative activity and ability to block androgen-dependent protein expression. Among the monoacylated analogues of dapsone, that offered limited impact on potency compared to **33**, the most potent inhibitor was the biphenylylamide **37** (IC_{50} = 1.5 μM) Fig. (**8**). Among the diacyl derivatives that showed potent enzyme inhibition as well as good cytotoxic activity, the most potent compound was the bis-chloroacetylated **38** with an IC_{50} of 1.5 μM in MCF7a and LNCaP cell lines. Several derivatives of compound **38** have been produced. They showed less activity than the lead compound with the exception of the 3,4-dichloro derivatives **39**, which showed an IC_{50} of 10.2 μM and modest antiproliferative activity (IC_{50} = 52 μM). More potency has been obtained from the sulfonamide analogue **40** that inhibited HRMT1 with an IC_{50} of 5 μM [146].

CONCLUSIONS

Epigenetic modifications causing gene transcriptional repression have been associated with malignant transformation and are interesting new targets in the treatment of cancer. Chromatin remodeling proteins have been well characterized both *in vitro* and *in vivo*. In particular, the histone methylation field is growing rapidly. Even though the field of epigenetic drug discovery is still at its beginning, a dynamic development can be anticipated and clearly further epigenetic drugs based on histone methyltransferases will be developed in the near future. A notable progress in the protein methylation field is the ever-growing evidence that methylation is found to occur broadly on a variety of non-histone proteins, thus out of the realm of chromatin biology. Histone methylation on chromatin templates has been considered to be intimately associated with transcriptional activation and repression. In this review,

we have highlighted the significant advances and functional insights on histone lysine and arginine methylation over the past few years. In particular we have pointed out the development of new HMT inhibitors and SAR studies. Although few histone methyltransferase inhibitors have been identified or synthesized, most of them have only micromolar activity. Further effort is required in order to obtain HKMT and HRMT inhibitors with potency at nano- or picomolar levels. Nonetheless, major issues remain to be addressed before these newer epigenetic agents become established therapeutics; in particular, a systematic evaluation of the pharmacological impact of new methyltransferase inhibitors in cancer cells and/or other disease models has to be performed, in order to validate the therapeutic potential of these compounds and to improve their selectivity.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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