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Bioorganic & Medicinal Chemistry Letters 18 (2008) 2788–2792
Bioorganic & Medicinal Chemistry Letters

Identification of long chain alkylidenemalonates as novel small molecule modulators of histone acetyltransferases

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Received 5 March 2008; revised 4 April 2008; accepted 6 April 2008
Available online 11 April 2008

Abstract—Pentadecylidenemalonate **1b**, a simplified analogue of anacardic acid, was identified as the first mixed activator/inhibitor of histone acetyltransferases (HATs). It potentiates PCAF HAT activity while inhibiting those of p300/CBP and recombinant CBP. The remarkable apoptotic effect together with the ability to selectively acetylate histone versus non-histone substrates appoint **1b** as a lead for the development of anticancer drugs.

The acetylation of proteins is a dynamic event involving the enzymatic activities of histone acetyltransferases (HATs)[1] and histone deacetylases (HDACs).[2–4] HATs primarily acetylate (and prevent positive charges from forming on) the ε-amino groups of specific lysines in histones,[5] as well as in transcription factors (i.e., p53)[6] and other nuclear proteins (i.e., α-tubulin),[7] thus regulating transcription and histone deposition during nucleosome assembly, DNA repair, and other genomic processes.[5]

The impairment of this cellular regulatory system (for example, after treatment with HDAC inhibitors) can have significant impact on cell functions, including altered gene expression, growth arrest, differentiation, and cell death, and is therefore a promising target for therapeutic development. As a matter of fact, a number of HDAC inhibitors are currently undergoing clinical evaluation for efficacy in the treatment of human tumors[4,8] and one of them, suberoylanilide hydroxamic acid (SAHA, vorinostat)[9] was approved by the U.S. Food and Drug Administration (FDA) on late 2006 for the treatment of advanced cutaneous T-cell lymphoma.

On the contrary, HAT proteins are less validated as chemotherapeutic targets. They have been categorized phylogenetically into several classes, including the GCN5-*N*-acetyltransferase (GNAT),[†] the MYST, and the p300/CBP families, each of which exhibits distinct histone lysine specificities.[2] Translocations and deletion mutations in HAT genes have been reported in a variety of solid tumors and hematologic malignancies.[10,11] A nuclear hormone receptor coactivator with HAT activity, AIB-1 (Amplified in Breast Cancer-1), is amplified and overexpressed in breast, ovarian, and gastric cancers.[12,13] In acute myeloid leukemia, HAT genes are translocated and several fusion proteins between p300/CBP enzymes and MYST family HATs (e.g., MOZ and MORF) are formed.[14,15] Mistargeted and deregulated

Keywords: Epigenetics; Histone acetyltransferase modulators; HAT; Antitumor agents; Anacardic acid.
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† *Abbreviations:* p300, E1A binding protein 300 kDa; CBP, CREB binding protein; CREB, *c*-AMP response element-binding; SRC, steroid receptor coactivator; TAF1, TBP-associated factor; TBP, TATA binding protein; ATF2, activated transcription factor; GCN5, general control of nitrogen metabolism-5; PCAF, p300/CBP-associated factor; MYST, MOZ, YBF2/SAS3, SAS2, TIP60 *N*-acetyltransferase; MOZ, monocytic leukemia zinc finger protein; GNAT, GCN5-*N*-acetyltransferase; MORF, MOZ-related factor.

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doi:10.1016/j.bmcl.2008.04.017

HAT activities of GCN5/PCAF and p300 have also been reported to play an important role in genetic diseases and in human colorectal, breast, and pancreatic cancers.[16–18] Selective modulators of these HATs may therefore exert useful therapeutic applications.[19,20]

A limited number of HAT inhibitors have been described so far (Chart 1), with various degrees of selectivity and cell permeability. Peptide-CoA conjugates (Lys-CoA and H3-CoA-20)[21] as well as a few natural small molecules such as anacardic acid,[22] garcinol,[23] and curcumin[24] were described as potent p300 or PCAF inhibitors. Isothiazolone derivatives were also disclosed as inhibitors of PCAF, but they exhibit significant off target activity in vivo due to their high chemical reactivity with thiol groups.[25] Recently, the γ-butyrolactone MB-3[26] and a few quinoline derivatives[27] were reported as small, cell-permeable GCN5 inhibitors. Moreover, the selective inhibition of p300 HAT by semi-synthetic derivatives of garcinol (i.e., LTK-14) was also described.[28] On the other side, only *N*-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-6-pentadecyl-benzamide (CTPB, Chart 1) was reported to selectively activate p300 HAT activity.[22]

Prompted by our interest in the discovery of small molecule modulators of epigenetic targets,[27,29–33] we prepared a series of molecules containing long alkyl chains (compounds **1**–**6**, Fig. 1), structurally related (as simplified analogues or heterocyclic analogues) to anacardic acid. As cell permeability is an important issue for modulators of histone modifying enzymes,[34] we decided to pre-screen compounds **1**–**6** for their effects on cell cycle, apoptosis induction, and granulocytic differentiation in the human leukemia U937 cell line and then to test the inhibiting capability of just the more active derivatives against HAT enzymes.

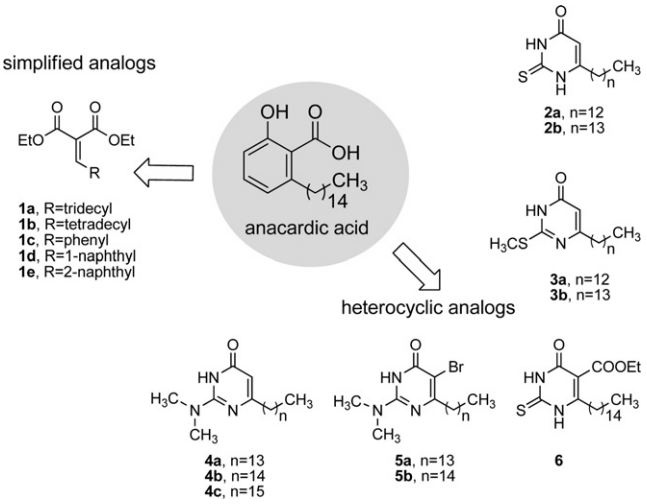
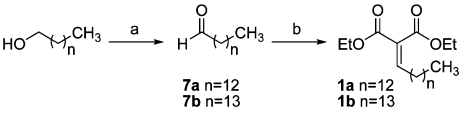


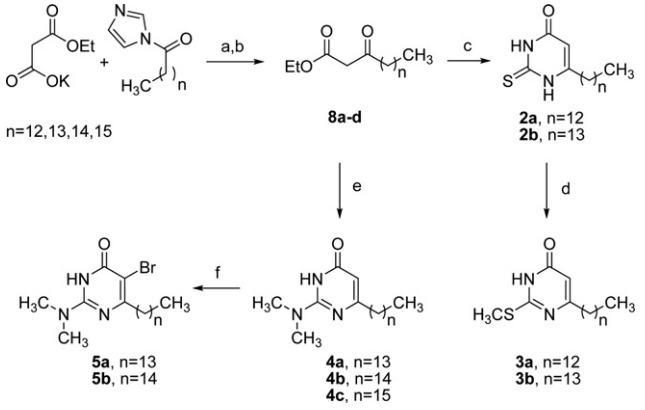
Figure 1. Compounds screened on U937 cells.

Derivatives **1a** and **1b** were prepared (Scheme 1) by Knoevenagel condensation of diethyl malonate with aldehydes **7a** or **7b**, respectively, obtained from tetradecan-1-ol and pentadecan-1-ol by oxidation with 1,1,1-triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1*H*)-one (Dess-Martin periodinane, DMP).[35] Derivatives **1c**–**e** were commercially available or prepared as previously reported.[36]

The acylation of potassium ethyl malonate with the appropriate acyl imidazolide in the presence of magne-



Scheme 1. Reagents and conditions: (a) DMP, dichloromethane, rt; (b) diethyl malonate, acetic acid, piperidine, dichloromethane, rt.



Scheme 2. Reagents and conditions: (a) $MgCl_2$, Et_3N, acetonitrile, rt; (b) 13% HCl, rt; (c) thiourea, EtONa, EtOH, reflux; (d) MeI, DMF, rt; (e) *N*,*N*-dimethylguanidine sulfate, EtONa, EtOH, reflux; (f) NBS, benzoyl peroxide, carbon tetrachloride, reflux.

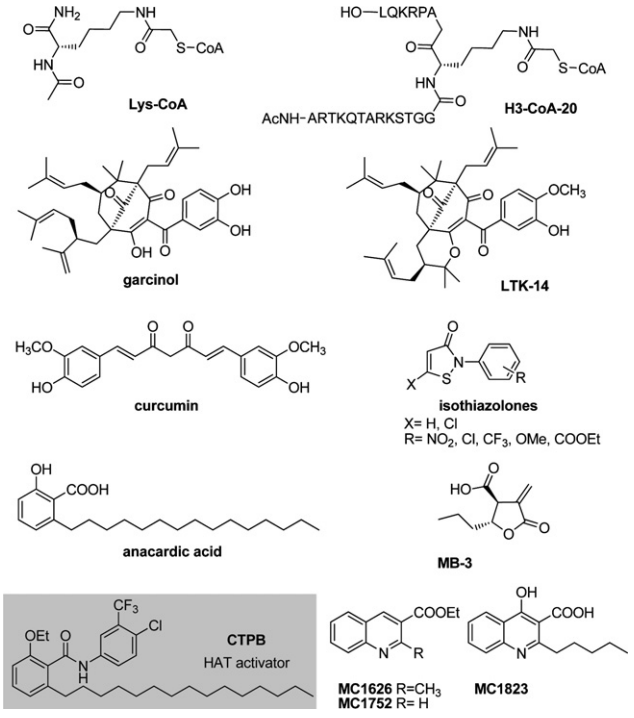
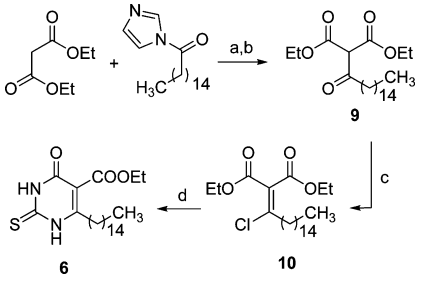


Chart 1. Known HAT modulators.

sium dichloride and triethylamine furnished the corresponding β-oxoesters, which were in turn condensed with thiourea and sodium ethoxide in ethanol to give the 2-thiouracils **2a**, **b** or with *N*,*N*-dimethylguanidine sulfate in the same alkaline medium to afford compounds **4a**–**c**. Following the treatment of **2a**, **b** with methyl iodide afforded the 2-methylthiopyrimidin-4(3*H*)-ones **3a**, **b**,[27] whereas the treatment of 2-(dimethylamino)-pyrimidin-4(3H)-ones **4a**–**c** with *N*-bromosuccinimide (NBS) yielded the corresponding 5-bromo derivatives **5a**, **b** (Scheme 2).



Scheme 3. Reagents and conditions: (a) $MgCl_2$, Et_3N, acetonitrile, rt; (b) 13% HCl, rt; (c) phosphoryl chloride, Et_3N, rt to 80 °C; (d) thiourea, EtONa, EtOH, reflux.

The ethyl 6-pentadecyl-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate **6** was obtained starting from the acylation of diethyl malonate with hexadecanoylimidazolide in the presence of magnesium dichloride and triethylamine. The resulting 2-(1-hydroxyhexadecylidene)malonate was treated with phosphoryl chloride in the presence of triethylamine to obtain the diethyl 2-(1-chlorohexadecylidene)malonate which was finally condensed with thiourea and sodium ethoxide in ethanol to give the 2-thiouracil **6** (Scheme 3).

Compounds **1**–**6** were tested for their effects on cell cycle and apoptosis induction, in human leukemia U937 cell line, using anacardic acid (AA)[22] as reference compound. After 42 h of treatment at 25 μM, only compounds **1a** and **1b** were able to arrest both the cell cycle in the S phase (Fig. 2A) and induce apoptosis (Fig. 2B; 23.8% and 23.9%, respectively), whereas the other derivatives had very weak effects on cell cycle and apoptosis induction in the same conditions (Fig. 2). Consistently with the reported low cytopermeability,[22] cells treated with anacardic acid were comparable to the control. The apoptotic effect of compounds **1a** and **1b** resulted even more evident after treatment at 50 μM, with a dramatic increase for compound **1b** (94.4%, Fig. 2B). None of the tested compounds **1**–**6**

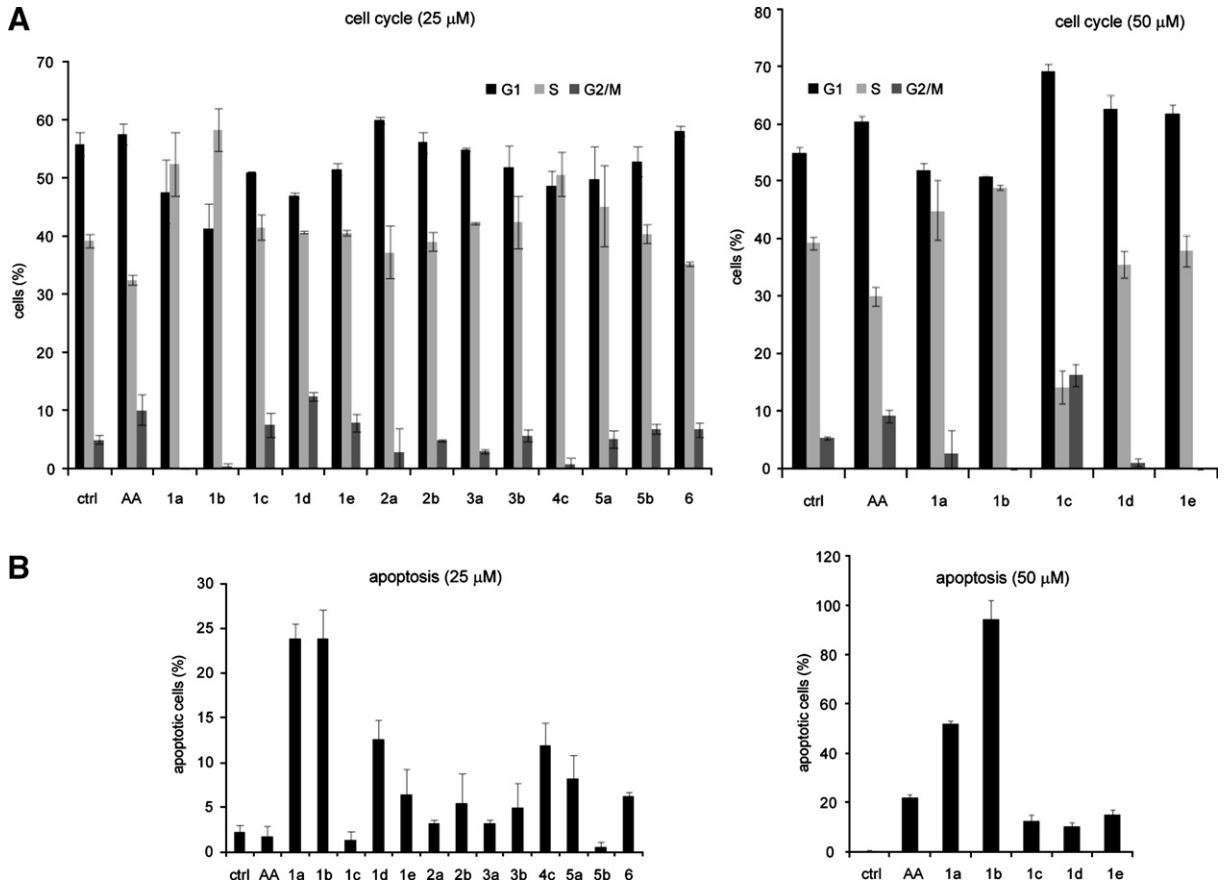


Figure 2. Cell cycle analysis (A) and analysis of apoptosis induction (B) in U937 cells by fluorescence-activated cell sorting (FACS). The U937 cells were treated with compounds **1**–**6** at 25 μM (left) or with compounds **1a**–**e** at 50 μM (right) for 42 h, then stained with propidium iodide and subjected to flow cytometric analysis to determine the cell distributions at each phase of the cell cycle (A) or double-stained with Annexin V/propidium iodide and subjected to flow cytometric analysis to determine the apoptotic subpopulations (B). Caspase 3 was also detected and quantified (not shown). Anacardic acid (AA) was used as a reference compound. Data are reported as means ± SD of three independent experiments.

showed any significant differentiating effect (see Supplementary data).

As derivative **1b** could be considered a simplified analogue of anacardic acid, we decided to explore its activity against a panel of HAT enzymes (p300, PCAF, CBP, and GCN5), using anacardic acid (AA),[22] curcumin,[24] and MB-3[26] (all at 50 μM, unless differently reported) as reference compounds.

As shown in Figure 3, **1b** showed a very good inhibitory effect (74% of inhibition at 50 μM) against immunoprecipitated p300/CBP from U937 cell nuclear extracts, thus being more potent than curcumin (46% of inhibition); moreover, **1b** displayed a strong inhibition against recombinant CBP (81%), thereby being much more powerful than AA (62%) and MB-3 (55% of inhibition at 50 μM) in this assay. We observed no inhibition of the HAT activity of GCN5 (Fig. 3B), but, surprisingly, we noticed a significant increase of the acetylating activity of PCAF (Fig. 3D). The effect of compound **1b** on the acetylation levels of H3 terminal tails was assessed in the human immortalized kidney epithelial cell line (HEK-TE, kind gift of Dr. W.C. Hahn). These cells are transformed but not tumorigenic and in respect to U937 cells show higher basal acetylation histone H3 levels. Cells were incubated with vehicle, compound **1b** (at 50 μM) or with the reference compounds curcumin (at 50 μM) and suberoylanilide hydroxamic acid (SAHA, at 5 μM), a well-known HDAC inhibitor[9] able to increase histone H3 as well as the non-histone substrate α-tubulin acetylation levels.

As shown in Figure 4A by Western blot analysis, compound **1b** did not induce hypoacetylation on histone

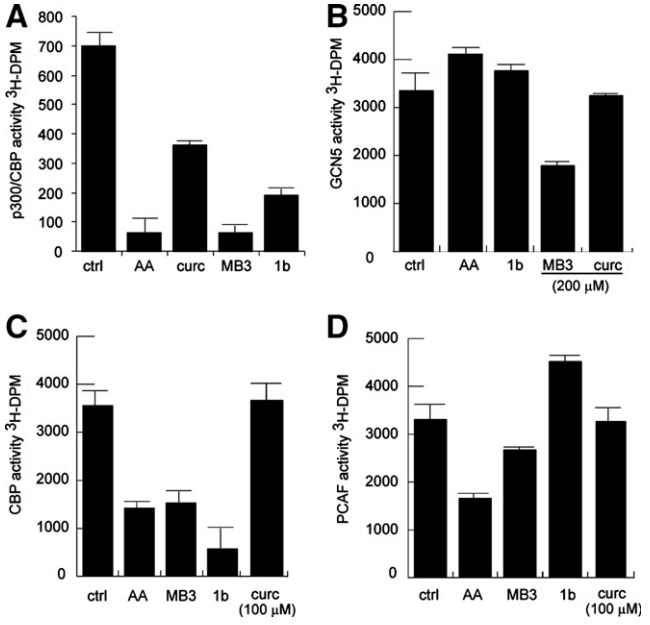


Figure 3. HAT assay performed with 50 μM **1b** on different HATs. (A) p300/CBP immunoprecipitated enzymes from U937 cell nuclear extracts; (B) GCN5; (C) CBP; (D) PCAF. Anacardic acid (AA), curcumin (curc), and MB-3 were used as reference compounds at 50 μM concentrations, except when differently indicated.

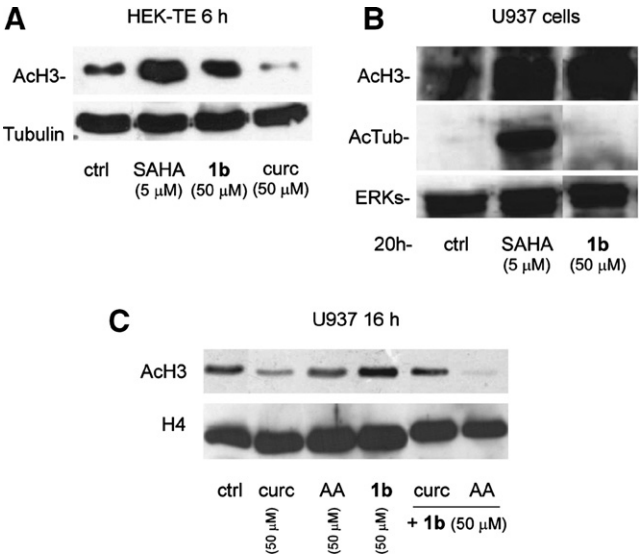


Figure 4. (A) Effects exerted by **1b** (50 μM) on H3 acetylation on Human Kidney Epithelial Cells (HEK-TE). (B) Effects exerted by **1b** (50 μM) on H3 acetylation and on α-tubulin acetylation on U937 leukemia cells. (C) Effects exerted by **1b** and p300 or PCAF inhibitors, alone or in combination, on H3 acetylation in histone extracts of U937 leukemia cells. Total tubulin, total extracellular signal-regulated kinases (ERKs), and total histone H4 were used, respectively, to normalize for equal loading. Anacardic acid (AA, 50 μM), curcumin (curc, 50 μM), and SAHA (5 μM) were used as reference compounds.

H3 as expected. On the contrary, it produced an unpredictable increase of the H3 acetylation level, similar (even if less pronounced) to the one induced by SAHA but without affecting HDAC activity (see Supplementary data).

Similar results were obtained in the human U937 leukemia cell line (Fig. 4B), thus corroborating the hyperacetylating action of **1b** in both cellular models. With the aim to ascertain if this outcome could be at least partially ascribable to the enhancement of PCAF activity emerged from the enzymatic assays, we also treated U937 cells with curcumin (p300 inhibitor)[24] and anacardic acid (p300 and PCAF inhibitor)[22] alone or in combination with 50 μM **1b**. As shown in Figure 4C, the co-treatment with a p300 inhibitor did not affect the H3 hyperacetylation induced by **1b** in this cell line, whereas this effect was barely visible after the co-treatment with a PCAF inhibitor. Note that (Fig. 4B), differently from SAHA that exhibited a marked hyperacetylating effect on both substrates, compound **1b** produced in the U937 cell line a significant increase of the H3 acetylation level but no detectable effect on the acetylation level of the non-histone substrate α-tubulin. This is particularly interesting as although acetylation of α-tubulin was found in mammalian cells almost two decades ago, the responsible acetyltransferase remains unidentified.[37]

In summary, we have identified pentadecylidenemalonate **1b**, a simplified analogue of anacardic acid, as the first activator/inhibitor of histone acetyltransferases. Although rather un-drug-like, its unique activity profile together with the powerful apoptotic effect (particularly notable if considering that anacardic acid is not cell per-

meable) designate this compound as a valuable biological tool to understand the mechanisms of HAT enzymes and may also prelude to a novel group of compounds for antineoplastic therapeutics.

Acknowledgments

This work was partially supported by Grants Regione Campania Grant 2003 LR 5/02 (G.S.), PRIN 2006052835_003 (L.A.), Regione Campania Grant 2005 LR 5/02 (L.A.), and AIRC 2007 (A.M.), PRIN 2006038137_005 (A.M.). We are grateful to Dr. W.C. Hahn for the kind gift of HEK-TE cells.

Supplementary data

Experimental chemical and biological procedures, characterization data for compounds **1**–**6** and for intermediates **7**–**9** and additional graphs. This material is available free of charge via the Internet at http://www.sciencedirect.com. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.04.017.

References and notes

1. A number of scientists have recently proposed to rationalize the nomenclature for all the characterized members of the families of lysine demethylases, acetyltransferases, and lysine methyltransferases. We agree with the reasons supporting this proposal, yet, to avoid confusion, in this paper we preferred to use the current nomenclature. For the new one see: Allis, C. D.; Berger, S. L.; Cote, J.; Dent, S.; Jenuwein, T.; Kouzarides, T.; Pillus, L.; Reinberg, D.; Shi, Y.; Shiekhattar, R.; Shilatifard, A.; Workman, J.; Zhang, Y. *Cell* **2007**, *131*, 633.
2. Lee, K. K.; Workman, J. L. *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 284.
3. Carrozza, M. J.; Utley, R. T.; Workman, J. L.; Cote, J. *Trends Genet.* **2003**, *19*, 321.
4. Minucci, S.; Pelicci, P. G. *Nat. Rev. Cancer* **2006**, *6*, 38.
5. Peterson, C. L.; Laniel, M. A. *Curr. Biol.* **2004**, *14*, R546.
6. Liu, Y.; Colosimo, A. L.; Yang, X.-J.; Liao, D. *Mol. Cell. Biol.* **2000**, *20*, 5540.
7. Hubbert, C.; Guardiola, A.; Shao, R.; Kawaguchi, Y.; Ito, A.; Nixon, A.; Yoshida, M.; Wang, X. F.; Yao, T. P. *Nature* **2002**, *417*, 455.
8. Mai, A.; Massa, S.; Rotili, D.; Cerbara, I.; Valente, S.; Pezzi, R.; Simeoni, S.; Ragno, R. *Med. Res. Rev.* **2005**, *25*, 261.
9. Richon, V. M.; Emiliani, S.; Verdin, E.; Webb, Y.; Breslow, R.; Rifkind, R. A.; Marks, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3003.
10. Kouzarides, T. *Curr. Opin. Genet. Dev.* **1999**, *9*, 40.
11. Timmermann, S.; Lehrmann, H.; Polesskaya, A.; Harel-Bellan, A. *Cell Mol. Life Sci.* **2001**, *58*, 728.
12. Anzick, S. L.; Kononen, J.; Walker, R. L.; Azorsa, D. O.; Tanner, M. M.; Guan, X. Y.; Sauter, G.; Kallioniemi, O. P.; Trent, J. M.; Meltzer, P. S. *Science* **1997**, *277*, 965.
13. Sakakura, C.; Hagiwara, A.; Yasuoka, R.; Fujita, Y.; Nakanishi, M.; Masuda, K.; Kimura, A.; Nakamura, Y.; Inazawa, J.; Abe, T.; Yamagishi, H. *Int. J. Cancer* **2000**, *89*, 217.
14. Aguiar, R. C.; Chase, A.; Coulthard, S.; Macdonald, D. H.; Carapeti, M.; Reiter, A.; Sohal, J.; Lennard, A.; Goldman, J. M.; Cross, N. C. *Blood* **1997**, *90*, 3130.
15. Panagopoulos, I.; Fioretos, T.; Isaksson, M.; Isaksson, M.; Samuelsson, U.; Billstrom, R.; Strombeck, B.; Mitelman, F.; Johansson, B. *Hum. Mol. Genet.* **2001**, *10*, 395.
16. Muraoka, M.; Konishi, M.; Kikuchi-Yanoshita, R.; Tanaka, K.; Shitara, N.; Chong, J. M.; Iwama, T.; Miyaki, M. *Oncogene* **1996**, *12*, 1565.
17. Gayther, S. A.; Batley, S. J.; Linger, L.; Bannister, A.; Thorpe, K.; Chin, S. F.; Daigo, Y.; Russell, P.; Wilson, A.; Sowter, H. M.; Delhanty, J. D.; Ponder, B. A.; Kouzarides, T.; Caldas, C. *Nat. Genet.* **2000**, *24*, 300.
18. Ionov, Y.; Matsui, S.; Cowell, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 1273.
19. Lehrmann, H.; Pritchard, L. L.; Harel-Bellan, A. *Adv. Cancer Res.* **2002**, *86*, 41.
20. Zheng, Y.; Thompson, P. R.; Cebrat, M.; Wang, L.; Devlin, M.; Alani, R. M.; Cole, P. A. *Methods Enzymol.* **2004**, *376*, 188.
21. Lau, O. D.; Kundu, T. K.; Soccio, R. E.; Ait-Si-Ali, S.; Khalil, E. M.; Vassilev, A.; Wolffe, A. P.; Nakatani, Y.; Roeder, R. G.; Cole, P. A. *Mol. Cell* **2000**, *5*, 589.
22. Balasubramanyam, K.; Swaminathan, V.; Ranganathan, A.; Kundu, T. K. *J. Biol. Chem.* **2003**, *278*, 19134.
23. Balasubramanyam, K.; Altaf, M.; Varier, R. A.; Swaminathan, V.; Ravindran, A.; Sadhale, P. P.; Kundu, T. K. *J. Biol. Chem.* **2004**, *79*, 33716.
24. Balasubramanyam, K.; Varier, R. A.; Altaf, M.; Swaminathan, V.; Siddappa, N. B.; Ranga, U.; Kundu, T. K. *J. Biol. Chem.* **2004**, *279*, 51163.
25. Stimson, L.; Rowlands, M. G.; Newbatt, Y. M.; Smith, N. F.; Raynaud, F. I.; Rogers, P.; Bavetsias, V.; Gorsuch, S.; Jarman, M.; Bannister, A.; Kouzarides, T.; McDonald, E.; Workman, P.; Aherne, G. W. *Mol. Cancer Ther.* **2005**, *4*, 1521.
26. Biel, M.; Kretsovali, A.; Karatzali, E.; Papamatheakis, J.; Giannis, A. *Angew. Chem. Int. Ed.* **2004**, *43*, 3974.
27. Mai, A.; Rotili, D.; Tarantino, D.; Ornaghi, P.; Tosi, F.; Vicidomini, C.; Sbardella, G.; Nebbioso, A.; Miceli, M.; Altucci, L.; Filetici, P. *J. Med. Chem.* **2006**, *49*, 6897.
28. Mantelingu, K.; Reddy, B. A. A.; Swaminathan, V.; Kishore, A. H.; Siddappa, N. B.; Kumar, G. V. P.; Nagashankar, G.; Natesh, N.; Roy, S.; Sadhale, P. P.; Ranga, U.; Narayana, C.; Kundu, T. K. *Chem. Biol.* **2007**, *14*, 645.
29. Massa, S.; Mai, A.; Sbardella, G.; Esposito, M.; Ragno, R.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2001**, *44*, 2069.
30. Mai, A.; Massa, S.; Ragno, R.; Esposito, M.; Sbardella, G.; Nocca, G.; Scatena, R.; Jesacher, F.; Loidl, P.; Brosch, G. *J. Med. Chem.* **2002**, *45*, 1778.
31. Bartolini, S.; Mai, A.; Artico, M.; Paesano, N.; Rotili, D.; Spadafora, C.; Sbardella, G. *J. Med. Chem.* **2005**, *48*, 6776.
32. Sbardella, G.; Bartolini, S.; Castellano, S.; Artico, M.; Paesano, N.; Rotili, D.; Spadafora, C.; Mai, A. *ChemMedChem* **2006**, *1*, 1073.
33. Ragno, R.; Simeoni, S.; Castellano, S.; Vicidomini, C.; Mai, A.; Caroli, A.; Tramontano, A.; Bonaccini, C.; Trojer, P.; Bauer, I.; Brosch, G.; Sbardella, G. *J. Med. Chem.* **2007**, *50*, 1241.
34. Varier, R. A.; Swaminathan, V.; Balasubramanyam, K.; Kundu, T. K. *Biochem. Pharmacol.* **2004**, *68*, 1215.
35. Dess, D. B.; Martin, J. C. *J. Am. Chem. Soc.* **1991**, *113*, 7277.
36. Allinson, G.; Bushby, R. J.; Jesudason, M. V.; Paillaud, J.-L.; Taylor, N. *J. Chem. Soc., Perkin Trans. 2* **1997**, *2*, 147.
37. Glozak, M. A.; Sengupta, N.; Zhang, X.; Seto, E. *Gene* **2005**, *363*, 15.