Epigenetic Multiple Ligands: Mixed Histone/Protein Methyltransferase, Acetyltransferase, and Class III Deacetylase (Sirtuin) Inhibitors

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A number of new compounds bearing two *ortho*-bromo- and *ortho*,*ortho*-dibromophenol moieties linked through a saturated/unsaturated, linear/(poly)cyclic spacer (compounds **1**–**9**) were prepared as simplified analogues of AMI-5 (eosin), a recently reported inhibitor of both protein arginine and histone lysine methyltransferases (PRMTs and HKMTs). Such compounds were tested against a panel of PRMTs (RmtA, PRMT1, and CARM1) and against human SET7 (a HKMT), using histone and nonhistone proteins as a substrate. They were also screened against HAT and SIRTs, because they are structurally related to some HAT and/or SIRT modulators. From the inhibitory data, some of tested compounds (**1b**, **1c**, **4b**, **4f**, **4j**, **4l**, **7b**, and **7f**) were able to inhibit PRMTs, HKMT, HAT, and SIRTs with similar potency, thus behaving as multiple ligands for these epigenetic targets (epi-MLs). When tested on the human leukemia U937 cell line, the epi-MLs induced high apoptosis levels [i.e., 40.7% (**4l**) and 42.6% (**7b**)] and/or massive, dose-dependent cytodifferentiation [i.e., 95.2% (**1c**) and 96.1% (**4j**)], whereas the single-target inhibitors eosin, curcumin, and sirtinol were ineffective or showed a weak effect.

Introduction

For many years, tumorigenesis has been believed to be the result of a multistep process involving genetic defects such as gene mutations and deletions or chromosomal abnormalities leading to either loss or gain of function of tumor suppressor genes or oncogenes, respectively. Such multistep process can involve an imbalance in the molecular signaling programs responsible for differentiation and proliferation. In addition, distinct gene expression programs are switched on or off during development, growth, and differentiation. Recently it has been demonstrated that epigenetic modifications play a key role in these processes.[1–3] Post-translational modification of core histones include serine/threonine phosphorylation, lysine/arginine methylation, lysine acetylation/deacetylation, ubiquitylation, and sumoylation. These covalent modifications crosstalk with each others, thus forming a complex network of signals that allows gene expression to be finely tuned to the requirements of the cell.[4–6]

Histone tails are modified by a wide group of chromatin-associated enzymes, including histone acetyltransferases (HATs) and the counteracting enzymes, histone deacetylases (HDACs), sirtuins (SIRTs,[a] class III HDACs), and by histone lysine methyltransferases (HKMTs), protein arginine methyltransferases (PRMTs), and histone demethylases (such as the lysine-specific demethylase LSD1 and the JmjC domain-containing demethylases).[4,5] In addition to histone substrates, such families of enzymes can also act on nonhistone proteins, such as transcription factors (i.e., GATA1, BCL6, STAT3, NF-κB, MyoD, YY1), tumor suppressors (p21, p53), cell cycle regulators (Rb, E2F), cytoskeletal proteins (α-tubulin), the chaperone heat shock protein 90 (Hsp90), and others.[7] All histone/nonhistone protein modifications, either directly or through the recruitment of regulatory protein complexes, can modulate a number of specific DNA-based processes such as transcription, DNA replication, DNA repair, cell cycle progression, and chromosome stability. Histone acetylation generally leads to activation of gene

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[a] Abbreviations: BCL6, B cells lymphoma 6; BL21, *Escherichia coli* B cells lack the Lon protease; CARM1, coactivator-associated arginine methyltransferase 1; CD11c, cluster differentiation 11c; E2F, gene E2 promoter specific factor; ELISA, enzyme linked immunosorbent assays; GATA1, erythroid transcription factor 1 or globin transcription factor 1; GST-RmtA, glutathione-*S*-transferase-fungal arginine methyltransferase A; HIV-1, human immunodeficiency virus 1; HSV1, herpes simplex virus 1; IgG1, immunoglobulin G1; IPTG, isopropyl-β-D-thiogalactopyranoside; JmjC, transcription factor jumonji domain-containing protein; LSD1, human histone lysine specific demethylase 1; Myo-D, myogenic differentiation factor; NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; NF-κB, nuclear factor κB; Npl3, nuclear shuttling protein; p300/CBP, CREB (cAMP-response element-binding protein) binding protein; PABP1, poly(A) binding protein 1; PBS, phosphate buffer saline; PBS-MLK, phosphate buffer saline-nonfat dry milk; PCAF, p300/CBP-associated factor; PVDF, polyvinylidene fluoride; Rb, retinoblastoma protein; RPMI medium, Roswell Park Memorial Institute medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SET7, three Drosophylia proteins that arbor the domain 7; su(var.), enhancer of zeste and trithorax; SIRT, silent mating type information regulation; STAT3, signal transducer and activator of transcription 3; TCA, trichloroacetic acid; YY1, ying yang transcription factor 1.

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expression, whereas deacetylation is related to gene silencing.[8] The role of histone methylation in regulating gene expression can be either positive or negative, depending on the context and the types of enzymes involved. According to the site and the extent of lysine methylation, this modification leads to either active or repressed chromatin. Thus, methylation of Lys9 and Lys27 in H3 and Lys20 in H4 are associated primarily with transcriptional silencing, whereas Lys4, Lys36, and Lys79 methylation of H3 correlate with transcriptional activation.[9,10]

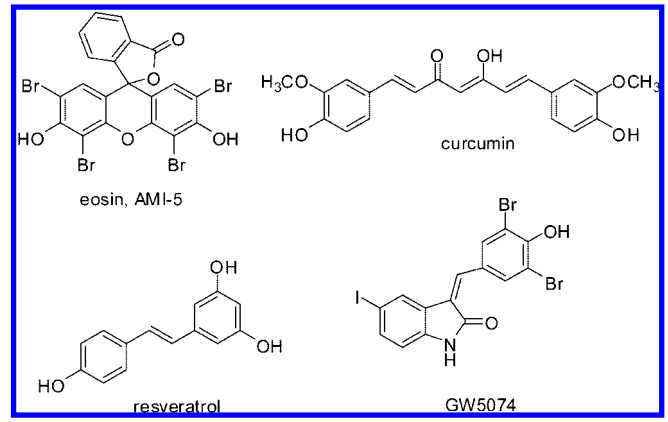
Differently from normal cells, cancer cells show aberrant epigenetic features (such as CpG island hypermethylation, global genomic hypomethylation, loss of acetylation at Lys16, and loss of trimethylation at Lys20 in H4) that play a key role in invasion, metastasis, chemotherapy resistance, and immune response of the tumor disease.[11,12] The cancer epigenome is currently being unravelled using the powerful approaches of ChIP-on-chip and ChIP-seq technologies.[13] Some epigenetic aberrations can be reverted by inhibition of histone-modifying enzymes (i.e., HDAC inhibitors (HDACi), HAT inhibitors (HATi), sirtuin inhibitors (SIRTi), methyltransferase inhibitors (HMTi)), restoring the normal epigenetic state of the cells.[4,14–16]

In cell-based studies, HDACi exhibited interesting antiproliferative, apoptotic, and/or cytodifferentiating properties. In preclinical studies, some of them have been found to have potent anticancer activities.[17–23] However, such studies have also highlighted the complexity of the molecular mechanism(s) involved in the antitumor action(s) of HDACi.[22,23] Actually, highly complex diseases, such as cancer and central nervous system disorders, easily involved a wide number of altered cellular pathways and signals, and many "*reductionist*" single-target chemotherapy approaches have proven to be largely fruitless.[24,25] Thus, the use of HDACi in combination with other anticancer agents (epi-drugs such as 5-aza-2′-deoxycytidine and retinoic acid, death-receptor–ligands, kinase inhibitors, regulators of proteasomal degradation, and conventional chemotherapeutic agents) seems to be a more promising application.[19,23] In particular, phase 1/2 studies of the combination of a DNA hypomethylating agent with a HDACi (and the eventual addition of all-*trans* retinoic acid) in patients with acute myeloid leukemia or high-risk myelodysplastic syndrome showed that this combination of epigenetic therapy was safe and active and was associated with transient reversal of aberrant epigenetic marks.[26–28]

An alternative to combination therapy is the development of a strategy based on "smart" drugs simultaneously able to modulate multiple targets (designed multiple ligands, DMLs).[25,29,30] This multitarget-directed drug design strategy has been successfully proposed for the treatment of neoplastic disorders[24,31–33] as well as neurodegenerative diseases.[34–38] The overall goal of the DML approach is to enhance the efficacy and/or improve the safety of the therapy, with respect to the drug combination. An advantage of the use of DMLs is the higher predictability of pharmacokinetic and pharmacodynamic parameters during therapy due to the administration of a single compound, as well as an improved patient compliance.

Since 1999, we have been engaged in design, synthesis, and biological evaluation of small molecule modulators of epigenetic targets. Up to now, we have described several series of HDACi,[39–54] a group of sirtinol analogues as SIRTi,[55] and some quinoline-based HATi,[56,57] evaluating their effects on cell cycle, proliferation, apoptosis, and cytodifferentiation on several leukemia cell lines. In an effort to discover chemical entities active against HMT enzymes, we undertook molecular modeling studies of a series of dyes and dye-like compounds (AMI-compounds) reported as regulators of PRMT activity.[58] Previously we had observed that eosin (reported as AMI-5, Chart 1) efficiently inhibited both Arg and Lys methyltranferases,[59] thus we designed some simplified eosin analogues bearing two pharmacophoric *ortho*-bromo- and *ortho*,*ortho*-dibromophenol moieties linked through a saturated/unsaturated, linear/(poly)-cyclic spacer (compounds **1**–**9**, Figure 1). We tested their activities against a panel of HMTs (PRMTs: fungal RmtA, human PRMT1, human CARM1; HKMTs: SET7). Western blot analyses were performed on histone treated with selected derivatives to determine the H3K4 and the H4R3/H3R17 methylation extents as markers of HKMT and PRMT inhibition, respectively, in the human leukemia U937 cell line. Preliminary results on the 1,5-diphenyl-1,4-pentadien-3-ones **1**[60] highlighted the key role of bromo and hydroxy substituents at the phenyl rings to obtain low-micromolar inhibiting activity, with the number and the position of the bromine atoms that discriminated for PRMT1 versus CARM1 or PRMT1 versus SET7 selectivity. In this article, we describe in detail the synthesis and biological evaluation of compounds **1**, and we report the synthesis and anti-HMT activities of the novel series of compounds **2**–**9**.

Chart 1. Small-Molecule Modulators of Epigenetic Targets



The chemical strategy used for designing compounds **1**–**9** led to a merger, into the structures of **1**–**9**, of some chemical features that are common to curcumin (Chart 1), a component of turmeric (*Curcuma longa*), which has been recently reported as HAT inhibitor,[61,62] and to resveratrol (Chart 1), a polyphenolic phytoalexin able to activate SIRT1.[63–65] Moreover, the *ortho*,*ortho*-dibromophenol moiety is shown by the indolinone GW5074 (Chart 1), recently reported to be endowed with SIRT2 inhibiting activity.[66] Thus, we tested selected derivatives **1**–**9** in human p300/CBP HAT assay and in human SIRT1 and SIRT2 assays to determine if such bromo- and dibromophenol-containing compounds could be multiple ligands for epigenetic targets (epi-MLs). In addition, the effects of selected compounds on cell cycle, apoptosis induction, and granulocytic differentiation on U937 cells were evaluated.

Chemistry

The eosin analogues **1**, **2**, and **4**–**7** were prepared by condensation of the appropriate (methoxymethoxy)- or hydroxybenzaldehydes with the suitable ketones or diketones in alkaline or acidic medium, followed by eventual hydrolysis of the methoxymethyl protection. Reaction between 3,5-dibromo-4-(methoxymethoxy)benzoic acid and the opportune amines followed by cleavage of the protecting ether furnished the amides **8**, while the anilides **9** were obtained by reaction of the reported dicarboxylic acyl chlorides with 3,5-dibromo-4-hydroxyaniline.

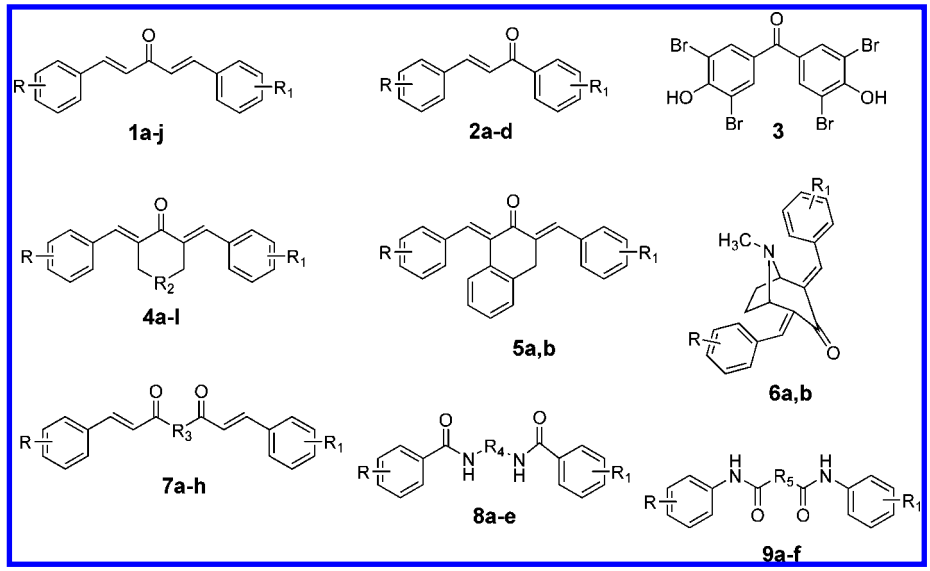


Figure 1. Novel simplified AMI-5 analogues.

The chemistry section is reported in detail as Supporting Information (see Schemes S1–S6, Tables S1–S3, and Experimental Procedures).

Results and Discussion

Part 1. HMT Assays. As we started our work by designing the compounds **1**–**9** as simplified analogues of AMI-5 (eosin), a recently reported inhibitor of both Lys and Arg methyltransferases,[59] we first investigated the activities of our compounds against such enzymes, using histone as well as nonhistone proteins as a substrate. Therefore, we tested compounds **1**–**9** against RmtA, a fungal PRMT acting on histone H4 substrate that was shown to be a useful, predictive model for studying PRMT inhibition in mammals.[58] Selected compounds were tested (50 μM) against human recombinant PRMT1 in vitro using histone H4 as a substrate to confirm their inhibitory activity. Afterward, we screened compounds **1**–**9** (100 μM) against two human PRMTs (PRMT1 and CARM1) by using nonhistone proteins as a substrate [the RNA-binding nuclear shuttling protein (Npl3) and the poly(A)binding protein 1 (PABP1), respectively], to observe the influence of substrates different from histones on the inhibiting activity. In addition, we tested our compounds against the HKMT SET7 using histone H3 as a substrate to assess their capability to also inhibit this Lys methyltransferase. Finally, to study the in vivo efficacy of our compounds to inhibit Lys and Arg methylation reactions, we performed Western blot analyses on human leukemia U937 cells using monomethyl-H3K4, monomethyl-H4R3, and dimethyl-H3R17 antibodies, and the methylation extent on such residues in U937 cells after treatment with selected compounds **1**–**9** (50 μM, 24 h) was determined.

A. Inhibitory Activity against RmtA. Compounds **1**–**9** were tested against *Aspergillus nidulans* RmtA, a fungal PRMT with significant sequence similarity to human PRMT1 and specific for methylation at Arg3 of histone H4.[67] The percent values of inhibition at a fixed dose (nearly 100 μM) were first determined (data not shown), and then the IC_{50} values for the active compounds were established (Table 1).

Structure–activity relationship on the effect of compounds **1** on the fungal RmtA has been recently reported by us as a Communication.[60] Briefly, the IC_{50} data (Table 1) highlighted the role of *ortho*-bromo- and *ortho*,*ortho*-dibromophenol moieties in inhibiting the enzyme, with **1b** and **1c** being the most potent compounds (IC_{50}s: 69 and 40 μM, respectively). It is noteworthy that the 3-carboxy-4-hydroxy substitution at the 1,5-diphenyl-1,4-pentandien-3-one scaffold furnished also a highly active compound (the new compound **1j**, IC_{50} = 55 μM). In the chalcone series **2**, the tribromo- and tetrabromo-containing compounds **2c** and **2d** showed the highest inhibitory activity (IC_{50}s: 40 and 29 μM, respectively), they being as potent as (**2c**) or 2.4-fold more potent than (**2d**) the corresponding 1,5-diphenyl-1,4-pentadien-3-ones **1c** and **1b**. On the other hand, the compound **3** was totally inactive against RmtA. Among the compounds **4** bearing a cyclic ketone as a linker between the two bromo-hydroxy- or dibromo-hydroxyphenyl moieties, the observed trend of activity was fully respected: the bis(dibromo-hydroxyphenyl) derivatives **4b**,**f**,**h**,**j**,**l** were always more potent than the corresponding bis(bromo-hydroxyphenyl) counterparts **4a**,**d**,**g**,**i**,**k** against the fungal RmtA. The highest activity was recorded with the introduction of cyclohexanone as a spacer (compound **4b**, IC_{50} = 19 μM, showing the same activity as AMI-5 used as reference drug). The insertion of heteroatom-containing cyclic ketones (i.e., *N*-methyl-4-piperidone, tetrahydro-4*H*-pyran-4-one, and tetrahydro-4*H*-thiopyran-4-one) led to a slight decrease of the potency (see compounds **4f**, IC_{50} = 39 μM; **4j**, IC_{50} = 29 μM; and **4l**, IC_{50} = 45 μM), whereas with the *N*-benzyl-4-piperidone, a barely active compound (**4h**, IC_{50} = 210 μM) was obtained. Compounds carrying bicyclic rings such as β-tetralone (**5a**,**b**) and 8-methyl-8-azabicyclo[3.2.1]octan-3-one (**6a**,**b**) as a connection between the two pharmacophore moieties showed up to 2-fold decrease of the RmtA inhibitory activity in comparison with the cyclohexanone-containing **4a**,**b** and the *N*-methyl-4-piperidone derivatives **4d**,**f**, respectively. The curcuminoids characterized by a benzene insertion at the R_3 position (compounds **7a**−**d**) displayed high potencies against RmtA, with the 1,1′-(1,3-phenylene)bis(3-(3,5-dibromo-4-hydroxyphenyl)prop-2-en-1-one) **7b** being the most effective compound (IC_{50} = 10 μM, 2-fold more active than AMI-5). The curcumin analogues **7e**,**f** were 10- to 5-times less active than **7b**, whereas the 4,4-dimethyl derivatives **7g**,**h** showed no or slight RmtA inhibition. In the bis(benzamide) series **8**, the IC_{50} data reported in Table 1 showed that compounds with the ethyl and *n*-propyl (**8a**,**b**) but not *n*-butyl (**8c**) chains connecting the two amide functions were tolerated for enzyme inhibition, and that the 1,3-substituted benzene spacer was 2-fold more efficient than the related 1,4-substituted ring. About the bis(a-

Table 1. RmtA Inhibitory Activity of Compounds **1–9**[a]

cmpd	R	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM) or % inhbtn
1a	3-Br-4-OH	3-Br-4-OH					162
1b	3-Br-4-OH	3,5-Br_2-4-OH					40
1c	3,5-Br_2-4-OH	3,5-Br_2-4-OH					69
1d	2-Br-4-OH	2-Br-4-OH					114
1e	2,6-Br_2-4-OH	2,6-Br_2-4-OH					215
1f	2,4-Br_2-6-OH	2,4-Br_2-6-OH					238
1g	3,5-Me_2-4-OH	3,5-Me_2-4-OH					206
1h	3-F-4-OH	3-F-4-OH					169.4
1i	3-NO_2-4-OH	3-NO_2-4-OH					249
1j	3-COOH-4-OH	3-COOH-4-OH					55
2a	3-Br-4-OH	H					190
2b	3,5-Br_2-4-OH	H					115
2c	3-Br-4-OH	3,5-Br_2-4-OH					40
2d	3,5-Br_2-4-OH	3,5-Br_2-4-OH					29
3							0 at 90 μM
4a	3-Br-4-OH	3-Br-4-OH	CH_2				90
4b	3,5-Br_2-4-OH	3,5-Br_2-4-OH	CH_2				14
4c	3-Br-4-OH	3-Br-4-OH	CH_2CH_2				0 at 89.4 μM
4d	3-Br-4-OH	3-Br-4-OH	NCH_3				161.6
4e	3-Br-4-OH	3,5-Br_2-4-OH	NCH_3				123.1
4f	3,5-Br_2-4-OH	3,5-Br_2-4-OH	NCH_3				39
4g	3-Br-4-OH	3-Br-4-OH	NCH_2Ph				0 at 77.0 μM
4h	3,5-Br_2-4-OH	3,5-Br_2-4-OH	NCH_2Ph				210
4i	3-Br-4-OH	3-Br-4-OH	O				132
4j	3,5-Br_2-4-OH	3,5-Br_2-4-OH	O				29
4k	3-Br-4-OH	3-Br-4-OH	S				0 at 88.5 μM
4l	3,5-Br_2-4-OH	3,5-Br_2-4-OH	S				45
5a	3-Br-4-OH	3-Br-4-OH					92
5b	3,5-Br_2-4-OH	3,5-Br_2-4-OH					37
6a	3-Br-4-OH	3-Br-4-OH					0 at 84.6 μM
6b	3,5-Br_2-4-OH	3,5-Br_2-4-OH					84
7a	3-Br-4-OH	3-Br-4-OH		1,3-Ph			59
7b	3,5-Br_2-4-OH	3,5-Br_2-4-OH		1,3-Ph			10
7c	3-Br-4-OH	3-Br-4-OH		1,4-Ph			47
7d	3,5-Br_2-4-OH	3,5-Br_2-4-OH		1,4-Ph			37
7e	3-Br-4-OH	3-Br-4-OH		CH_2			109
7f	3,5-Br_2-4-OH	3,5-Br_2-4-OH		CH_2			48
7g	3-Br-4-OH	3-Br-4-OH		$C(CH_3)_2$			0 at 91.8 μM
7h	3,5-Br_2-4-OH	3,5-Br_2-4-OH		$C(CH_3)_2$			156.4
8a	3,5-Br_2-4-OH	3,5-Br_2-4-OH			$(CH_2)_2$		89.7
8b	3,5-Br_2-4-OH	3,5-Br_2-4-OH			$(CH_2)_3$		75.4
8c	3,5-Br_2-4-OH	3,5-Br_2-4-OH			$(CH_2)_4$		616
8d	3,5-Br_2-4-OH	3,5-Br_2-4-OH			1,3-Ph		61.4
8e	3,5-Br_2-4-OH	3,5-Br_2-4-OH			1,4-Ph		122.3
9a	3,5-Br_2-4-OH	3,5-Br_2-4-OH				none	32.6
9b	3,5-Br_2-4-OH	3,5-Br_2-4-OH				CH_2	101.7
9c	3,5-Br_2-4-OH	3,5-Br_2-4-OH				$(CH_2)_2$	156.1
9d	3,5-Br_2-4-OH	3,5-Br_2-4-OH				$(CH_2)_3$	172.6
9e	3,5-Br_2-4-OH	3,5-Br_2-4-OH				1,3-Ph	13.8
9f	3,5-Br_2-4-OH	3,5-Br_2-4-OH				1,4-Ph	0 at 70.4 μM
AMI-5							18

[a] Values are means determined for at least two separate experiments.

nilides) **9**, the oxalyl derivative **9a** was the most potent compound among those with aliphatic spacers (**9a**−**d**), and the increasing insertion of methylene units (compounds **9b**−**d**) led to a constant decrease of the inhibiting activity. In the phthalic anilide series (compounds **9e**,**f**), the benzene 1,3-substitution assured the highest RmtA inhibiting activity (see compound **9e**, IC_{50} = 13.8 μM).

B. Human PRMT1/H4 Assay. Selected compounds were tested at 50 μM against human recombinant PRMT1, using histone H4 as a substrate (Figure S1 in Supporting Information). Data depicted in Figure S1 confirmed that the PRMT1 inhibitory potency of the derivatives depends on the extension of bromination at the phenyl rings (the more bromine atoms the more potent the compound, see **1a**−**c** and **2c**,**d**), and the feasibility of the chalcone scaffold as an alternative to the 1,4-diphenyl-1,5-pentadien-3-one for the design of new PRMTi. Among compounds **4** bearing a cyclic ketone as a spacer connecting the two (di)bromo-hydroxyphenyl moieties, the tetrahydro-4*H*-pyran-4-one derivative **4j** showed the highest inhibitory activity (91.1% of inhibition), followed by the thiopyranone and the *N*-methyl-4-piperidone analogues **4f** and **4l** (72.8 and 72.7% of inhibition). The cyclohexanone-containing **4b**, which was the most effective in inhibiting the fungal RmtA, inhibited the human PRMT1 activity of 44.2% at 50 μM. The 1,3-disubstituted-benzene curcuminoid **7b** was 2-fold more efficient than the 1,4-disubstituted counterpart **7d** in inhibiting PRMT1, and the bromo-analogue of curcumin **7f** showed 70.2% of inhibition in this assay. It is noteworthy that curcumin has been reported inactive against G9a and other HMTs,[61] and in our hand it displayed millimolar inhibiting activity against both RmtA and PRMT1.[58] Among the bis(benzamide) and bis(anilide) series **8** and **9**, compound **8d** was highly potent in inhibiting PRMT1 (86.1% of inhibition), whereas the corresponding bis(anilide) **9e** displayed a drop of activity.

C. PRMT1/Npl3p, CARM1/PABP1, and SET7/H3 Inhibitory Activity. Compounds **1**, **2**, and **4**–**9** were screened

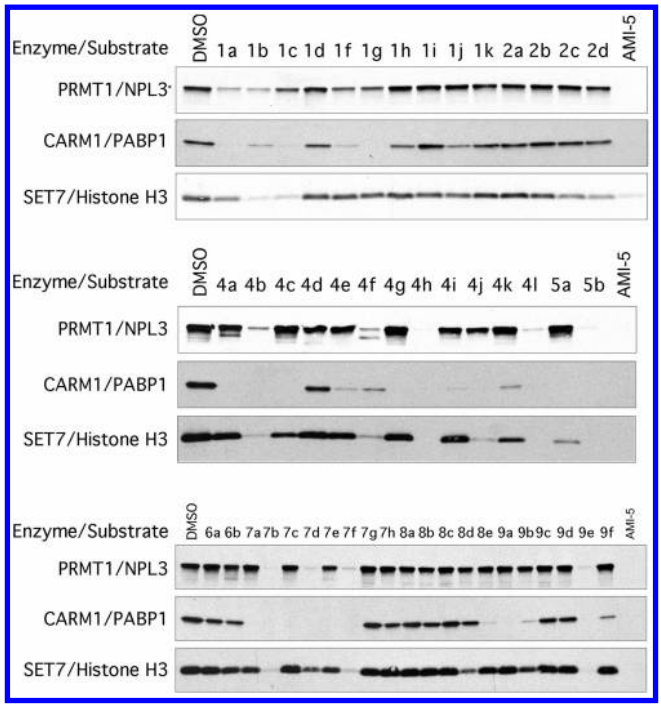


Figure 2. Inhibitory activities of compounds **1**, **2**, **4**–**9** (100 μM) against PRMT1 using Npl3p as a nonhistone substrate, CARM1 using PABP1 as a nonhistone substrate, and SET7 using histone H3 as a substrate.

at 100 μM (fluorograph analysis) against human PRMT1, using as a substrate the heterogeneous nuclear ribonucleoprotein (hnRNP) Npl3p, an in vivo substrate of HMT1 from *Saccharomyces cerevisiae*,[68] against human CARM1 with the poly-(A)binding protein 1 (PABP1) as a substrate[69] and against the HKMT SET7 using histone H3 as a substrate (Figure 2). As a rule, the tetrabromo-substituted compounds endowed with inhibitory activity displayed high potency against all three tested enzymes. Exceptions are **4j**, which showed low activity against PRMT1, **7d**, which was less active against SET7, and **8e**, **9a**, and to a lesser extent **9b** and **9f**, which exhibited a degree of selectivity toward CARM1. In the bis(phenyl)pentadienone series, the dibromo compound **1a** was particularly active against CARM1 and, to a lesser extent, PRMT1, and the addition of a third bromine atom increased the potency of the compound (**1b**) against SET7. The 1,4-bis(2,6-dibromo-4-hydroxyphenyl) and the 1,4-bis(2,4-dibromo-6-hydroxyphenyl) analogues **1e** and **1f** showed inhibitory activities against the PRMTs but not against SET7, while the dinitro-derivative **1i** was to some extent CARM1-selective. Compounds belonging to the chalcone series **2** were inactive in this assay, with **2c**,**d** showing low inhibition against only SET7. In the **4**, **5**, and **7** series, the termini bearing two bromine atoms in their structures (**4a**, **4c**, **4g**, **4i**, **4k**, **5a**, **7a**, **7c**, and **7e**) generally displayed low or no activity against PRMT1 and SET7, whereas they were able to inhibit CARM1. When the spacer is represented by the *N*-methylpiperidone, the dibromo-derivative **4d** failed in inhibiting the tested enzymes, and a third bromine atom (**4e**) is required to furnish a CARM1-inhibitory activity. The 8-methyl-8-azabicyclo[3.2.1]octan-3-one derivatives **6a**,**b**, as well as the 4,4-dimethylhepta-1,6-diene-3,5-diones **7g**,**h**, were inactive in this fluorograph assay. Among the bis(benzamides) **8**, while those with alkyldiamino spacers (**8a**−**c**) showed no inhibiting activity, the 1,3-phenylenediamino derivative **8d** displayed a slight effect against SET7, and the 1,4-phenylenediamino counterpart **8e** displayed high CARM1 inhibition. In the bis(anilide) series **9**, the oxalyl (**9a**), malonyl

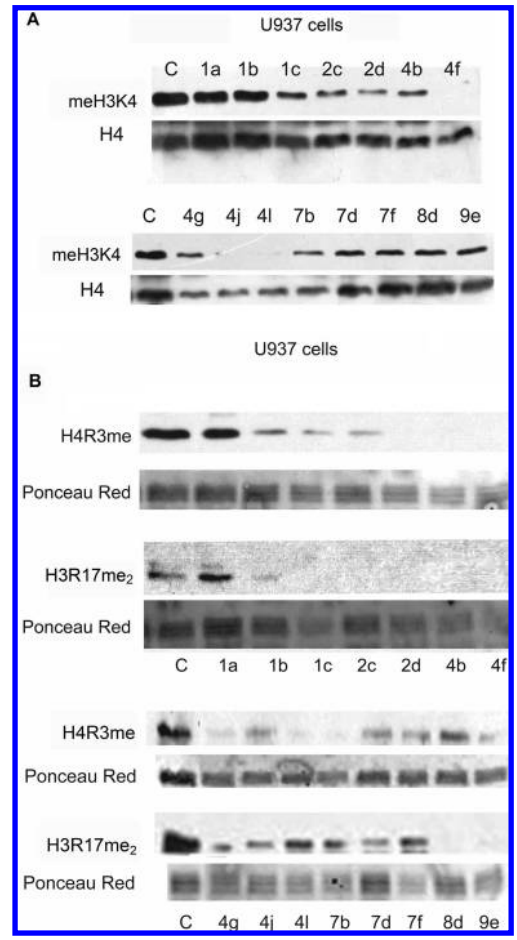


Figure 3. Western blot analyses performed with selected compounds **1**–**9** (at 50 μM for 24 h) on H3K4 (A) and H4R3/H3R17 (B) methylation. The strong signal corresponds to methylation of the appropriate lysine (A) or arginine (B), whereas a weaker signal shows a decrease of the methylation in comparison with the control. As control for equal loading total histone H4 (A) or the Ponceau Red staining of histones (B) have been used, respectively.

(**9b**), and to a lesser extent 1,4-benzenedicarboxylyl (**9f**) derivatives were selectively active against CARM1, whereas the 1,3-benzenedicarboxylyl analogue **9e** highly inhibited all three tested enzymes.

D. Western Blot Analyses. Selected compounds **1**–**9** were subjected to Western blot analyses on human leukemia U937 cells (at 50 μM for 24 h), to study their effects on H3K4 and H4R3/H3R17 methylation. Data depicted in Figure 3 show that tested compounds from **1c** to **7b** were efficient in inhibiting H3K4 methylation in U937 cells, with the bis(benzylidene)heterocycloalkanones **4f**, **4j**, and **4l** being the most potent. Compounds **7d**, **7f**, **8d**, and **9e** gave a less evident histone hypomethylation, whereas **1a** and **1b** were inactive in the H3K4 assay. In the Arg methylation assays (part B of Figure 3), all the tested compounds with the exception of **1a** clearly inhibited H4R3 and H3R17 methylation, showing somehow a different extent of inhibition according to their chemical structure and/or the specific antibody (anti-H4R3 or anti-H3R17) used in the Western blots. Thus, the pentadienones **1b**,**c**, the chalcones **2c**,**d**, and the bis(benzylidene)-cyclohexanone and -*N*-methyl-4-piperidone **4b**,**f** gave almost the same degree of inhibition against both the Arg residues, whereas the *N*-benzyl-4-piperidone **4g**, the thiopyranone **4l**, and the 1,1′-(1,3-phenylene)bis(prop-2-en-1-one) **7b** displayed the highest activity against the H4R3 methylation. The amides **8d** and **9e** selectively inhibited the methylation of H3R17.

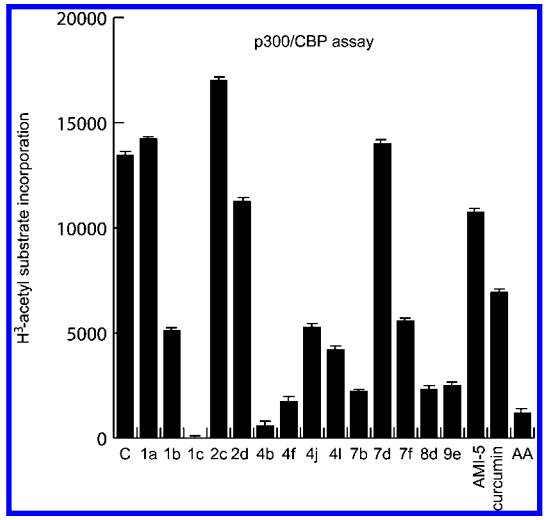


Figure 4. p300/CBP HAT assay performed on selected compounds **1–9** (at 50 μM). An indirect ELISA assay has been performed for the detection of acetyl residues on histone H3 substrate using 10 μg of U937 cell nuclear extract per assay as a source of p300/CBP enzymes. The incubations with DMSO alone (control) or with selected compounds **1–9**, AMI-5, curcumin, and anacardic acid (AA; all at 50 μM) have been carried out for 90 min. Data have been expressed as ^{3}H-acetyl substrate incorporation activity.

Part 2. p300/CBP (HAT) Assay. Compounds bearing a bis(phenyl)pentadienone, chalcone, bis(benzylidene)(hetero)cycloalkanone, or bis(phenyl)eptandione structure (curcuminoids) are strictly related to the natural spice curcumin and have been widely investigated as antitumor (both in vitro and in vivo),[70–74] antiangiogenic (vascular endothelial cell proliferation, capillary tube formation, and growth inhibitors),[75,76] and chemoprotective (phase 2 enzyme inducers and radical scavengers) agents.[77] In addition, curcuminoids have been reported to inhibit HIV-1 integrase[78] and to block HSV-1 infection.[79] Curcumin has been recently identified as a p300/CBP HAT specific inhibitor, also able to repress the p300-mediated acetylation of p53 in vivo, and of HIV-Tat protein in vitro, thus inhibiting the HIV proliferation.[61]

As our bis(dibromophenol)-containing compounds resembled some chemical features of curcumin and curcuminoids, we tested some representative samples of **1–9** at 50 μM against p300/CBP immunoprecipitate (IP) in U937 cells to determine their potential anti-HAT effect. Curcumin and anacardic acid (AA),[80] a well-known p300/CBP and PCAF inhibitor, were used as reference drugs. As a result (Figure 4), the fully brominated bis(phenyl)pentadienone **1c** showed the highest HAT inhibitory activity (100.0% of inhibition), whereas the dibromo analogue **1a** was ineffective. The 1-(3-bromo-4-hydroxyphenyl)-5-(3,5-dibromo-4-hydroxyphenyl)penta-1,4-dien-3-one **1b** had an intermediate behavior (61.9% of inhibition). Chalcone derivatives **2c**,**d** were either slightly or not active in this assay, whereas all the bis(benzylidene)(hetero)cycloalkanone compounds **4** exhibited 61.0% (**4j**) to 95.6% (**4b**) of inhibition of the p300/CBP IP. Among the curcumin analogues **7b**,**d**,**f**, the compounds **7b** and **7f** inhibited the p300/CBP activity more efficiently than curcumin, whereas **7d** was ineffective. Finally, the bis(dibromo)benzamide **8d** and the bis(dibromo)anilide **9e** were highly active in this HAT inhibitory assay, whereas AMI-5 showed only a weak inhibition.[81]

Part 3. SIRT1 and SIRT2 Assays. Because the chemical scaffolds used for compounds **1–9** resembled some characteristic features showed by the plant phytoalexin resveratrol, a known SIRT1 activator,[63–65] and by the indolinone GW5074, which

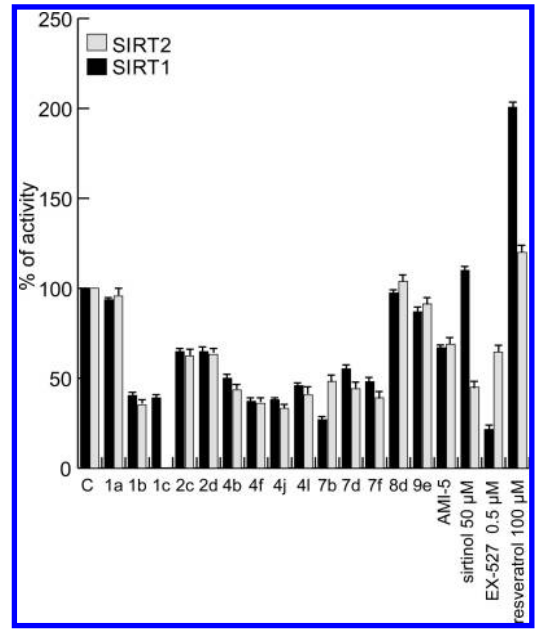


Figure 5. SIRT1 and SIRT2 assays performed on selected compounds **1–9** (at 25 μM). First (deacetylation phase), the SIRT1 or SIRT2 enzymes were incubated with the substrate Fluor de Lys-SIRT1 or -SIRT2 in the presence of NAD^+ and various concentrations of tested compounds (sirtuins activators or inhibitors). The second stage is initiated by the addition of the Developer II, including nicotinamide (NAM), a sirtuin inhibitor that stops the SIRT1/2 activity, and the fluorescent signal is produced. Data are expressed as % of activity.

exhibited SIRT2 inhibiting activity,[66] selected compounds **1–9** plus AMI-5 were tested at 25 μM against SIRT1 and SIRT2. EX-527 (0.5 μM),[82] a recently reported SIRT1-selective inhibitor active at submicromolar level, and sirtinol (50 μM),[55] more efficient in inhibiting SIRT2 than SIRT1, were used as a control for SIRT1 and SIRT2 inhibition, respectively, and resveratrol (100 μM) was added as a control for SIRT1 activation. Data depicted in Figure 5 clearly shows that in the bis(phenyl)pentadienone series, the compound **1a**, carrying two bromine atoms at the phenyl rings, was totally inactive in inhibiting SIRT1 and SIRT2. The further introduction of one bromine atom (compound **1b**) elicited a fine inhibitory activity against both of the enzymes (60% and 65% of inhibition against SIRT1 and SIRT2 at 25 μM), and the compound with four bromine atoms at the phenyl rings (**1c**) showed 61% inhibition against SIRT1 and totally inhibited the activity of SIRT2 at the tested concentration. Compounds belonging to the cyclohexanone- and cyclohexanone-like-containing series (**4**), as well as the curcuminoids **7**, showed in general >50% of inhibition against both SIRT1 and SIRT2 at 25 μM, the most potent being the 1,1′-(1,3-phenylene)bis(3-(3,5-dibromo-4-hydroxyphenyl)prop-2-en-1-one) **7b** against SIRT1 (73% of inhibition). AMI-5 and the chalcones **2** displayed 30% of inhibition against the two enzymes, whereas the bis(benzamide) **8d** and its bis(anilide) isomer **9e** were totally ineffective in these assays.

Part 4. In-Cell Evaluation. Effects on Cell Cycle, Apoptosis Induction, and Granulocytic Differentiation on Human Leukemia U937 Cell Line. From the data reported here on the activities of selected compounds **1–9** against HMTs (Part 1), HAT (Part 2), and SIRT (Part 3), it seems feasible that some of them could act as multiple ligands by inhibiting at the same time and with similar potencies several epigenetic targets involved in regulation of gene expression and transcription (epigenetic multiple ligands, epi-MLs). To date, the most studied application of epi-drugs is the treatment of cancer

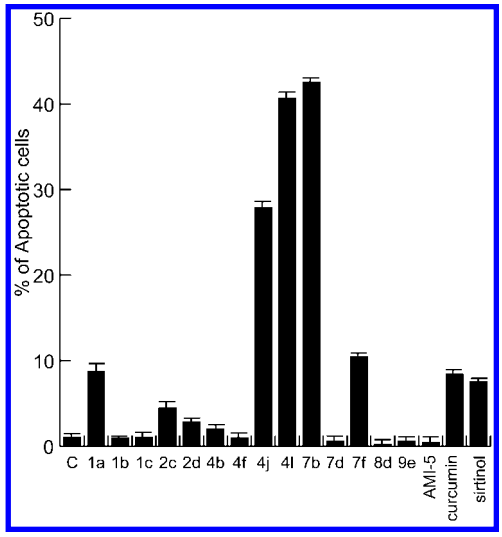


Figure 6. Apoptosis induction exerted by selected compounds **1**–**9** (at 25 μM) in human leukaemia U937 cell line. Apoptosis measured as caspase 3 activity has been taken as readout of the action of selected compounds.

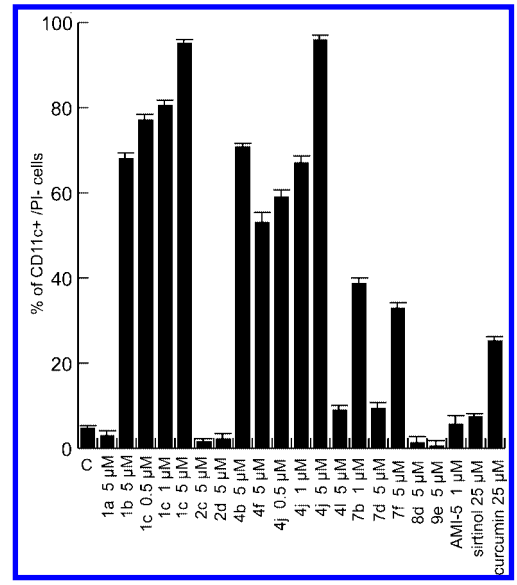


Figure 7. Granulocytic differentiation showed by selected **1**–**9** on human leukemia U937 cells. Granulocytic differentiation is represented by the CD11c positive cells. PI positive cells, which represent dead cells, have been excluded from the analysis. The concentrations are indicated.

diseases. We thus tested our selected compounds **1**–**9** on human leukemia U937 cells by determining their effects on cell cycle progression, apoptosis induction, and granulocytic differentiation.

For a comparison purpose, the HMTi AMI-5, the HATi curcumin, and the SIRTi sirtinol were added to the assays. All the compounds were tested at 25 μM to study their effects on cell cycle (the analysis was determined after 30 h of treatment) and apoptosis induction (measured as caspase 3 activation by FACS analysis and checked after 30 h of treatment). To evaluate granulocytic differentiation on U937 cells, the CD11c expression levels upon 30 h of stimulation were determined. In this assay, the highest testable dose for the selected compounds **1**–**9** and AMI-5 was 5 μM or lower because their deeply colored solutions interfered with the assay at higher concentrations. In the same assay, the reference drugs curcumin and sirtinol were tested at 25 μM, as for cell cycle and apoptosis studies.

Figure S2 in Supporting Information shows the effect of the selected compounds (tested at 25 μM) on cell cycle phases in the U937 cells. Compounds **1b**, **2c**, **4f**, **4l**, and **7b** showed a high increase of percent of G2 phase cells, in two cases (**4f** and **7b**) with a total absence of cells in S phase, whereas compounds **4b** and **7f** as well as sirtinol displayed a total lack of cells in G2 phase.

The tetrahydro-4*H*-pyran-4-one **4j** showed a high level of apoptosis (28%) when tested in the U937 cell line to determine the apoptosis induction (Figure 6) at 25 μM for 30 h of treatment. More importantly, its thio-analogue **4l** and the 1,1′-(1,3-phenylene)bis(3-(3,5-dibromo-4-hydroxyphenyl)prop-2-en-1-one) **7b** were highly more effective (**4l**, 40.7%; **7b**, 42.6% of apoptosis). A 10.5% of apoptosis was detected after treatment of U937 cells with **7f**. The pentadienone **1a**, as well as curcumin and sirtinol, showed <10% of apoptosis, and AMI-5 was totally inactive in this assay.

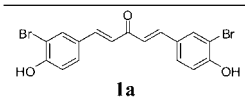
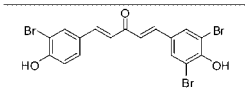
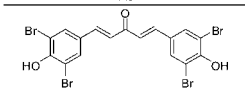
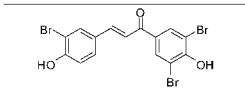
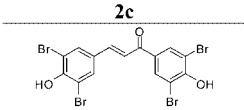
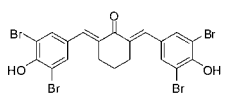
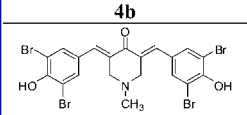
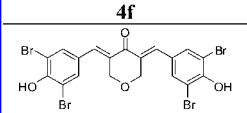
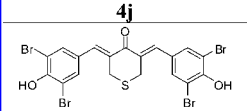
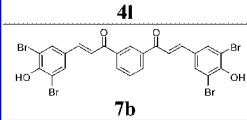
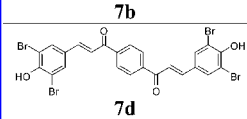
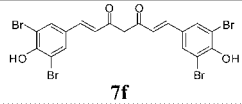
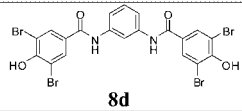
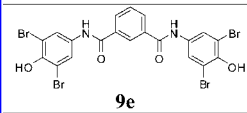
Granulocytic differentiation has been evaluated in human leukemia U937 cells by determining the percent values of CD11c positive/propidium iodide (PI) negative cells after 30 h of treatment with selected compounds **1**–**9** at 5 μM (**1a**−**c**, **2c**,**d**, **4b**,**f**,**j**,**l**, **7d**,**f**, **8d**, and **9e**), or 1 μM (**7b** and AMI-5) (Figure 7). Higher doses of the chosen compounds gave interference with the reading of the results because of the deeply colored solutions obtained. Curcumin and sirtinol were tested at the same concentration (25 μM) used in cell cycle and apoptosis assays. In these conditions, the 1,5-bis(3,5-dibromo-4-hydroxyphenyl)-penta-1,4-dien-3-one **1c** and the 3,5-bis(3,5-dibromo-4-hydroxybenzylidene)dihydro-2*H*-pyran-4(3*H*)-one **4j** displayed a massive, dose-dependent differentiating effect, with nearly 100% of CD11c positive/PI negative cells. When tested at 0.5 and 1 μM, **1c** and **4j** retained high values of cell differentiation activity [77–81% (**1c**); 59–67% (**4j**)]. At the tested doses (5 μM), also the **1c**-related compound **1b** and the **4j**-analogues **4b** and **4f** showed high cell differentiation (68, 71, and 53% CD11c positive/PI negative cells, respectively), whereas the thiopyran-4(3*H*)-one **4l** was ineffective. Among the tested curcuminoids **7b**,**d**,**f** the compounds **7b** and **7f** displayed a granulocytic differentiation effect (39 and 33% CD11c positive/PI negative cells, respectively), whereas **7d** failed to yield such effect. The bis(benzamide) **8d** and the bis(anilide) **9e** were also inactive in this assay. Among the reference compounds, only curcumin (at 25 μM) showed a moderate differentiation effect on U937 cells (25% CD11c positive/PI negative cells).

Conclusion

In this article we have described the synthesis of a number of 1,5-diphenyl-1,4-pentadien-3-ones (**1a**−**j**), chalcones (**2a**−**d**), benzophenone (**3**), bis(benzylidene)(hetero)cycloalkanones (**4a**−**l**) and bicyclic analogues (**5a**,**b** and **6a**,**b**), 1,1′-(1,3- and 1,4-phenylene)bis(prop-2-en-1-ones) (**7a**−**d**), 1,7-bis(phenyl)-hepta-1,6-diene-3,5-ones (**7e**−**h**), bis(benzamides) (**8a**−**e**), and bis(anilides) (**9a**−**f**) as simplified analogues of AMI-5 (eosin), a recently reported HMTi. Such derivatives were tested against a panel of HMTs, including both PRMTs (the fungal RmtA, human PRMT1, and human CARM1) and HKMT (human SET7), using histone and nonhistone proteins as a substrate. They were also screened for studying their potential HAT and SIRT modulating activities, because they resemble in their structures some chemical features typical of curcumin, a known p300 HAT inhibitor, resveratrol, recently reported as SIRT1 activator, and GW5074, able to inhibit SIRT2.

Taken all together, the anti-HMT, anti-HAT, and anti-SIRT data for selected compounds **1**–**9** (Table 2) showed that the

Table 2. Summary of Activities of Selected Compounds **1–9** Toward the Investigated Epigenetic Targets

compd	PRMT inhbtn[a]	HKMT inhbtn[b]	HAT inhbtn[c]	SIRT1 inhbtn[d]	SIRT2 inhbtn[d]	epi-ML[e]
 **1a**	IC_{50}^{RmtA} = 162 μM PRMT1/H4: NA[f] PRMT1/Npl3: + CARM1/PABP1: ++	SET7: ±	NA	NA	NA	N
 **1b**	IC_{50}^{RmtA} = 40 μM PRMT1/H4: 17% PRMT1/Npl3: + CARM1/PABP1: +	SET7: ++	62%	60%	65%	Y
 **1c**	IC_{50}^{RmtA} = 69 μM PRMT1/H4: 76% PRMT1/Npl3: ± CARM1/PABP1: ++	SET7: ++	100%	61%	100%	Y
 **2c**	IC_{50}^{RmtA} = 40 μM PRMT1/H4: 36% PRMT1/Npl3: - CARM1/PABP1: -	SET7: ±	0%	35%	38%	N
 **2d**	IC_{50}^{RmtA} = 29 μM PRMT1/H4: 61% PRMT1/Npl3: - CARM1/PABP1: -	SET7: ±	16%	35%	37%	N
 **4b**	IC_{50}^{RmtA} = 14 μM PRMT1/H4: 44% PRMT1/Npl3: ++ CARM1/PABP1: ++	SET7: ++	96%	50%	56%	Y
 **4f**	IC_{50}^{RmtA} = 39 μM PRMT1/H4: 73% PRMT1/Npl3: ++ CARM1/PABP1: ++	SET7: ++	87%	63%	64%	Y
 **4j**	IC_{50}^{RmtA} = 29 μM PRMT1/H4: 91% PRMT1/Npl3: ± CARM1/PABP1: ++	SET7: ++	61%	62%	67%	Y
 **4l**	IC_{50}^{RmtA} = 45 μM PRMT1/H4: 73% PRMT1/Npl3: ++ CARM1/PABP1: ++	SET7: ++	69%	54%	59%	Y
 **7b**	IC_{50}^{RmtA} = 10 μM PRMT1/H4: 60% PRMT1/Npl3: ++ CARM1/PABP1: ++	SET7: ++	83%	73%	48%	Y
 **7d**	IC_{50}^{RmtA} = 37 μM PRMT1/H4: 31% PRMT1/Npl3: ++ CARM1/PABP1: ++	SET7: ±	0%	45%	56%	N
 **7f**	IC_{50}^{RmtA} = 48 μM PRMT1/H4: 70% PRMT1/Npl3: ++ CARM1/PABP1: ++	SET7: ++	58%	52%	61%	Y
 **8d**	IC_{50}^{RmtA} = 61.4 μM PRMT1/H4: 86% PRMT1/Npl3: ± CARM1/PABP1: ±	SET7: +	83%	2%	0%	N
 **9e**	IC_{50}^{RmtA} = 13.8 μM PRMT1/H4: 38% PRMT1/Npl3: ++ CARM1/PABP1: ++	SET7: ++	81%	13%	9%	N
AMI-5	IC_{50}^{RmtA} = 18 μM PRMT1/H4: 96% PRMT1/Npl3: ++ CARM1/PABP1: ++	SET7: ++	20%	33%	31%	N

[a] See Part 1, *A*, *B*, and *C*. [b] See Part 1, *C*. [c] See Part 2. [d] See Part 3. [e] Y, yes; N, no. [f] NA, not active.

bis(dibromophenol) motif linked through an unsaturated, (di)-oxo-containing spacer could be useful for designing new epigenetic multiple ligands (epi-MLs) active against HMTs, p300 HAT, SIRT1, and SIRT2 at the same time. To date, in epigenetics, only the psammaplins[83] were reported endowed with dual anti-DNMT and anti-HDAC activities, and the selective PRMTi AMI-1 has been recently found also active against SIRT1.[84] In particular, bis(dibromophenol) moieties connected through a penta-1,4-dien-3-one (**1c**), a 2,6-dimethylene(hetero)cycloalkanone (**4b**, **4f**, **4j**, **4l**), a 1,1′-(1,3-phe-

Table 3. Summary of Cellular Activities Observed with Epi-MLs on Human Leukemia U937 Cells[a]

compd	cell cycle	apoptosis induction	differentiation
1b	arrest in S phase	1.0%	68.1%
1c	arrest in G_2 phase	1.1%	95.2%
4b	arrest in S phase	2.0%	70.9%
4f	arrest in G_2 phase	1.0%	53.2%
4j	arrest in S phase	27.9%	96.1%
4l	arrest in S/G_2 phase	40.7%	9.1%
7b	arrest in G_1/G_2 phase	42.6%	38.8%
7f	arrest in S phase	10.5%	33.0%
AMI-5	no effect	0.5%	5.7%
curcumin	no effect	8.5%	25.3%
sirtinol	weak arrest in S phase	7.5%	7.5%

[a] See Part 4.

nylene)diprop-2-en-1-one (**7b**), and a hepta-1,6-diene-3,5-dione linker (**7f**) showed RmtA inhibiting activity in the range 10 to 69 μM were able to inhibit >50% of activity of human PRMT1 at 50 μM, gave p300 HAT inhibition ranging from 60 to 100% at 50 μM and were highly efficient (50 to 100% inhibition) in inhibiting both SIRT1 and SIRT2 at 25 μM. Compounds carrying less than four bromine atoms (i.e., **1a**, **1b**, and **2c**) were less or not active against the tested enzymes: only the **1c**-related 1-(3-bromo-4-hydroxyphenyl)-5-(3,5-dibromo-4-hydroxyphenyl)-penta-1,4-dien-3-one **1b** among them showed significant inhibition of all the tested enzymes. The tetrabromo-chalcone **2d** was able to inhibit the fungal RmtA and the human PRMT1 using a histone substrate. Nevertheless, it was inactive against HAT and showed only moderate SIRT inhibition. The 1,1′-(1,4-phenylene)diprop-2-en-1-one derivative **7d** displayed good HMT and SIRT inhibitory activities, but was unable to inhibit HAT at 50 μM. The *N*,*N*′-(1,3-phenylene)dibenzamide **8d** well inhibited RmtA and PRMT1 tested with a histone substrate, but showed very low or no inhibition against PRMT1 and CARM1 with nonhistone proteins and against SET7. Conversely, the related N^1,N^3-diphenylisophthalamide **9e** gave high PRMT inhibition using nonhistone substrate and was less potent in the PRMT1/H4 assay. When tested against HAT and SIRTs, **8d** and **9e** showed high HAT inhibition but no action toward the tested sirtuins. The reference compound AMI-5 in our hands displayed a good inhibition of HMTs but only a slight effect against HAT and SIRTs.

Tested in the human leukemia U937 cell line to determine their effects on apoptosis induction and granulocytic differentiation, only the epi-MLs **1b**, **1c**, **4b**, **4f**, **4j**, **4l**, **7b**, and **7f** showed interesting apoptotic and/or differentiating properties, much more evident than those displayed by the single HMT (AMI-5), HAT (curcumin), and SIRT (sirtinol) inhibitors (Table 3). In particular, **4j**, **4l**, and **7b** induced up to 43% apoptosis at 25 μM, and **1c** and **4j** increased the CD11c levels of >90% at 5 μM, thus showing a massive, dose-dependent cytodifferentiating effect in the U937 cells. In this latter assay, **1b**, **4b**, **4f**, **7b**, and **7f** also showed high differentiating activities ranging from 33% to 71% of CD11c positive PI negative cells. Thus, it is feasible that the epi-MLs **1b**, **1c**, **4b**, **4f**, **4j**, **4l**, **7b**, and **7f**, by acting at the same time against several epigenetic targets such as PRMTs, HKMTs, HAT, and SIRTs that interplay each other in modulation of gene expression and transcription, could show high important effects such as apoptosis and differentiation in the U937 cells. Indeed, in the same assays the single-target inhibitors curcumin and sirtinol gave <10% of apoptosis at 25 μM, and only curcumin (25 μM) showed a slight cytodifferentiating effect (Table 3). Further biological in vitro and in vivo studies are in progress to better characterize the observed properties of these epi-MLs.

Experimental Section

Chemistry. Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker AC 400 spectrometer; chemical shifts are reported in δ (ppm) units relative to the internal reference tetramethylsilane (Me_4Si). EI MS spectra were recorded with a Fisons Trio 1000 spectrometer; only molecular ions (M^+) and base peaks are given. All compounds were routinely checked by TLC and ^{1}H NMR. TLC was performed on aluminum-backed silica gel plates (Merck DC, Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light. All solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of solutions after reactions and extractions involved the use of a rotary evaporator operating at a reduced pressure of about 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within ±0.40% of the theoretical values. All chemicals were purchased from Aldrich Chimica, Milan (Italy), or from Lancaster Synthesis GmbH, Milan (Italy), and were of the highest purity.

General procedures as well as description of the reactions used to obtain compounds **1**–**22** are reported in detail as Supporting Information (Schemes S1–S6, Tables S1–S3, and Experimental Section).

Biochemistry. Preparation of GST-RmtA Fusion Proteins. The coding sequence of RmtA[67] was cloned into a pGEX-5X-1 expression vector (Amersham Pharmacia Biotech). RmtA-Protein was expressed in BL21 cells in LB-medium. 250 mL cultures with an A_{600} of 0.4 were induced with a final concentration of 1 mM IPTG and grown for 4 h at 37 °C. After centrifugation of cells at 4000*g*, the pellet was resuspended in 6 mL of GST-binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing one protease inhibitor tablet (Complete, Roche, Mannheim, Germany) for 50 mL of buffer. For cell lysis, lysozyme was added at a final concentration of 5 mg/mL binding buffer and cells were passed through a french press with a pressure setting of 1000 psi. The resulting lysate was centrifuged at 20000*g* for 10 min at 4 °C. GST fusion protein was purified from soluble extracts by binding to a GST-HiTrap column (Amersham Pharmacia Biotech). Proteins were eluted with 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0, and assayed for histone methyltransferase activity.

RmtA Inhibitory Assay. For inhibition assays, affinity purified GST-RmtA fusion proteins were used as the enzyme source. HMT activities were assayed using chicken erythrocyte core histones as substrates. GST-RmtA fusion proteins (500 ng) were incubated with different concentrations of compounds for 15 min at room temperature. A total of 20 μg of chicken core histones and 0.55 μCi of [^{3}H]-*S*-adenosyl-L-methionine ([^{3}H]AdoMet) were added. This mixture was incubated for 30 min at 30 °C. Reaction was stopped by trichloroacetic acid (TCA) precipitation (25% final concentration) and samples were kept on ice for 20 min. Whole sample volumes were collected onto glass fiber filters (Whatman GF/F) preincubated with 25% TCA. Filters were washed three times with 3 mL of 25% TCA and then three times with 1 mL of ethanol. After drying the filters for 10 min at 70 °C, radioactivity was measured by liquid scintillation spectrophotometry (3 mL scintillation cocktail). In the IC_{50}s determination, the SD values were within ±5%.

PRMT1, CARM1/PRMT4, and SET7 Inhibitory Assays. In vitro methylation reactions have been described in detail previously.[59] Briefly, all methylation reactions were carried out in the presence of [^{3}H]AdoMet (79 μCi from a 12.6 μM stock solution in dilute HCl/ethanol 9:1, pH 2.0–2.5, Amersham Biosciences) and PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). To determine the specificity of the small molecules, compounds were incubated with GST-PRMT1 and Npl3p, GST-PRMT4 and PABP1, GST-SET7 and histone H3,

respectively. Substrates (0.5 μg) were incubated with recombinant enzymes (0.2 μg) in the presence of 0.5 μM [^{3}H]AdoMet and 100 μM concentration of each of the compounds for 90 min at 30 °C in a final volume of 30 μL PBS. Reactions were run on a 10% SDS-PAGE, transferred to a PVDF membrane, sprayed with Enhance, and exposed to film overnight. Reactions were performed in the presence of DMSO at 3.3% v/v.

Western Blot Analysis. Histone Extraction Protocol. U937 cells were harvested and washed twice with ice-cold PBS 1× . Then cells were lysed in Triton Extraction Buffer (TEB: PBS containing 0.5% Triton X 100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02% (w/v) NaN_3) at a cellular density of 10^7 cells per mL for 10 min on ice, with gentle stirring. After a brief centrifugation at 2000 rpm at 4 °C, the supernatant was removed and the pellet was washed in half the volume of TEB and centrifugated as before. The pellet was resuspended in 0.2 M HCl at a cell density of 4×10^7 cells per mL and acid extraction was proceeded overnight at 4 °C on a rolling table. The day after the samples were centrifugated at 2000 rpm for 10 min at 4 °C, the supernatant was removed and its protein content was established using the Bradford assay.

Immunoblot Protocol. About 3–10 μg of acid-extracted proteins were loaded on 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. The blotted nitrocellulose was washed twice with water and then blocked in freshly prepared PBS, containing 3% nonfat dry milk (PBS-MLK) for one hour at room temperature with constant agitation. The nitrocellulose was incubated with 1:500 dilution of anti monomethyl-H3K4 (Abcam), monomethyl-H4R3 (Abcam) or dimethyl-H3R17 (Abcam) antibodies in freshly prepared PBS-MLK, overnight at 4 °C in agitation. The day after, the nitrocellulose was washed three times with water and incubated in the secondary reagent of choice in PBS-MLK for 1.5 h at room temperature in agitation. The nitrocellulose was washed with water three times and once in PBS-0.05% Tween 20 for 5 min and then rinsed 4–5 times with water. At the end, the ECL detection method (Amersham) was used.

p300/CBP HAT Assay. HAT inhibition assay has been performed as recommended by the suppliers (Upstate) and as reported earlier.[57] Briefly, an indirect ELISA assay has been performed for the detection of acetyl residues on histone H3 substrate using 10 μg of U937 cell nuclear extract (prepared according to Nebbioso et al.)[85] per assay as a source of HAT enzymes. The incubations with DMSO alone (control) or with selected compounds **1–9**, AMI-5, curcumin, and anacardic acid (AA; all at 50 μM) have been carried out for 90 min. Acetylated histone H3 peptides (Upstate) have been included as positive controls and have been used to make standard curves for the assay quantization. Data have been expressed in ^{3}H-acetyl substrate incorporation activity.

SIRT1 and SIRT2 Assays. The SIRT activity assay was performed using the SIRT1 and SIRT2 fluorescent activity assay/drug discovery kit (AK-555 and AK-556 BIOMOL Research Laboratories, respectively). The assay was performed in two stages: in the first one, that is the deacetylation phase, the SIRT1 (1 U/well) or SIRT2 (5 U/well), respectively, were incubated (37 °C for 2 h) with the substrate Fluor de Lys-SIRT1/2 in the presence of NAD^+ and various concentrations of tested compounds (sirtuins activators or inhibitors). As internal controls, suramin sodium, a sirtuin inhibitor, and resveratrol, a SIRT1 activator, have been included. The second stage is initiated by the addition of the Developer II, including nicotinamide (NAM), a sirtuin inhibitor which stops the SIRT1/2 activity, and the fluorescent signal is produced. The fluorescence was measured on a fluorometric reader (Inphinite 200 TECAN) with excitation set at 360 nm and emission detection set at 460 nm.

Biology Cellular Assays. Cell Lines and Cultures. U937 cell line was cultured in RPMI with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin-B, 10 mM HEPES, and 2 mM glutamine. U937 cells were kept at the constant concentration of 200000 cells per milliliter of culture medium.

FACS Analysis of Apoptosis on U937 Cells. Apoptosis was measured by caspase 3 activation detection (B-BRIDGE) as recommended by the suppliers; samples were analyzed by FACS with Cell Quest technology (Becton Dickinson) as previously reported.

Granulocytic Differentiation on U937 Cells. Granulocytic differentiation was carried out as previously described.[86] Briefly, U937 cells were harvested and resuspended in 10 μL phycoerythrine-conjugated CD11c (CD11c-PE). Control samples were incubated with 10 μL PE conjugated mouse IgG1, incubated for 30 min at 4 °C in the dark, washed in PBS, and resuspended in 500 μL PBS containing PI (0.25 μg/mL). Samples were analyzed by FACS with Cell Quest technology (Becton Dickinson). PI positive cells have been excluded from the analysis.

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Supporting Information Available: Detailed chemistry section with synthetic procedures used for obtaing the title compounds (Schemes S1–S6), characterization data for compounds **1–22** (Tables S1−S3), PRMT1/H4 assay (Figure S1), cell cycle effect of selected compounds **1–9** on the U937 cells (Figure S2), and experimental procedures (chemistry, PRMT1/H4 assay, cell cycle analysis). This material is available free of charge via the Internet at http://pubs.acs.org.

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