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# Synthesis and Biological Validation of Novel Synthetic Histone/Protein Methyltransferase Inhibitors

Antonello Mai,*[a] Sergio Valente,[a] Donghang Cheng,[b] Andrea Perrone,[a] Rino Ragno,[a] Silvia Simeoni,[a] Gianluca Sbardella,[c] Gerald Brosch,[d] Angela Nebbioso,[e] Mariarosaria Conte,[e] Lucia Altucci,*[e] and Mark T. Bedford*[b]

In eukaryotic cells, genes are complexed with core histones and other chromosomal proteins to form the chromatin. The basic unit of chromatin is the nucleosome, a nucleoprotein particle that consists of 147 base pairs of DNA wrapped around a core of histones (H2A, H2B, H3, and H4).[1] The histone lysine- and arginine-rich N-terminal tails protrude out of the histone core and are the sites of many types of post-translational modifications such as acetylation, methylation, and phosphorylation. The post-translational modification of histone tails regulates the level of chromatin condensation, and is in turn important for gene transcription.[2] Histone acetylation is one of the best understood histone modifications. The highly regulated activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) are responsible for the control of specific acetylation levels. Indeed, actively transcribed regions of chromatin (euchromatin) are hyperacetylated in comparison with condensed regions (heterochromatin), which are not accessible to transcription factors.[3] In this scenario, small molecule inhibitors of HDACs can affect the heritable changes in gene expression of specific genes, and are used as drugs for cancer therapy.[4]

Histone methylation has also been shown to be important in establishing stable gene-expression patterns. Histone methylation does not alter the overall charge of the histone tails, but has an influence on basicity, hydrophobicity, and on the affinity for anionic molecules such as DNA.[5] Histone tails can be mono-, di-, and trimethylated on the $\varepsilon$-amino group of lysine residues, and either mono- or dimethylated on arginine residues. Depending on the context, lysine methylation provides either activating or repressing modification. Thus, trimethylation of Lys9 in histone H3 is associated primarily with transcriptional silencing, whereas Lys4 methylation correlates with transcriptional activation.[6] Moreover, aberrant histone methylation has been linked to a number of human diseases such as cancer.[7]

Protein arginine methyltransferases (PRMTs) are grouped into two major classes, type I enzymes catalyzing the formation of asymmetric $\omega$-$N^G$,$N^G$-dimethylarginine tails, and type II enzymes catalyzing the formation of symmetric $\omega$-$N^G$,$N^{G'}$-dimethylarginine tails. To date, no mutations have been identified in PRMTs in tumour cells. However, the coactivator-associated arginine methyltransferase (CARM1/PRMT4) is over-expressed in both grade-3 breast tumours and in hormone-dependent prostate tumours.[8] In addition to their role in histone modification, PRMTs target several proteins involved in cell proliferation, signal transduction, mRNA splicing, RNA transport, and protein–protein interactions.[9]

PRMT1 regulates the nuclear cytoplasmic shuttling of the heterogeneous nuclear ribonuleoprotein (hnRNP) Npl3p, and methylates Arg3 in H4 facilitating acetylation of H4 by the HAT p300, which leads to transcriptional activation. CARM1 binds the p160 family of nuclear hormone receptor coactivators, and enhances the nuclear receptor-mediated transcription activation through methylation of H3.[9] Whereas studies on PRMTs are in their infancy, it is likely that they hold crucial roles in chromatin remodelling with regulation of gene expression and cellular processes. As such, PRMTs are likely to provide useful targets in the design of new anticancer agents.[9]

In 2004, a series of dyes and dye-like compounds were evaluated as small molecule modulators of PRMT and histone lysine methyltransferase (HKMT) activity. In this screen, AMI-1 was described as the first specific PRMT inhibitor, and AMI-5 was one of the most potent, though less selective, compounds (Figure 1).[10] Recently, the fungal metabolite chaetocin was identified and characterized as the first specific inhibitor of the HKMT SU(VAR)3-9 (Figure 1).[11] As a part of our medicinal chemistry project aimed at discovering new entities as small molecule modulators of epigenetic targets,[12] we chose the AMI-5 chemical structure as a template and designed a new series of simplified analogues starting from a pharmacophore hypothesis. In this hypothesis, we identified the presence of two *o*-bromo- or *o,o*-dibromophenol moieties as crucial for having antimethyltransferase activity, and inserted a hydrophobic spacer between the above fragments. In particular, we prepared a series of substituted 1,5-diphenyl-1,4-pentadien-3-ones **1**–**12** (Figure 2), in which some of them share two or more bro-

[a] *Prof. A. Mai, Dr. S. Valente, Dr. A. Perrone, Dr. R. Ragno, Dr. S. Simeoni*
*Istituto Pasteur-Fondazione Cenci Bolognetti, Dipartimento di Studi Farmaceutici*
*Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, 00185 Roma (Italy)*
*Fax: (+39) 06-491491*
*E-mail: antonello.mai@uniroma1.it*

[b] *Dr. D. Cheng, Prof. M. T. Bedford*
*University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, Texas 78957 (USA)*
*Fax: (+1) 512-237-2475*
*E-mail: mtbedford@mdanderson.org*

[c] *Prof. G. Sbardella*
*Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte Don Melillo, 84084 Fisciano (SA) (Italy)*

[d] *Prof. G. Brosch*
*Division of Molecular Biology, Biocenter, Innsbruck Medical University, Fritz-Preglstrasse 3, 6020 Innsbruck (Austria)*

[e] *Dr. A. Nebbioso, Dr. M. Conte, Prof. L. Altucci*
*Dipartimento di Patologia Generale, Seconda Università degli Studi di Napoli, vico L. De Crecchio 7, 80138 Napoli (Italy)*
*Fax: (+39) 081-450-169*
*E-mail: lucia.altucci@unina2.it*

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author. Supporting information includes experimental procedures, characterization data for compounds **1**–**14**, molecular modelling investigation, and further biological data on U937 cell line.

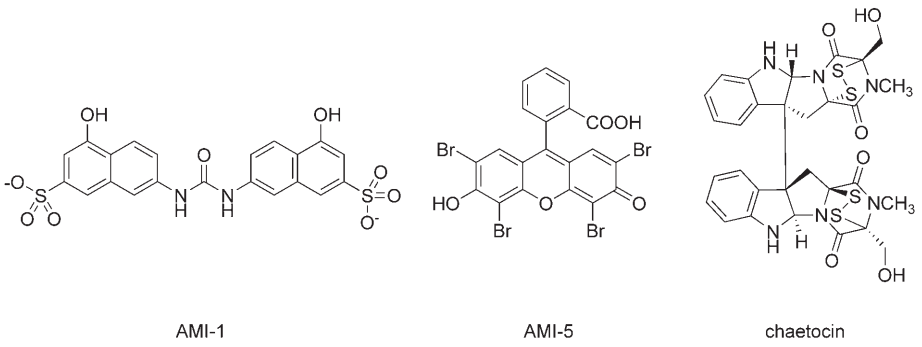



**Figure 1.** Known PRMT and HKMT inhibitors.

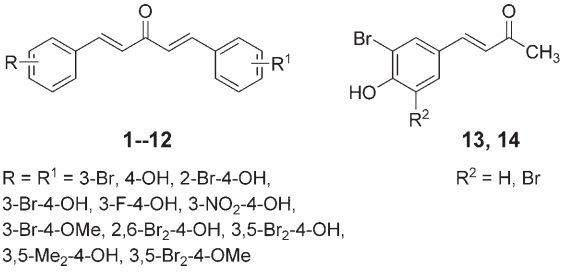
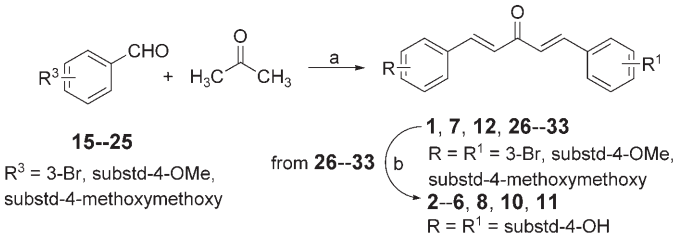



**Figure 2.** Novel designed PRMT/HKMT inhibitors.




**Scheme 1.** Reagents and conditions: a) barium hydroxide, MeOH, RT; b) 3 N HCl, MeOH, 70 °C.

mine atoms at the C2- or C3-benzene positions, and two hydroxy groups at the C4-benzene position (compounds **3**, **4**, **8**–**10**). To confirm our pharmacophore hypothesis, we also prepared compounds lacking the hydroxy (**1**) or the bromine (**2**) substituent, as well as bromo-containing compounds in which the 4-hydroxy group has been replaced by the methoxy one (**7** and **12**).

Moreover, compounds bearing varying substituents other than bromine at C3- (such as fluorine (**5**) and nitro (**6**)) or C3,C5-benzene position (such as methyl (**10**)) were synthesised. Finally, the 3-bromo- and 3,5-dibromo-4-hydroxyphenyl monomers **13** and **14**, harbouring just one *o*-bromophenol moiety, were included for comparison purposes (Figure 2).

Compounds **1**, **7**, and **12** were prepared by reacting the 3-bromo-, 3-bromo-4-methoxy-, and 3,5-dibromo-4-methoxybenzaldehydes (**15**–**17**) with 2-propanone (0.5 eq) in the presence of barium hydroxide. Following the same procedure, condensation of the properly substituted 4-(methoxymethoxy)benzaldehydes **18**–**25** (see Supporting Information) with 2-propanone (0.5 equiv) afforded, after acidic hydrolysis of the 1,5-bis(4-methoxymethoxyphenyl)-1,4-pentadien-3-ones **26**–**33**, the desired compounds **2**–**6**, **8**, **10**, and **11** (Scheme 1).

The 4-(3-bromo- and 3,5-dibromo-4-hydroxyphenyl)but-3-en-2-ones **13** and **14** and the asymmetric 1-(3-bromo-4-hydroxyphenyl)-5-(3,5-dibromo-4-hydroxyphenyl)-1,4-pentadien-3-one **9** were prepared following the same procedure as described above using a 1:1 ratio of aldehyde/2-propanone (**13** and **14**) or aldehyde/phenylbut-3-en-2-one (**9**) (see Supporting Information).

Compounds **1**–**14** were tested against RmtA, an arginine methyltransferase from *Aspergillus nidulans* with significant sequence homology to human PRMT1 and specific for methylation at Arg3 of H4.[13] Histones H4 were used as substrate. The results have been reported as percent of inhibition at a fixed dose or $IC_{50}$ (50% inhibitory concentration) values (Table 1). According to our pharmacophore hypothesis, the bromo-containing 1,5-bis(4-hydroxyphenyl)-1,4-pentadien-3-ones **3**, **4**, **8**–**10** showed PRMT (RmtA) inhibiting activity. The $IC_{50}$ values ranged from 40 to 215 μM depending on the number and the position of the bromine atoms, the highest activity being associated to the 3,5,3′-tribromo (**9**, 40 μM) or 3,5,3′,5′-tetrabromo (**10**, 69 μM) substitution. Compounds lacking the hydroxy (**1**) or the bromine (**2**) substituents and those with a 4-methoxy group replacing the 4-hydroxy one (**7** and **12**) were unable to inhibit RmtA up to 90 μM. The insertion of a fluorine (**5**), nitro (**6**), or methyl (**11**) group at the C3-benzene position of the 1,5-bis(4-hydroxyphenyl)-1,4-pentadien-3-one skeleton was still tolerated for displaying RmtA inhibiting activity to some extent, though the corresponding $IC_{50}$ values were higher than those of **9** and **10**. Finally, the 4-(3-bromo- and 3,5-dibromo-4-hydroxyphenyl)but-3-en-2-ones **13** and **14**

**Table 1.** RmtA inhibitory activity of compounds **1**–**14**[a]

| cpd | R | $R^1$ | $R^2$ | $IC_{50}$ (μM) or % inhbtn |
|---|---|---|---|---|
| **1** | 3-Br | 3-Br | | 0 at 90 μM |
| **2** | 4-OH | 4-OH | | 0 at 90 μM |
| **3** | 2-Br-4-OH | 2-Br-4-OH | | 114 |
| **4** | 3-Br-4-OH | 3-Br-4-OH | | 162 |
| **5** | 3-F-4-OH | 3-F-4-OH | | 10 at 90 μM |
| **6** | 3-$NO_2$-4-OH | 3-$NO_2$-4-OH | | 249 |
| **7** | 3-Br-4-OMe | 3-Br-4-OMe | | 0 at 90 μM |
| **8** | 2,6-$Br_2$-4-OH | 2,6-$Br_2$-4-OH | | 215 |
| **9** | 3-Br-4-OH | 3,5-$Br_2$-4-OH | | 40 |
| **10** | 3,5-$Br_2$-4-OH | 3,5-$Br_2$-4-OH | | 69 |
| **11** | 3,5-$Me_2$-4-OH | 3,5-$Me_2$-4-OH | | 206 |
| **12** | 3,5-$Br_2$-4-OMe | 3,5-$Br_2$-4-OMe | | 0 at 90 μM |
| **13** | | | H | 0 at 90 μM |
| **14** | | | Br | 0 at 90 μM |
| AMI-5 | | | | 18 |

[a] Data represent mean values of at least three separate experiments.

showing only one pharmacophore moiety were totally inactive up to 90 μM.

To acquire information on their effect and selectivity on human PRMTs and HKMTs, compounds **1**–**12**, and **14** were tested at 100 μM against human recombinant PRMT1 using the *Saccharomyces cerevisiae* RNA-binding protein Npl3p as a substrate, against human recombinant CARM1/PRMT4 using poly(A)binding protein 1 (PABP1) as a substrate, and against the lysine methyltransferase SET7 with histones H3 as a substrate (Figure 3). As depicted in Figure 3, the 1,5-bis(3-bromo-4-hydroxyphenyl)-1,4-pentadien-3-one **4** was efficient and selective in inhibiting PRMTs, whereas it was less effective against the HKMT SET7. The introduction of further bromine atoms at the 5 or 5,5′ position (compound **9** or **10**) increased the inhibiting potency of the derivatives but abated the selectivity. By shifting the bromine from C3- to C2-benzene position a substantially inactive compound (**3**) was obtained. Nevertheless, the introduction of bromine atoms at both 2,6 and 2′,6′ positions gave a another molecule (**8**) which was highly active and selective against PRMTs. The 1,5-bis(3-bromophenyl)-1,4-pentadien-3-one **1** and the 1,5-bis(4-hydroxyphenyl)-1,4-pentadien-3-one **2** were ineffective in inhibiting all the three tested HMTs. The replacement of the 4-hydroxy group of **4** with the 4-methoxy one (compound **7**) gave similar results. Interestingly, the 1,5-bis(3,5-dibromo-4-methoxy) analogue **12** behaved as a very potent and selective CARM1 inhibitor (% inhibition: 0 (PRMT1), 83.9 (CARM1), 0 (SET7)). The replacement of 3-bromo with 3-fluoro or 3,5-dimethyl substituent (compounds **5** and **11**) furnished inactive derivatives, whereas the 1,5-bis(3-nitro-4-hydroxyphenyl)-1,4-pentadien-3-one **6** showed high activity and selectivity against CARM1. Finally, the monomer 4-(3,5-dibromo-4-hydroxyphenyl)but-3-en-2-one **14** failed to inhibit all the tested enzymes.

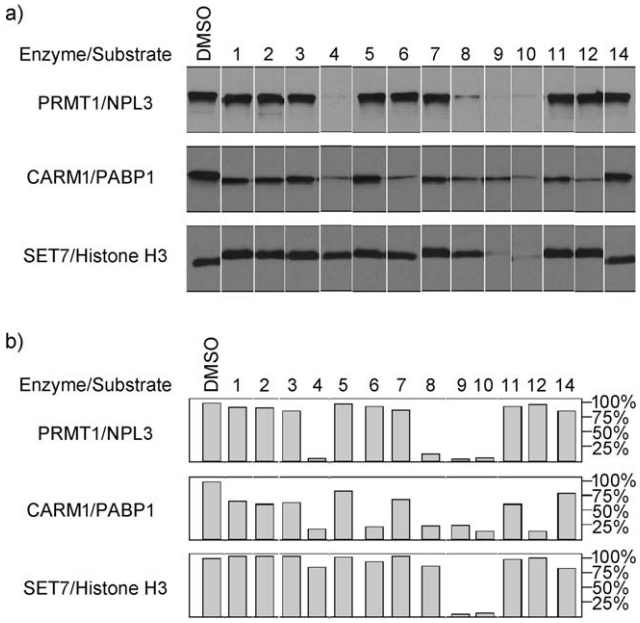



**Figure 3.** Inhibiting activities of compounds **1**–**12** and **14** (100 μM) on PRMTs (PRMT1 and CARM1) and HKMT (SET7).

The $IC_{50}$ values of selected compounds were determined against PRMT1, CARM1, and SET7 (Table 2). These data show that the 1,5-bis(3-bromo-4-hydroxyphenyl)-1,4-pentadien-3-one **4** showed high PRMT1 inhibiting activity (11.8 μM), and was moderately active against CARM1 and SET7. By adding one or two bromine atoms to its structure (compounds **9** and **10**), an increase in potency against PRMT1 and SET7 was observed. In particular, **9** showed a $IC_{50} = 3.0$ μM against PRMT1, fourfold more potent than **4** and threefold more potent than AMI-5, tested as reference drug. In contrast to what was observed in the anti-RmtA assay, the bis(2,6-dibromo) substituted compound **8** was highly potent against PRMT1 ($IC_{50} = 3.4$ μM), and showed tenfold decrease in activity against both CARM1 and SET7. Finally, compounds **6** and **12** were confirmed to be CARM1-selective inhibitors, as assessed by the fluorograph assay.

**Table 2.** $IC_{50}$ values of selected compounds against PRMT1, CARM1, and SET7.[a]

| cpd | R | $R^1$ | $IC_{50}$ [μM] PRMT1 | CARM1 | SET7 |
|---|---|---|---|---|---|
| **4** | 3-Br-4-OH | 3-Br-4-OH | 11.8 | 52.7 | 63.6 |
| **6** | 3-$NO_2$-4-OH | 3-$NO_2$-4-OH | 614.3 | 96.6 | 201.5 |
| **8** | 2,6-$Br_2$-4-OH | 2,6-$Br_2$-4-OH | 3.4 | 35.7 | 37.1 |
| **9** | 3-Br-4-OH | 3,5-$Br_2$-4-OH | 3.0 | 100.1 | 34.6 |
| **10** | 3,5-$Br_2$-4-OH | 3,5-$Br_2$-4-OH | 9.9 | 40.8 | 19.1 |
| **12** | 3,5-$Br_2$-4-OMe | 3,5-$Br_2$-4-OMe | 433.9 | 27.0 | 2738 |
| AMI-5 | | | 9.8 | 53.7 | ND[b] |

[a] Data represent mean values of at least three separate experiments. [b] ND, not determined.

To elucidate the binding possibilities of selected HMT inhibitors, compounds **4** (PRMT1-selective), **10** (non selective), and **12** (CARM1-selective) were docked (AutoDock program)[14] into the modeled structures of human PRMT1 and CARM1 (manuscript in preparation), and into the SET7 crystal structure.[15] The analysis of the AutoDock-proposed binding modes for **4**, **10**, and **12** revealed that the compounds are able to bind in the *S*-adenosylmethionine (AdoMet) or in the Arg/Lys substrate binding sites (Figure 4). The three compounds were docked in the AdoMet binding pocket (Figure 4a and 4b) of the two PRMTs (PRMT1 and CARM1), whereas for SET7, compounds **4** and **10** occupied both the lysine and AdoMet binding sites, and **12** only occupied the AdoMet pocket (Figure 4c). A deeper inspection of **4**, **10**, and **12** binding modes revealed different interaction profiles that are in agreement with the observed experiments (see Supporting information).

Selected compounds, **2**, **9**, **10**, and **12**, were subjected to Western blot analysis (at 50 μM for 24 h) to determine the H3K4 and the H3R17 methylation extents as markers of HKMT and PRMT inhibition in human leukaemia U937 cells, respectively.

As depicted in Figure 5, compounds **9** and **10** showed clear hypomethylation effects in both the assays with respect to the control, whereas **12** was highly effective only against the

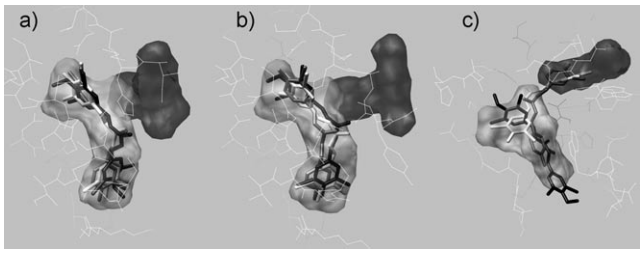



**Figure 4.** Compounds **4** (light grey), **10** (dark grey), and **12** (black) docked into a) PRMT1, b) CARM1, and c) SET7. The Arg/Lys binding sites is the darker area to the fight of each structure and the AdoMet binding site is the lighter grey shaded area. The enzymes residues within 5.0 Å from the substrates are reported in white wire. For the sake of clarity hydrogen atoms are not displayed.

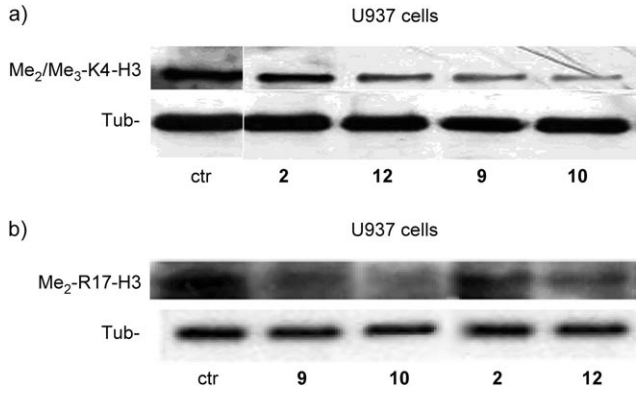



**Figure 5.** a) H3 K4 methylation assay by compounds **2**, **9**, **10**, and **12** on U937 cells (50 μM, 24 h). b) H3R17 methylation assay by the same compounds on U937 cells (50 μM, 24 h).

H3R17 methylation, and **2** was scarcely active or totally inactive in both assays.

Selected compounds **2**, **4**, **9**, **10**, and **12** were then tested on the U937 cell line to determine their effects on cell cycle, apoptosis induction, and granulocytic differentiation. After 24 h of treatment with the compounds at 5 μM, the cell cycle analysis did not show any alteration. The apoptosis, measured as Annexin V/propidium iodide (PI) double staining by FACS analysis, showed % values similar to the control (see Supporting Information). To determine granulocytic differentiation of the U937 cells, CD11c expression levels were detected after 24 h of stimulation with **2**, **4**, **9**, **10**, and **12** (all at 5 μM). As reported in Figure 6, compounds **2**, **4**, and **12** were ineffective in inducing granulocytic differentiation, whereas **9** and **10** showed a very strong effect, 68% and 92% of CD11c positive PI negative cells, respectively. This high cytodifferentiating effect could be explained, in part, by the HKMT inhibition, as **9** and **10** are the most active derivatives against SET7 among those tested.

Nevertheless, because of the extension (92% for **10** at 5 μM) and the rapidity (24 h) of the observed granulocytic differentiation, the involvement of alternative mechanism(s) cannot be ruled out.

In conclusion, a novel series of substituted 1,5-diphenyl-1,4-pentadien-3-ones as synthetic PRMT/HKMT inhibitors is reported herein. The bromo-hydroxy-containing compounds **3**, **4**, **9**, and **10** were the most potent in inhibiting RmtA, a PRMT from

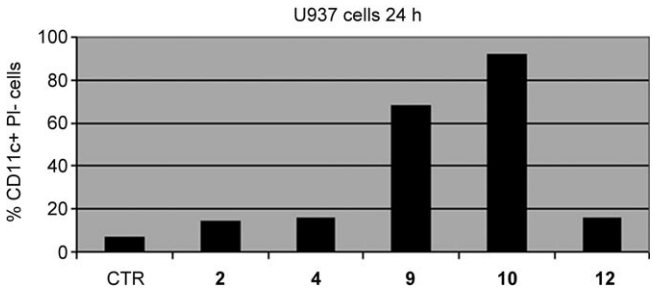



**Figure 6.** Granulocytic differentiation showed by compounds **2**, **4**, **9**, **10**, and **12** on U937 cells.

*A. nidulans* and the homologue of PRMT1. The removal of either hydroxy (**1**) or bromo (**2**) substituent from the pharmacophore moiety as well as the replacement of the hydroxy with a methoxy group (**7** and **12**) abated the inhibitory activity of the derivatives. The introduction of fluoro (**5**), nitro (**6**), or methyl (**11**) substituents instead of bromo afforded barely active compounds. Compounds bearing only one pharmacophore group (**13** and **14**) were totally inactive.

RmtA inhibitory data largely agree with those against human recombinant PRMT1, with few differences possibly due to the use of different substrates (histones H4 for RmtA, the nonhistone protein Npl3p for PRMT1). The 1,5-bis(2,6-dibromo-4-hydroxyphenyl)- (**8**) and the 1-(3-bromo-4-hydroxyphenyl)-5-(3,5-dibromo-4-hydroxyphenyl)- (**9**) 1,4-pentadien-3-ones were the most potent PRMT1 inhibitors ($IC_{50}$ values = 3.4 and 3.0 μM, respectively), being threefold more active than the reference AMI-5. The 1,5-bis(3-bromo) and -(3,5-dibromo) analogues **4** and **10** showed the same PRMT1 inhibitory activity ($IC_{50}$ values = 11.8 and 9.9 μM, respectively) as AMI-5, **4** being barely active against SET7. Finally, the 1,5-bis(3-nitro-4-hydroxy) derivative **6** and the 1,5-bis(3,5-dibromo-4-methoxy) derivative **12** were highly selective CARM1 inhibitors, as they are inactive against PRMT1 and SET7.

When tested on the human leukaemia U937 cell line, compounds **9** and **10** showed a high granulocytic differentiating effect (68% and 92% of CD11c positive PI negative cells, respectively) after 24 h of treatment at 5 μM, whereas **2**, **4**, and **12**, poorly active or totally inactive against SET7, were ineffective.

Further synthetic and biological studies are underway on the pharmacophore motif and the linker connecting the two phenyl rings, to improve both potency and selectivity of the derivatives. In addition, molecular biology efforts will be made to elucidate the mechanism(s) involved in the potent differentiating effect observed with **10**.

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**Keywords:** 1,5-diphenyl-1,4-pentadien-3-ones · bromophenol · chromatin remodeling · histone lysine methyltransferase (HKMT) · protein arginine methyltransferase (PRMT)

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