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Research paper

Identification of PR-SET7 and EZH2 selective inhibitors inducing cell death in human leukemia U937 cells

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ABSTRACT

Chemical manipulations undertaken on some bis(bromo- and dibromo-phenol) compounds previously reported by us as wide-spectrum epigenetic inhibitors let us to identify bis (bromo- and dibromo-methoxyphenyl) derivatives highly selective for PR-SET7 and EZH2 (compounds **4**, **5**, **9**, and **10**). Western blot analyses were carried out in U937 cells to determine the effects of such compounds on the methyl marks related to the tested enzymes (H3K4me1, H3K9me2, H4H20me1, and H3K27me3). The 1,5-bis(3-bromo-4-methoxyphenyl)penta-1,4-dien-3-one **4** (EC_{50} vs EZH2 = 74.9 μM), tested in U937 cells at 50 μM, induced massive cell death and 28% of granulocytic differentiation, highlighting the potential use of EZH2 inhibitors in cancer.

1. Introduction

Reversible covalent modification of histones is one of the epigenetic mechanisms involved in control of gene expression and transcription. In particular, acetylation or methylation reactions can occur at the lysine (Lys) residues on histone tails, to switch chromatin from its transcriptionally silent (heterochromatin) to its transcriptionally active (euchromatin) form, and vice versa [1]. Enzymes involved in chromatin remodeling can add, remove, or read such covalent modifications, and are now classified into "writers", "erasers", or "readers" [2,3]. Histone acetyltransferases (HATs) and histone Lys methyltransferases (HKMTs), able to add acetyl and methyl groups, respectively, at histone tails are writer enzymes. Histone deacetylases (HDACs) and histone Lys demethylases (HKDMs), catalyzing the opposite reaction, represent examples of erasers. Another group of proteins, which possess effector domains including plant homeodomain (PHD), tudor, chromo or bromo domains to recognize specific modified residues, are the readers of epigenetic information. The writers/erasers-driven chemical modifications display different effects on chromatin, depending on the type of modification. Lys acetylation is usually related with transcription activation, while deacetylation typically lead to gene silencing. Differently, Lys methylation can be associated to transcription activation or repression depending on the specific Lys residue and the extension of the resulting methylation (mono-, di-, or tri-methylation). Accordingly, methylation of histone H3 at Lys residues 9 or 27 (H3K9 or H3K27) or histone H4 at Lys 20 (H4K20) is associated with gene silencing, while H3K4, H3K36 and H3K79 methylation correlates with gene activity. An altered expression and/or function of writers, readers or erasers of epigenetic marks can lead to the development of tumors, by activation of oncogenes or by silencing tumor-suppressor proteins [4–8].

From the report by Cheng et al. indicating some dye-like small molecules as inhibitors of protein arginine methyltransferases (PRMTs) and/or HKMTs [9], we chose AMI-1 as PRMT1-selective prototype to develop a novel series of PRMT-targeted inhibitors [10,11], and AMI-5 (Fig. 1) as unselective lead compound to design new simplified analogs to test against PRMTs and HKMTs [12]. We

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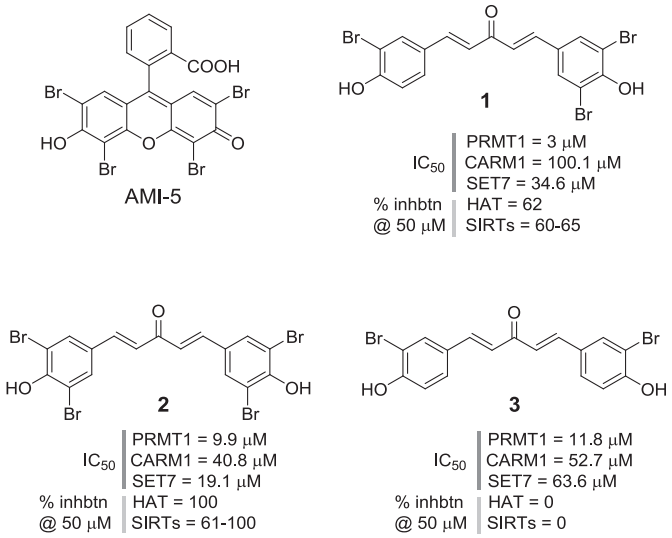


Fig. 1. Bromo-hydroxy-containing compounds acting as epi-MLs or non specific PRMT/HKMT inhibitors.

were intrigued by the presence of two dibromo-hydroxy-phenyl moieties in the AMI-5 structure, and we postulated these fragments to be crucial for PRMT and/or HKMT inhibiting activity. Thus we prepared a large series of compounds bearing two bromo- or dibromo-hydroxyphenyl portions (or their analogs) separated by variously substituted spacers, and we tested them against two PRMTs, PRMT1 and CARM1, and against SET7 as an example of HKMT. In addition, since the structure of the designed compounds reminded of chemical features typical of HAT and SIRT modulators, we tested some of them against p300 HAT and SIRT1/2 enzymes [13–15]. From these assays, it resulted that compounds carrying two 3,5-dibromo-4-hydroxyphenyl moieties linked through a penta-1,4-dien-3-one, 2,6-dimethylene(hetero)cycloalkanone, 1,1-(1,3-phenylene)diprop-2-en-1-one, or hepta-1,6-diene-3,5-dione spacer behaved as epigenetic multiple ligands (epi-MLs), inhibiting at the same time all the tested PRMT, HAT, and SIRT enzymes, as well as SET7 [14].

Among the 1,4-diphenylpenta-1,4-dien-3-one derivatives, those showing either a 3-bromo-4-hydroxy/3,5-dibromo-4-hydroxy or a bis(3,5-dibromo-4-hydroxy) substitution at the phenyl rings (compounds **1** and **2**, Fig. 1) were epi-MLs, while the bis(3-bromo-4-hydroxy) analog (**3**) showed PRMT1, CARM1, and SET7 inhibition, but was totally inactive against HATs and SIRTs (Fig. 1).

To further investigate the effect of such compounds against HKMTs, we tested **1**–**3** against PR-SET7, G9a, and SET7/9, three HKMTs different from SET7. In particular, PR-SET7 is a H4K20 lysine methyltransferase highly involved in cell cycle regulation and progression [16,17], G9a [18] acts on H3K9 and has been found expressed in aggressive lung cancer cells, with its elevated expression related to poor prognosis [19], and SET7/9 [20] (with its epigenetic mark H3K4me1) has been associated to inflammatory diseases and diabetes [21]. As expected, **1**–**3** confirmed their wide inhibitory spectrum against epigenetic targets, they being able to inhibit the three enzymes in the range 4.3–62.5 μM (Table S1 in Supplementary material). Then, with the aim to identify selective HKMT inhibitors among this library of compounds, we noticed that analogs of derivatives **1**–**3** which bear methoxy instead of hydroxy group on the two phenyl wings of the penta-1,4-dien-3-one scaffold (**4** and **5**, Fig. 2) as well as simplified products such as the bis(3,5-dibromo-4-hydroxyphenyl)methanone **6** and the 4-(3-bromo- and 3,5-dibromo-4-hydroxyphenyl)but-3-en-2-ones **7** and **8** (Fig. 2), led to compounds inactive against PRMT1, CARM1, and SET7, or endowed with slight CARM1 inhibiting activity (compound **5**) [12]. Thus, we prepared the 2,6-bis(3-bromo- and 3,5-dibromo-4-methoxybenzylidene)cyclohexanones **9** and **10** as constrained analogs of **4** and **5**, and we tested the derivatives **4**–**10** against PR-SET7, G9a, and SET7/9. In addition, selected bis(bromo- or dibromo-methoxyphenyl) compounds **4**, **5**, **9**, and **10** were tested against Enhancer of Zeste Homolog 2 (EZH2), to ascertain their capability to inhibit its enzymatic activity. EZH2 catalyzes di- and trimethylation of H3K27 residues, is the enzimatically active component of the multiprotein Polycomb Repressor Complex 2 (PRC2), and is involved in stem cell identity, pluripotency, and cancer [22–24]. In cancer, EZH2 mediates epigenetic silencing of tumor-suppressor genes in many contexts, and has been found overexpressed in breast and prostate cancers [25–27], Ewing's sarcoma [28], glioblastoma [29], and neuroblastoma [30].

In addition to enzymatic assays, compounds **4**, **5**, **9**, and **10** were tested in human leukemia U937 cells to determine their effects on some methylation marks, H4K20me1, H3K9me2, H3K4me1, and H3K27me3, related to PR-SET7, G9a, SET7/9, and EZH2 activity, respectively. Moreover, such compounds have been tested in the same cellular model to study their outcome on cell cycle, cell death induction and granulocytic differentiation.

2. Materials and methods

2.1. Chemistry

2.1.1. General

Melting points were determined on a Buchi 530 melting point apparatus and are uncorrected. Infrared (IR) spectra (KBr) were recorded on a Perkin–Elmer Spectrum One instrument. ^{1}H-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). Electronic impact mass spectrometry (EI-MS) was performed on a Finnigan LCQ DECA Termo-Quest (San Josè, California, USA) mass spectrometer. 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 or using a $KMnO_4$ alkaline solution. 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 reduced pressure of ca. 20 Torr. Organic solutions were dried over anhydrous sodium sulfate. Analytical results are within 0.40% of the theoretical values (see Supplementary material). All chemicals were purchased from Aldrich Chimica, Milan (Italy), or from Lancaster Synthesis GmbH, Milan (Italy), and were of the highest purity. As a rule, samples prepared for physical and biological studies were dried in high vacuum over P_2O_5 for 20 h at temperatures ranging from 25 to 110 °C, depending on the sample melting point. Syntheses and chemical and physical data of **4**–**8** have been previously described [12].

2.1.2. General Procedure for the synthesis of the 2,6-bis(3-bromo- and 3,5-dibromo-4-methoxybenzylidene)cyclohexanones (*9* and *10*). example: synthesis of 2,6-bis(3-bromo-4-methoxybenzylidene) cyclohexanone (*9*)

Cyclohexanone (1.15 mmol, 0.12 mL) was added to a suspension of barium hydroxide octahydrate (4.6 mmol, 1.45 g) in methanol (20 mL), and the mixture was stirred for 5 min. Then a solution of 3-bromo-4-methoxybenzaldehyde (2.3 mmol, 0.5 g) in methanol (10 mL) was added, and the resultant mixture was stirred for 2 h at room temperature. The precipitate was filtered, washed with water, dried and recrystallized by acetonitrile to afford the pure product.

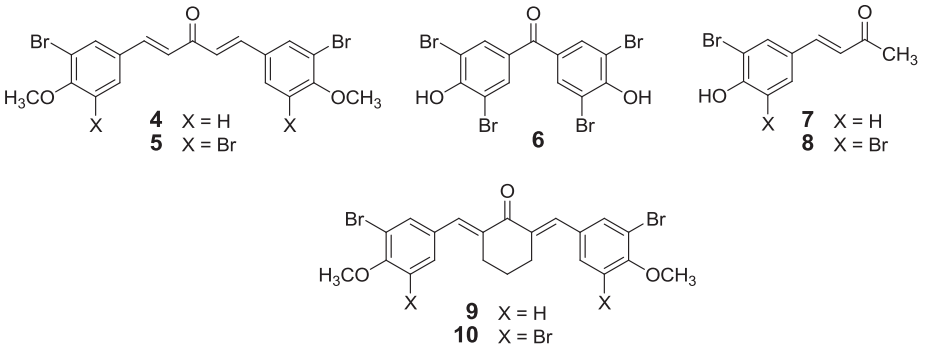


Fig. 2. Bromo-hydroxy- and –methoxy-phenyl derivatives.

Melting point: 170–172 °C; yield: 82%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.72–1.75 (m, 2H, cyclohexanone protons), 2.86–2.89 (m, 4H, cyclohexanone protons), 3.90 (s, 6H, OC*H*$_3$), 7.19–7.21(d, 2H, benzene protons), 7.55–7.59 (m, 4H, benzene protons and PhC*H* = CCO), 7.78 (s, 2H, benzene protons) ppm; ^{13}C NMR (400 MHz, DMSO-d_6) δ 25.1, 26.1 (2C), 55.1 (2C), 112.1 (2C), 112.2 (2C), 128.4 (2C), 129.2 (2C), 130.9 (2C), 132.2 (2C), 137.1 (2C), 156.2 (2C), 190.4 ppm; *m*/*z* [*M*]$^+$: 491.9759.

2.1.3. 2,6-bis(3,5-dibromo-4-methoxybenzylidene)cyclohexanone (**10**)

Recrystallized by: acetonitrile; melting point: 204–206 °C; yield: 78%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.73–1.76 (m, 2H, cyclohexanone protons), 2.84–2.87 (m, 4H, cyclohexanone protons), 3.84 (s, 6H, OC*H*$_3$), 7.51 (s, 2H, PhC*H* = CCO), 7.83 (s, 4H, benzene protons) ppm; ^{13}C NMR (400 MHz, DMSO-d_6) δ 25.1, 26.1 (2C), 60.8 (2C), 118.3 (4C), 129.9 (4C), 131.9 (2C), 132.2 (2C), 137.1 (2C), 154.2 (2C), 190.4 ppm; *m*/*z* [*M*]$^+$: 649.7949.

2.2. Biochemical assays

2.2.1. PR-SET7, G9a, and SET7/9 inhibitory assays

Assays were performed essentially as described previously for PR-SET7 [16], G9a [18], and SET7/9 [20]. Briefly, the samples were incubated at 30 °C for 10–60 min in a reaction buffer containing 50 mM Tris–HCl (pH 8.5), 5 mM $MgCl_2$, 4 mM DTT, and 1 μM *S*-adenosyl-L-[methyl-^{3}H]methionine (Amersham Pharmacia Biotech). Two micrograms of octamer, oligonucleosomes, or mononucleosome were used as substrates. The total volume of the reaction mixture was adjusted to 25 μL. The reaction was stopped by addition of SDS sample buffer and then fractionated on 15% SDS-PAGE. Separated histones were then transferred onto an Immobilon-P membrane (Millipore) and visualized by CBB staining. The membrane was sprayed with EN3HANCE (NEN), and exposed to Kodak XAR film overnight.

2.2.2. EZH2 inhibitory assay

2.2.2.1. Reagent. Reaction buffer; 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% DMSO.

2.2.2.2. Reaction conditions. EZH2: Complex of human EZH2 (GenBank Accession No. NM_004456), (amino acids 2-end) with N-terminal His tag, MW = 86 kDa, human EED (NM_003797) (a-a 2-end) with N-terminal Flag tag, MW = 51 kDa, human SUZ12 (NM_015355) (a-a 2-end) with N-terminal His tag, MW = 87 kDa, Human AEBP2 (NM_153207) (a-a 2-end) with N-terminal His tag, MW = 53 kDa, and human RbAp48 (NM_005610) (a-a 2-end) with N-terminal His tag, MW = 48 kDa, co-expressed in baculovirus expression system. Substrate: 5 μM Histone H3. Methyl donor: 1 μM *S*-adenosyl-L-[methyl-^{3}H]methionine. Enzyme: 100 nM EZH2 complex. Reaction Procedure: prepare indicated substrate in freshly prepared Reaction Buffer; deliver EZH2 into the substrate solution and mix gently; deliver compounds in DMSO into the EZH2 reaction mixture by using Acoustic Technology (Echo 550, LabCyte Inc. Sunnyvale, CA) in nanoliter range, incubate for 15 min; deliver ^{3}H-SAM into the reaction mixture to initiate the reaction; incubate for 1 h at 30 °C; deliver reaction mixture to filter-paper for detection.

2.3. Cellular assays

2.3.1. Compounds

All compounds were dissolved in DMSO (Sigma–Aldrich) and used at 25 μM or 50 μM.

2.3.2. Cell lines

U937 (human leukemic monocyte lymphoma cell line-ATCC) were grown in RPMI 1640 medium (Euroclone) supplemented with 10% heat-inactivated FBS (Euroclone), 1% glutamin (Lonza), 1% penicillin/streptomycin (Euroclone) and 0.1% gentamycin (Lonza), at 37 °C in air and 5% CO_2.

2.3.3. Cell cycle analysis

2.5 × 10^5 U937 cells were collected by centrifugation after 30 h stimulation with compounds at 25 μM and 50 μM. The cells were resuspended in 500 μL of hypotonic buffer (0.1% NP-40, 0.1% sodium citrate, 50 μg/mL PI, RNAse A) and incubated in the dark for 30 min. The analysis was performed by FACS-Calibur (Becton Dickinson) using the Cell Quest Pro software (Becton Dickinson) and ModFit LT version 3 software (Verity). The experiment was performed in triplicate.

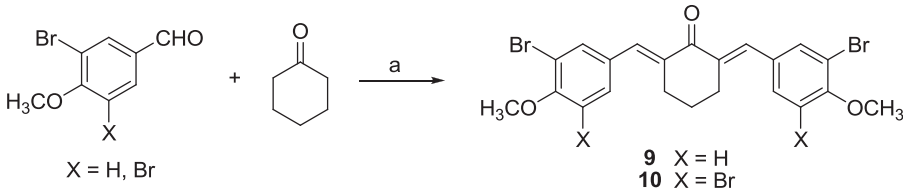
2.3.4. Granulocytic differentiation analysis

2.5 × 10^5 U937 cells were collected by centrifugation after 30 h stimulation with compounds at 25 μM and 50 μM. The cells were

Table 1
Inhibitory activities of compounds **4**–**10** against PR-SET7, G9a, and SET7/9.[a]

Compd	PR-SET7	G9a	SET7/9
	EC_{50}, μM		
4	9.0 ± 0.4	>250	>250
5	3.3 ± 0.2	>250	>250
6	38.8 ± 3.0	>250	>250
7	>250	>250	>250
8	>250	>250	>250
9	10.2 ± 0.5	>250	>250
10	2.6 ± 0.1	>250	164.4 ± 11.0

[a] Values are means ± SD determined from at least three experiments.



Scheme 1. Synthesis of compounds **9** and **10**; reagents and conditions: $Ba(OH)_2$, CH_3OH, room temperature, 2 h, 78–82%.

washed with PBS and incubated in the dark at 4 °C for 30 min with 10 μL of PE-conjugated anti-CD11c surface antigen antibody or with 10 μL of PE-conjugated IgG, in order to define the background signal. At the end of the incubation the samples were washed again and resuspended in 500 μL of PBS containing 0.25 μg/mL PI. The analysis was performed by FACS-Calibur (Becton Dickinson) using the Cell Quest Pro software (Becton Dickinson). The experiment was performed in triplicate and PI positive cells were excluded from the analysis.

2.3.5. Histone extraction

After stimulation with compounds, the cells were collected by centrifugation and washed two times with PBS. Then the samples were resuspended in Triton extraction buffer (PBS containing 0.5% Triton X 100 (v/v), 2 mM PMSF, 0.02% (w/v) NaN_3), and the lysis was performed for 10 min at 4 °C. Next, the samples were centrifugated at 2000 rpm for 10 min at 4 °C, and the pellets were washed in TEB (half the volume). After a new centrifugation under the same conditions, the samples were resuspended in 0.2 N HCl and the acid histone extraction was carried out overnight at 4 °C. The supernatant was recovered by centrifugation and the protein content was ensured by BCA™ Protein Assay (Pierce).

2.3.6. Western blot

5 μg of histonic extracts were loaded on 15% polyacrylamide gel. The histone H3 methylation was assessed with anti-dimethyl-K9-histone H3 antibody (Abcam), with anti-monomethyl K20 histone H4 (Diagenode), with anti-monomethyl K4 histone H3 (Diagenode), and with anti-trimethyl-K27-histone H3 antibody (Diagenode). To check for equal loading, Ponceau Red (Sigma) staining and the anti-histone H4 antibody (Abcam) were used.

3. Results

3.1. Chemistry

The 2,6-bis(3-bromo- and 3,5-dibromo-4-methoxybenzylidene) cyclohexanones **9** and **10** were prepared by condensation of cyclohexanone with 2 equivalents of the properly substituted benzaldehyde in the presence of barium hydroxide in methanol at room temperature (Scheme 1).

3.2. Histone methyltransferase (PR-SET7, G9a, SET7/9, and EZH2) inhibition

Compounds **4**–**10** (Fig. 2) were tested against PR-SET7 using nucleosome as a substrate, and against G9a and SET7/9 using in both cases the histone octamer as a substrate.

The resulting EC_{50} (effective compound concentration able to inhibit 50% of the enzyme activity) values are reported in Table 1. The bis(bromo- and dibromo-methoxyphenyl) derivatives **4**, **5**, **9**, and **10** as well as the bis(3,5-dibromo-4-hydroxyphenyl)methanone **6** were able to selectively inhibit PR-SET7, whereas the 4-phenylbut-3-en-2-ones **7** and **8** were totally ineffective. Against PR-SET7, the bis(3,5-dibromo-4-methoxyphenyl) analogs **5** and **10** displayed the highest potency (also higher than **1**–**3**), while the benzophenone **6** was the less potent.

Selected compounds **4**, **5**, **9**, and **10** were then tested against EZH2 (Table 2). In this assay, the human 5-component PRC2 (containing EZH2, EED, SUZ12, RBAP48, and AEBP2) was used as the enzyme source, and histone H3 was used as a substrate. *S*-adenosyl-L-homocysteine (SAH), a known methyltransferase inhibitor, was used as a reference drug. Against EZH2, the bis(3-bromo-4-methoxyphenyl) derivatives **4** and, to a lesser extent, **9** were the most effective inhibitors, suggesting that in this case the bis(3,5-dibromo) substitution, respect to the bis(monobromo) substitution, is detrimental for the inhibiting activity.

3.3. Western blot analysis

Western blot analyses were performed with **4**, **5**, **9**, and **10** at 50 μM in human leukemia U937 cells treated for 24 h (Fig. 3). H3 methylation marks, H3K4me1 H3K9me2 and H3K27me3, and H4K20me1 have been evaluated using specific antibodies. Concerning H4K20me1 compound **5** and, to a lesser extent, **9** and **10** displayed a signal reduction, in agreement with the PR-SET7 inhibitory data. Differently, H3K9me2 and H3K4me1 expression levels appear unmodified after treatment with **4**, **5**, **9**, and **10**, according to their lack of G9a and SET7/9 inhibitory activity. On the other hand, H3K27me3 strongly decreased after treatment with **4** and **9**, and was less evident with **5** and **10**, in accordance with their different degree of EZH2 inhibition.

3.4. Cell cycle and differentiation effects on human leukemia U937 cells

Compounds **4**, **5**, **9**, and **10** were tested at 25 and 50 μM in human leukemia U937 cells for 30 h, to determine their effect on cell cycle, cell death (pre-G1 peak), and granulocytic differentiation (Fig. 4).

In the tested conditions, only derivatives **4** (at 25 μM) and **5** (at 25 and 50 μM) affected cell cycle, inducing a slight increase of the cells at the G1 phase. At 50 μM it was not possible to analyze the cell cycle effect of **4** due to the extensive cell death caused. As regards cell death induction (pre-G1 peak), at 50 μM compound **4** displayed a massive effect (near 100%, we considered a cut-off of 90%), and **5** induced 41% of cell death. Granulocytic differentiation was evaluated by determining the number of CD11c positive cells with subtraction

Table 2
Inhibitory activity of **4**, **5**, **9**, and **10** against EZH2.[a]

Compd	EC_{50} (μM) or % inhibition at 75 μM
4	74.9 ± 4.0
5	8.7%
9	313.8 ± 15.0
10	6.2%
SAH	66.8 ± 3.5

[a] Values are means ± SD determined from at least three experiments.

Fig. 3. Western blot analyses for compounds **4**, **5**, **9**, and **10** in U937 cells: levels of H3K4me1, H3K9me2, H4K20me1, and H3K27me3 methylation. Ponceau Red (PR) staining of histones or total histone H4 were used for equal loading. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

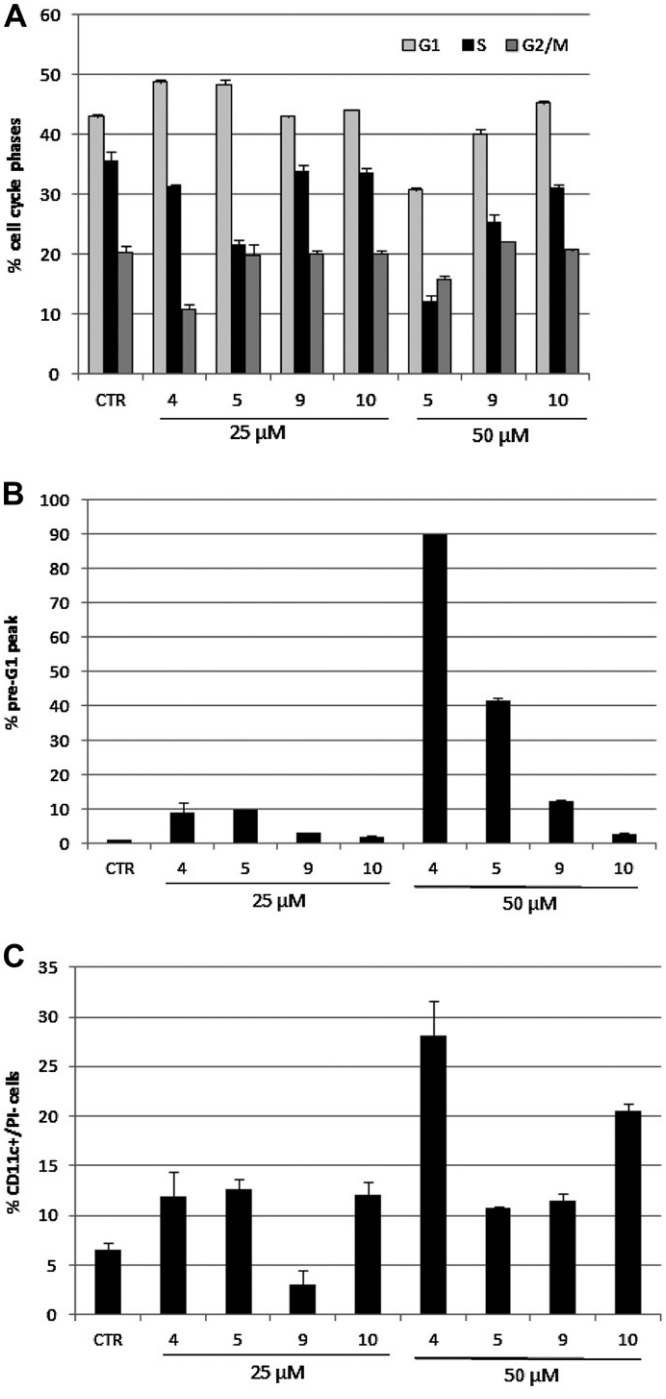


Fig. 4. Effects of treatment of U937 cells with **4**, **5**, **9**, and **10** for 30 h at the indicated concentrations: A) Cell cycle effect; B) Cell death induction (pre-G1 peak); C) Granulocytic differentiation (CD11c method).

of the propidium iodide (PI) positive cells (% CD11c+/PI- cells). In such assay, compounds **4** and **10** at 50 μM showed high differentiation effects, with 28 and 20% of CD11c positive cells.

4. Discussion

In conclusion, while bromo- and dibromo-hydroxyphenyl substituents at the penta-1,4-dien-3-one scaffold (compounds **1**–**3**) led to unselective inhibitors of epigenetic targets including PR-SET7, G9a, and SET7/9, bromo-methoxy substitution at either the 1,5-diphenylpenta-1,4-dien-3-one or 2,6-bis(benzylidene) cyclohexanone scaffold (compounds **4** and **9**) afforded specific PR-SET7 and EZH2 inhibitors. The insertion in such compounds of additional bromine atoms (**5** and **10**) increases their inhibitory action against PR-SET7 and decreases the effect against EZH2. In Western blot analyses (U937 cells, 24 h) at 50 μM compound **5** and, to a lesser extent, **9** and **10** reduced the signal for H4K20me1, according to their inhibition of PR-SET7, and **4** and **9** strongly decreased the level of H3K27me3, confirming their EZH2 inhibition. Methylation levels of H3K4me1 and H3K9me2, used as markers of the SET7/9 and G9a enzymatic activity, respectively, remained unchanged respect to the control, in agreement with the lack of inhibition observed with the tested compounds. In leukemia U937 cell assays, compounds **4** and **5** showed a slight increase of the cells at the G1 phase, in part due to their PR-SET7 (**4** and **5**) and/or EZH2 (**4**) inhibition. In addition, **4** tested at 50 μM in U937 cells induced massive cell death, and gave a considerable increase of CD11c positive cells, of about 28%, thus validating the use of EZH2 inhibitors as apoptotic and/or cytodifferentiating agents in cancer.

Acknowledgments

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biochi.2012.06.003.

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