Amino Acids (2014) 46:585–594
DOI 10.1007/s00726-013-1485-1

ORIGINAL ARTICLE

The re-expression of the epigenetically silenced *e-cadherin* gene by a polyamine analogue lysine-specific demethylase-1 (LSD1) inhibitor in human acute myeloid leukemia cell lines

Tracy Murray-Stewart · Patrick M. Woster · Robert A. Casero Jr.

Received: 15 January 2013 / Accepted: 26 February 2013 / Published online: 19 March 2013

Abstract Aberrant epigenetic silencing of tumor suppressor genes is a common feature observed during the transformation process of many cancers, including those of hematologic origin. Histone modifications, including acetylation, phosphorylation, and methylation, collaborate with DNA CpG island methylation to regulate gene expression. The dynamic process of histone methylation is the latest of these epigenetic modifications to be described, and the identification and characterization of LSD1 as a demethylase of lysine 4 of histone H3 (H3K4) has confirmed that both the enzyme and the modified histone play important roles as regulators of gene expression. LSD1 activity contributes to the suppression of gene expression by demethylating promoter-region mono- and dimethyl-H3K4 histone marks that are associated with active gene expression. As most post-translational modifications are reversible, the enzymes involved in the modification of histones have become targets for chemotherapeutic intervention. In this study, we examined the effects of the polyamine analogue LSD1 inhibitor **2d** (1,15-bis{N^5-[3,3-(diphenyl)propyl]-N^1-biguanido}-4,12-diazapentadecane) in human acute myeloid leukemia (AML) cell lines. In each line studied, **2d** evoked cytotoxicity and inhibited LSD1 activity, as evidenced by increases in the global levels of mono- and di-methylated H3K4 proteins. Global increases in other chromatin modifications were also observed following exposure to **2d,** suggesting a broad response to this compound with respect to chromatin regulation. On a gene-specific level, treatment with **2d** resulted in the re-expression of *e-cadherin*, a tumor suppressor gene frequently silenced by epigenetic modification in AML. Quantitative chromatin immunoprecipitation analysis of the *e-cadherin* promoter further confirmed that this re-expression was concurrent with changes in both active and repressive histone marks that were consistent with LSD1 inhibition. As hematologic malignancies have demonstrated promising clinical responses to agents targeting epigenetic silencing, this polyamine analogue LSD1 inhibitor presents an exciting new avenue for the development of novel therapeutic agents for the treatment of AML.

Keywords Histone · Methylation · Chromatin · Epigenetic · CDH-1 · Polyamine

T. Murray-Stewart · R. A. Casero Jr. (✉)
The Sidney Kimmel Comprehensive Cancer Center,
Johns Hopkins University, Bunting Blaustein Bldg,
Room 551, 1650 Orleans Street, Baltimore, MD 21287, USA
e-mail: rcasero@jhmi.edu

P. M. Woster
The Department of Drug Discovery and Biomedical Sciences,
The Medical University of South Carolina,
Charleston, SC 29425, USA

Introduction

Epigenetic regulation of gene expression involves hypermethylation of DNA promoter region CpG islands with subsequent binding of methyl-CpG binding proteins and recruitment of histone-modifying proteins. These histone-modifying enzymes catalyze post-translational modifications of specific residues on the N-terminal tails of histone proteins, including phosphorylation, acetylation, ubiquitination, and methylation, thereby affecting the accessibility of local chromatin structure. The combination of these histone "marks" at a given promoter, along with DNA methylation, ultimately regulates gene transcription (Lachner et al. 2003; Jenuwein and Allis 2001), and tumor

cells have been shown to alter these modifications as a means to evade growth, repair, and death control mechanisms. Aberrant epigenetic silencing of tumor suppressor genes is associated with tumorigenesis in most cancer types and is one of the most common molecular changes associated with hematopoietic neoplasia. These observations, along with the fact that epigenetic changes are typically the result of reversible post-translational modifications, suggest the utility of strategies reversing these modifications in the treatment of cancer. Therefore, several classes of "epi-drugs" have been developed to target specific enzymes that modify chromatin with the goal of restoring the natural growth regulatory pathways of tumor cells through the re-expression of tumor suppressor genes, rather than through overt tumor cell toxicity.

The lysine-specific histone demethylase LSD1 (KDM1, AOF2/BHC110/KIAA0601) is a member of a growing family of proteins that mediate the removal of methyl groups from specific lysine residues on the N-terminal tail of histone protein H3. Recent studies have suggested that LSD1 can act as either a repressor or facilitator of transcription, in a context-dependent manner (Wang et al. 2007). Originally identified as a member of several corepressor complexes (Humphrey et al. 2001; Shi et al. 2003; Hakimi et al. 2002; Tong et al. 1998; You et al. 2001), LSD1 specifically catalyzes the demethylation of mono- and dimethyl lysine 4 of histone H3 (H3K4me1 and H3K4me2) via an FAD-dependent amine oxidase reaction (Shi et al. 2004, 2005; Lee et al. 2005). Because H3K4me2 is a positive chromatin mark associated with the promoters of active genes (Liang et al. 2004; Schneider et al. 2004), LSD1 activity in this capacity is correlated with transcriptional repression, and pharmacologic inhibition of LSD1 in human colon, lung, and breast cancer cell lines has confirmed this correlation (Huang et al. 2007; Sharma et al. 2010; Zhu et al. 2012). In contrast, evidence also exists linking LSD1 activity to transcriptional activation via nuclear hormone receptors (Garcia-Bassets et al. 2007; Metzger et al. 2005; Wissmann et al. 2007). In this context, LSD1 has been proposed to function with the receptor complex to demethylate lysine 9 of histone H3 (H3K9), resulting in a facilitation of the transcription of nuclear receptor-mediated target genes. In either regard, it is evident that LSD1 has the potential to be a critical regulator of gene expression via modulation of chromatin structure. Importantly, LSD1 expression and activity have been found to be elevated in multiple cancers, again underscoring the potential of LSD1 as an antitumor target (Lim et al. 2010; Schulte et al. 2009).

The role of LSD1 as a repressor of transcription and the fact that in cancer, one of its substrates, H3K4me2, is depleted in the promoters of genes silenced by aberrant DNA methylation (McGarvey et al. 2006) suggests the potential of inhibiting LSD1 activity as a means of re-expressing these genes for therapeutic intervention. Because the LSD1 protein is significantly homologous to the FAD-dependent polyamine oxidases (Shi et al. 2004; Wang et al. 2001), polyamine analogue-based LSD1 inhibitors have been developed using the structures of known polyamine oxidase inhibitors as starting points (Federico et al. 2001; Bianchi et al. 2006; Sharma et al. 2010). The recently described biguanide compound **2d** (1,15-bis{N^5-[3,3-(diphenyl)propyl]-N^1-biguanido}-4,12-diazapentadecane) (Fig. 1a) has proven to be an effective inhibitor of LSD1 in human colon cancer cell lines, with treatment resulting in increased activating marks (H3K4me1 and H3K4me2) at the promoters of several epigenetically silenced genes important for cancer development. Importantly, these activating chromatin marks accompanied an ultimate re-expression of several aberrantly silenced genes, including members of the *GATA* family of transcription factors, as well as the secreted frizzled-related proteins (*SFRP*s), which are antagonists of the Wnt signaling pathway (Huang et al. 2007).

Epigenetic changes are common in hematopoietic malignancies, where the clinical effects of potential epi-drug therapies have been more comprehensively studied than in solid tumors. Acute myeloid leukemia (AML) is a highly malignant hematopoietic neoplasm that is the leading cause of death due to cancer in children and persons younger than 39 years of age. AML is the most common leukemia in adults and has the lowest survival rate of all leukemia types, with prognosis worsening with age of diagnosis (median survival without treatment of 11–20 weeks) (Deschler and Lubbert 2006). The frequent, aberrant, epigenetic silencing of multiple genes by promoter CpG island hypermethylation, including tumor suppressor genes, genes involved in cell cycle regulation, and adhesion molecule genes, has been well-documented in patients with myeloid leukemia (Toyota et al. 2001), and oncogenic fusion proteins that occur frequently as a cause of acute leukemia have been linked to the aberrant recruitment of DNA methyltransferases (DMNTs) (Di Croce et al. 2002) and histone deacetylases (HDACs) (Wang et al. 1998; Heibert et al. 2001) to target promoters. AML clinical trials with previously identified epi-drugs, including DNMT and HDAC inhibitors, have provided promising results, but with significant limitations. We therefore sought to investigate the therapeutic potential of the cellular inhibition of LSD1 by the polyamine analogue **2d** in established AML cell lines.

Materials and methods

Cell lines, culture conditions, and chemicals

The human AML cell lines ML-1, HNT-34, and KG1a and the acute promyelocytic leukemia cell line (APL) HL-60

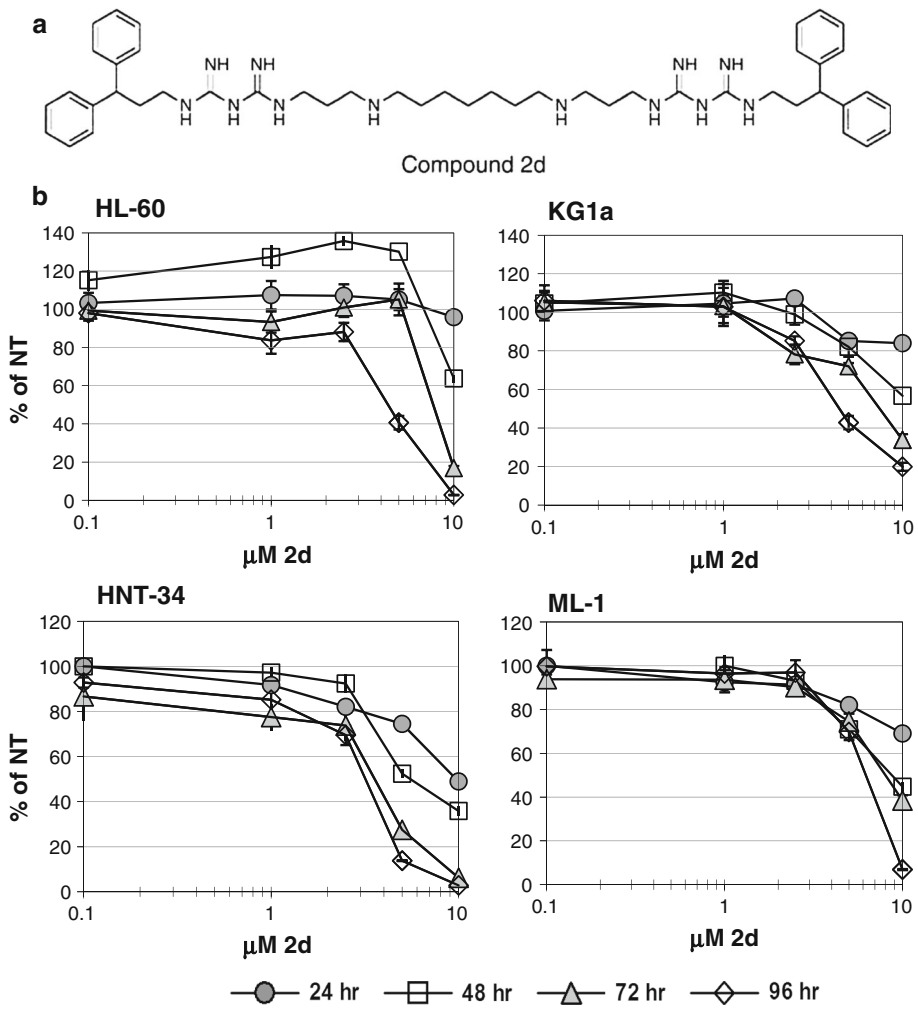


Fig. 1 **a** Chemical structure of compound **2d**. **b** Effects of **2d** on AML cell growth in culture. HL-60, KG1a, HNT-34, and ML-1 cells were seeded and treated in quadruplicate wells of 96-well plates and incubated for 24, 48, 72, or 96 h. At the specified times, plates were incubated with MTS reagent at 37 °C in 5 % CO_2 atmosphere for 1.5 h, and absorbance in individual wells was determined at A_{490}. Results are presented as the percentage of viable cells relative to cells not receiving treatment with **2d**, with each point representing the average of 2 separate biological experiments ± range

were maintained in RPMI 1640 medium containing 9 % fetal bovine serum, penicillin, and streptomycin, at 37 °C, 5 % CO_2. Compound **2d** was synthesized as previously described (Bi et al. 2006). Custom primers were synthesized by Invitrogen (Carlsbad, CA).

Cytoproliferation assays

HL-60, HNT-34, and ML-1 cells were seeded in quadruplicate at 5,000 cells per well of 96-well plates in 50 μL of medium. KG1a cells were seeded at 10,000 cells per well. At the appropriate time points, 50 μL of fresh medium containing the appropriate concentration of **2d** was added per well. Following incubation, 20 μL of CellTiter96 aqueous one solution cell proliferation assay reagent (Promega, Madison, WI) was added per well, plates were incubated 1.5 h at 37 °C, and absorbance was measured at 490 nm. Wells containing medium with **2d**, but without cells, were used as background controls.

Western blotting

ML-1, HL-60, KG1a, and HNT-34 cells were seeded at 2×10^5 cells/mL of medium. The LSD1 inhibitor **2d** was added at concentrations of 5 and 10 μM and incubated at 37 °C for 24 or 48 h. For Western blot analysis of specific histone modifications, nuclear protein was harvested using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL), and protein was quantified using the BioRad DC assay (Hercules, CA) with absorbance measured at 750 nm. Absorbance was converted to protein concentration using interpolation on a bovine serum albumin standard curve. Nuclear proteins (20 μg per lane) were separated on pre-cast 10 % Bis–Tris Novex gels with 1× MES running buffer (Invitrogen) and transferred onto Immun-Blot PVDF membranes (BioRad). Blots were blocked overnight at 4 °C in Odyssey blocking buffer (LI-COR, Lincoln, NE), and proteins of interest were visualized using the following antibodies: H3K4me1, H3K4me2,

H3K4me3, H3K9me1, H3K9me2, H3K9me3, AcH3K9, and H3K36me3 (all from Millipore, Billerica, MA), with Proliferating Cell Nuclear Antigen (PCNA) (Calbiochem, La Jolla, CA) as a loading control in all experiments except for the 48-h treatment of HL-60 cells with **2d**. Following washes, blots were incubated with species-specific, fluorophore-conjugated secondary antibodies to allow the visualization and quantification of immunoreactive proteins using the Odyssey infrared detection system and software (LI-COR).

RNA extraction and gene expression analysis

For gene re-expression studies using RT-PCR, cells were seeded and treated in parallel with those described above for nuclear protein. Following incubation with **2d**, total RNA was extracted using TRIzol reagent (Invitrogen) according to the provided protocol. RNA was treated with DNAse I, and cDNA was synthesized using the SuperScript III First Strand Synthesis System (Invitrogen) with oligo-$d(T)_{20}$ as the primer. PCR primers were as follows: *CDH-1,* 5′-CAA TCC CAC CAC GTA CAA G-3′ (sense) and 5′-CCT GGG CAG TGT AGG ATG TGA-3′ (antisense); and *GapDH,* 5′-GAA GAT GGT GAT GGG ATT TC-3′ (sense) and 5′-GAA GGT GAA GGT CGG AGT C-3′ (antisense). A total of 40 cycles of amplification were performed for each of the RT-PCR experiments. *GapDH* was amplified as an internal control. Amplified products were analyzed on 2 % agarose gels with GelStar staining (Lonza, Walkersville, MD).

Quantitative ChIP analysis of gene promoter-specific chromatin marks

HL-60 and KG1a cells were seeded and treated with 10 μM **2d** for 24 or 48 h, respectively. Following incubation, the total cell number of each condition was determined using Trypan blue exclusion. Cells were exposed to formaldehyde (30 min at room temperature) to cross-link proteins, rinsed with PBS, pelleted, and frozen at −80 °C. For the assay, cell pellets were thawed on ice and resuspended in lysis buffer at a concentration of 1×10^7 cells/mL. Aliquots of 400 μL were sonicated eight times for 10 s each using a duty setting of 2.5 and 40 % output. The sonicated lysates were divided into 100-μL aliquots for ChIP assays (1×10^6 cells per IP) using the reagents and protocol provided in the EZ-ChIP Assay Kit (Millipore). All modified histone antibodies for immunoprecipitation of DNA–protein complexes were used at concentrations of 1 μg per IP and were the same as those described for Western blotting. Chromatin eluted from IPs with IgG was used as a negative control, and chromatin immunoprecipitated with an antibody to pan histone H3 (Abcam, Cambridge, MA) was used as a positive control for normalization. Four previously described primer pairs (Li et al. 2006; Ting et al. 2005) tiling −568 toof the transcriptional start site of the *CDH-1* gene were used for SYBR green-mediated qPCR (Quanta Biosciences, Gaithersburg, MD) detection and quantification of eluted DNA on a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System. PCR products were also visualized on 2 % agarose gels using GelStar stain and KODAK Digital Science Image Analysis Software (Rochester, NY).

Analysis of the polyamine metabolic pathway in response to **2d**

HL-60 and KG1a cells were treated for 24 and 48 h with 10 μM **2d**. Cells were collected and assayed for ornithine decarboxylase (ODC) enzymatic activity as previously described (Seely and Pegg 1983). Samples were also assayed for total protein content using the method of Bradford (Bradford 1976), and intracellular polyamine concentrations were determined by HPLC following pre-column dansylation as described by Kabra et al. (1986).

Results

Cytoproliferative responses of AML cells to **2d** exposure

The representative AML cell lines HL-60, KG1a, HNT-34, and ML-1 were treated with increasing doses of **2d**, and growth response was evaluated every 24 h over a 96-h period (Fig. 1b). Each of the four cell lines exhibited significant growth inhibition over the 96-h exposure, with HNT-34 appearing to be the most sensitive to the anti-proliferative effects of **2d**. In HL-60 cells, no growth inhibition was detected within 24 h, and at 48 h, only the maximum dose (10 μM) of **2d** produced any effect (~40 % reduction in viable cells). It should be noted, however, that the HL-60 cells consistently demonstrated slightly increased proliferation in response to lower doses of **2d**. All cell lines exhibited nearly complete cytotoxicity by the end of the 96-h treatment.

Global epigenetic chromatin modifications increase in AML cell lines following treatment with **2d**

To determine if compound **2d** is capable of LSD1 inhibition in cells of AML origin, each of the four cell lines were treated with increasing doses of the compound for 24 h. In each cell line, this exposure resulted in significant increases in H3K4me2 protein within 24 h (Fig. 2a). The HNT-34 and ML-1 lysates consistently demonstrated 2 bands

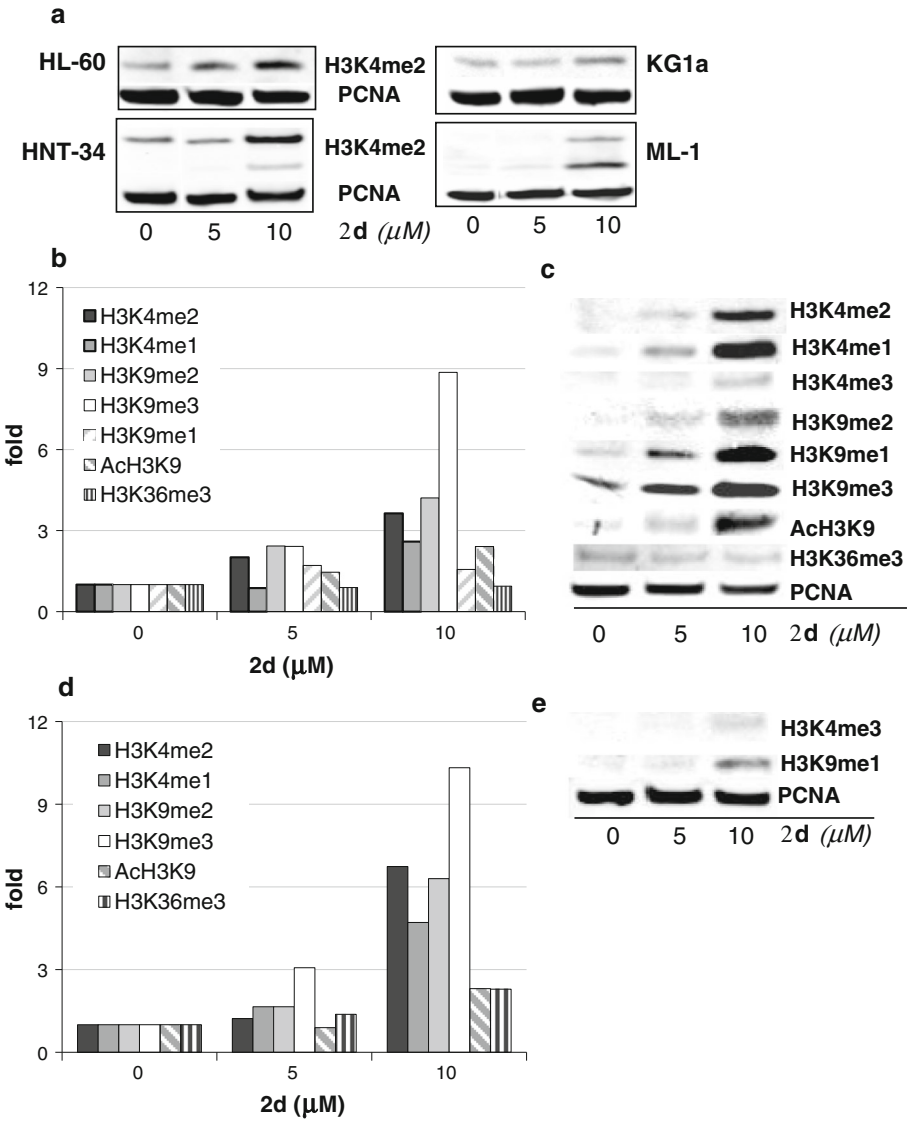


Fig. 2 Treatment with **2d** alters global chromatin marks in HL-60, KG1a, HNT-34, and ML-1 cells. Cells were treated for 24 or 48 h with 5 or 10 μM **2d** and harvested for nuclear protein. Twenty micrograms of protein were separated per lane, transferred to PVDF, and immunoblotted with antibodies specific for the individual chromatin modifications. **a** Western blots depict increases in global levels of dimethylated H3K4 following 24 h of treatment with **2d** in each cell line. An antibody to PCNA is used as a loading control. **b** The histogram represents global induction of epigenetically modified histone proteins in HL-60 cells following 24-h **2d** treatment as fold increase over untreated cells, relative to PCNA, in a representative experiment. Results were quantified using Odyssey infrared detection with fluorophore-conjugated secondary antibodies on Western blots. **c** Histone modification immunoblots of HL-60 nuclear lysates following treatment with **2d** for 48 h. **d** Histogram representing quantitative Western blots of global changes in histone H3 modifications following 48-h exposure of KG1a cells to **2d**. **e** Representative Western blots of KG1a lysates in **d**, showing global increases in additional epigenetic chromatin modifications that were not detectable prior to **2d** exposure, and therefore could not be quantified. All Western blots were repeated at least 2 times with similar results

corresponding to H3K4me2, possibly due to a degradation product. The HL-60 and KG1a cell lines were therefore chosen for further studies, and lysates were evaluated for additional epigenetically modified histone proteins, as indicated in Fig. 2, panels **b** through **e**. Surprisingly, in addition to the expected LSD1 substrates H3K4me2 and H3K4me1, treatment of HL-60 (Fig. 2b, c) or KG1a (Fig. 2d, e) cells resulted in substantially increased levels of acetylated, and mono-, di-, and trimethylated H3K9. Levels of H3K4me3 were not detectable in HL-60 cells at 24 h, but extending the treatment time to 48 h led to a detectable increase (Fig. 2c). HL-60 cells exhibited dramatic increases in nearly all histone modifications analyzed at 48 h; however, it should be noted that significant decreases in PCNA levels were detected at this time point, suggesting an association with cell death. Levels of β-actin

were analyzed as a potential alternative loading control, however, these also decreased with treatment at 48 h. The levels of the histone modifications in HL-60 cells at 48 h were therefore not quantified and representative blots are presented as Fig. 2c. Levels of PCNA were unchanged in all other experiments. These data are in contrast to those previously reported in HCT116 human colon carcinoma cells treated with **2d**, which demonstrated no change in global levels of methylated or acetylated H3K9 over a 48-h treatment period (Huang et al. 2007).

Re-expression of the aberrantly silenced e-cadherin gene by **2d**

Because H3K4me2 is frequently depleted in the promoter regions of transcriptionally inactive genes (McGarvey et al. 2006), we sought to determine if the increase in global H3K4 methylation observed following **2d** exposure correlated with the re-expression of a candidate epigenetically silenced gene in these AML cell lines. The *CDH-1* gene, which encodes e-cadherin, is aberrantly silenced by CpG island methylation in many types of cancers including leukemia (Melki et al. 1999, 2000; Corn et al. 2000), and RT-PCR analysis failed to detect a CDH-1 transcript in untreated KG1a or HL-60 cells. However, treatment of each cell line with 10 μM **2d** revealed a substantial re-expression of CDH-1 mRNA within 24 h for HL-60 cells and within 48 h for KG1a cells (Fig. 3). This re-expression was diminished in HL-60 cells when exposure time was increased to 48 h, possibly relating to the growth inhibitory and/or cytotoxic effects suggested by the decreased PCNA levels observed at this time (Fig. 2c).

Altered promoter occupancy by active and repressive chromatin marks following treatment with **2d**

To further confirm that the re-expression of CDH-1 by **2d** is related to its ability to inhibit LSD1, we performed quantitative ChIP assays to determine the changes in histone H3 modifications at various loci across the *CDH-1* promoter of HL-60 cells (Fig. 4a). As shown in Fig. 4b, a 24-h exposure to **2d** significantly increased *CDH-1* promoter occupancy by the active transcription mark and substrate of LSD1 H3K4me2. Measurable amounts of H3K4me1 were not detectable in untreated HL-60 cells; however, treatment with **2d** resulted in enrichment of this activating mark, also a substrate of LSD1, as indicated by the detection of a band at promoter region "D" (Fig. 4c). Furthermore, the transcriptionally repressive chromatin mark H3K9me2 was detected at substantial levels in untreated cells at promoter region "D". Treatment with **2d** diminished occupancy by this mark to a point not accurately quantifiable by qPCR. No significant changes in occupancy were observed with H3K4me3 or AcH3K9. These results provide evidence that treatment with **2d** alters the chromatin architecture of the *CDH-1* gene promoter to a more transcriptionally permissive state than that of untreated cells, and these chromatin changes are consistent with the inhibition of LSD1.

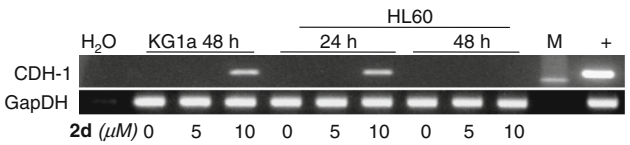


Fig. 3 Re-expression of the cancer-related *CDH-1* gene following exposure to **2d**. HL-60 and KG1a cells were treated for 24 or 48 h with 5 and 10 μM **2d**. RNA was extracted, and cDNA was produced for RT-PCR analysis of CDH-1 expression. GapDH mRNA expression is included as an internal control. "H_2O" refers to RT-PCR without template, and the "+" lane indicates a CDH-1-expressing colon cancer cell line, HCT116, as a positive control. "M" = DNA marker. Results represent a single experiment, repeated at least 3 times with similar results

Changes in polyamine metabolism following exposure to **2d**

Certain polyamine analogues exert their anti-tumor effects via a dramatic up-regulation of polyamine catabolism that is accompanied by a down-regulation of polyamine biosynthesis and transport, consequently depleting the pools of natural polyamines essential for continued proliferation. In addition to regulating growth, depletion of the longer-chain, cationic polyamines spermidine, and spermine has the potential to alter chromatin structure, as these are most commonly found bound to negatively charged molecules such as nucleic acids. We therefore analyzed the effects of treatment with **2d** on polyamine metabolism and the resulting changes in intracellular polyamine pools. As shown in Fig. 5a, 24-h **2d** exposure resulted in a down-regulation of the basal levels of one of the rate-limiting polyamine biosynthetic enzymes, ODC, in both HL-60 and KG1a cell lines, similar to results previously reported using HCT116 colon adenocarcinoma cells (Huang et al. 2007). However, this down-regulation of ODC had little effect on polyamine pools (Fig. 5b). In addition, the spermidine and spermine levels remaining near basal levels throughout the 48-h treatment indicated that not only were the polyamine pools unaffected by the down-regulation of ODC, but polyamine catabolism was not induced by **2d**. These results provide evidence that the cytokinetic effects observed with **2d** exposure are not due to depletion of the natural polyamines, and it is not likely that altered polyamine concentrations are contributing to the observed chromatin changes.

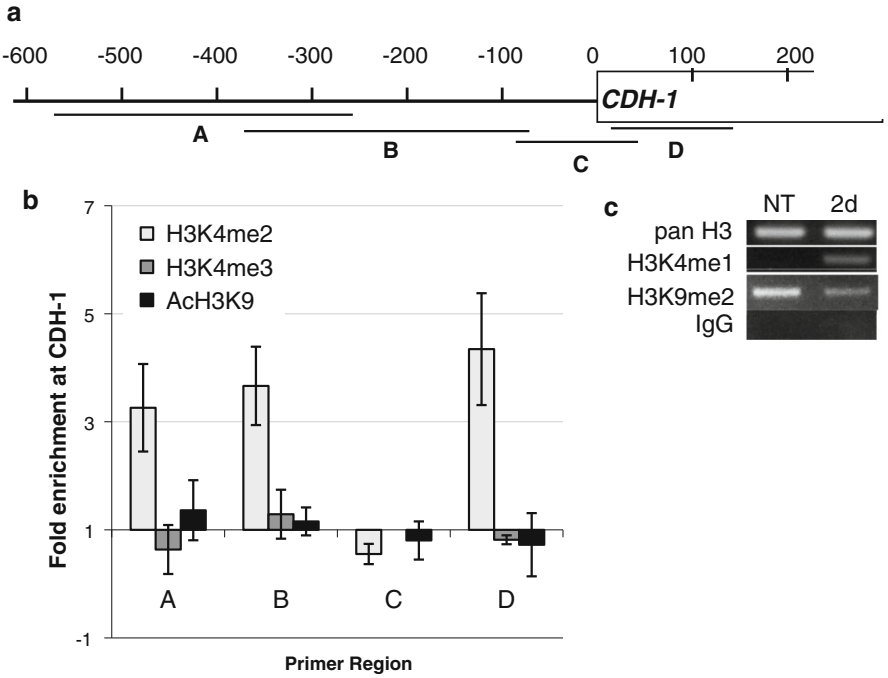
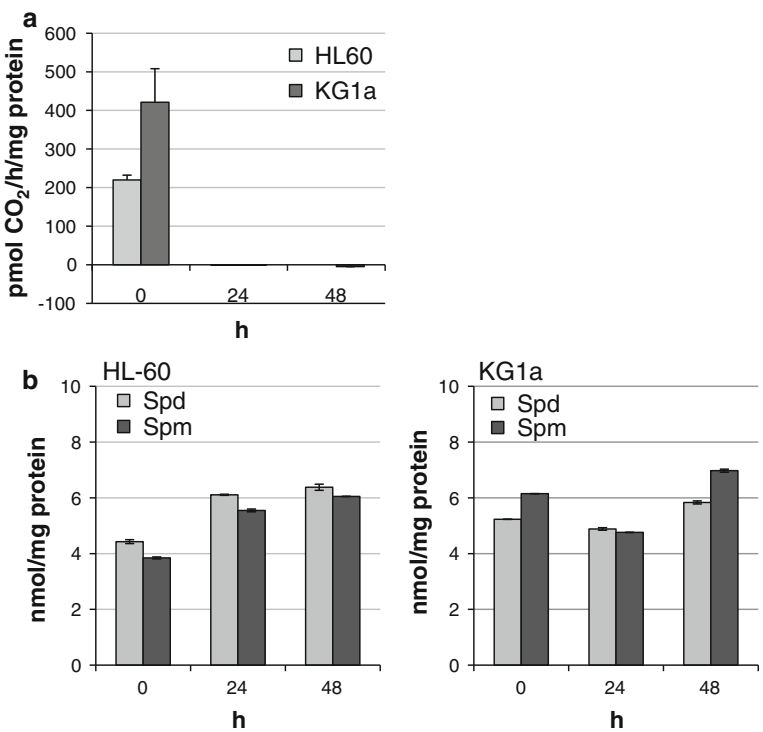


Fig. 4 Treatment with **2d** alters chromatin marks at the *CDH-1* promoter in HL-60 cells. **a** *CDH-1* promoter-tiling primer locations and sizes. ChIP was performed with antibodies to specific histone modifications and quantitative PCR of the *CDH-1* promoter region DNA was performed using primer pairs spanning the following regions: region A (−568 to −280 from the transcriptional start site of *CDH-1*); region B (−362 to −73); region C (−73 to +30); and region D (+13 to +150). (**b**) Results of *CDH-1* ChIP qPCR indicate fold enrichment, relative to untreated cells, of specific chromatin marks at each promoter region following 24-h treatment of HL-60 cells with 10 μM **2d**. **c** Representative agarose gel electrophoresis of HL-60 DNA immunoprecipitated with the indicated antibodies and amplified using primers corresponding to region D of *CDH-1*

Fig. 5 ODC activity is depleted in AML cells treated with **2d**. HL60 and KG1a cells were treated with 10 μM **2d** for 24 or 48 h, respectively. Cells were quick-frozen and assayed for ODC enzyme activity (**a**) and intracellular polyamine pool concentrations (**b**). ODC activity results in **a** represent mean activity levels as pmol of CO_2 generated per milligram of total cellular protein, with error bars depicting standard deviation. Intracellular concentrations of the higher level natural polyamines, spermidine and spermine, are presented in **b** as nmol of polyamine per milligram of total cellular protein. *Error bars* indicate the ranges of samples measured in duplicate



Discussion

The aberrant silencing of tumor suppressor and growth regulatory genes is an attractive target for anticancer therapy as it has become apparent that this silencing is reversible. With the discovery that LSD1 is one of the enzymes responsible for removing transcriptional activating marks and thus has the potential to broadly and aberrantly repress gene expression, it has become a logical target for anticancer therapy (Shi et al. 2004; Liang et al. 2004). In addition, because the active sight of LSD1 is both structurally and catalytically very similar to that of the polyamine oxidases (Wang et al. 2001; Shi et al. 2004), we hypothesized that compounds similar to the polyamine analogues capable of inhibiting the polyamine oxidases would also be capable of inhibiting LSD1; this hypothesis tested true (Huang et al. 2009).

Previous studies with compound **2d** have demonstrated that it effectively inhibited the H3K4 demethylase activity of purified LSD1 protein. Using structurally related, but minimally effective inhibitors for comparison, evaluation of colon cancer cell lines treated with **2d** confirmed the correlation between cellular inhibition of LSD1 and the consequent increase in global H3K4me1/2 levels and gene re-expression (Huang et al. 2007). In the current study, the AML cell lines HL-60 and KG1a also responded to treatment with **2d** by exhibiting substantial global increases in mono- and dimethylated H3K4 protein. However, in contrast to the colon cancer cell lines previously studied, both AML cell lines also demonstrated increases in dimethyl H3K9 protein, a repressive chromatin mark associated with silenced transcription (Daniel et al. 2005). In the MCF-7 breast cancer cell line, it has been shown that LSD1 can act as a demethylase of H3K9me2 when recruited to the promoters of specific estrogen receptor (ERα) target genes (Garcia-Bassets et al. 2007). In addition, when in complex with the androgen receptor (AR), LSD1 has been proposed to be part of a complex that demethylates H3K9me2, thereby facilitating transcription of AR target genes (Metzger et al. 2005; Wissmann et al. 2007). Thus, it might be possible that inhibiting LSD1 with **2d** results in an increase in the H3K9me2 chromatin mark and transcriptional repression of the target genes. It is therefore possible that LSD1 inhibition by **2d** is increasing both H3K4me2 and H3K9me2 abundance when considering the overall changes in total nuclear proteins. These changes are separated however, at the local level, where our ChIP experiments clearly demonstrate that only the H3K4me1/2 substrates are enriched at the *CDH-1* promoter following **2d** exposure, whereas H3K9me2 is diminished. These changes in promoter-region chromatin architecture are entirely consistent with the observed re-expression of e-cadherin mRNA.

At the global protein level, **2d** treatment also increased the abundance of the active chromatin mark AcH3K9. These results were also observed following pharmacologic inhibition or siRNA targeting of LSD1 in breast cancer cells (Huang et al. 2012) and represent the intimate protein interactions between specific members of the HDAC family and LSD1 (Lee et al. 2005; Shi et al. 2005; Lan et al. 2007).

Although well established as a suppressor of metastasis in epithelial cancers (Karayiannakis et al. 1998; Sulzer et al. 1998; Zheng et al. 1999), the precise role of e-cadherin in hematopoiesis and AML has yet to be fully determined. It is clear however that aberrant hypermethylation of the *e-cadherin* gene is a common event in AML (Corn et al. 2000; Melki et al. 1999, 2000; Shimamoto et al. 2005; Ekmekci et al. 2004). Shimamoto et al. (2005) have demonstrated significantly elevated numbers of leukemic cells in AML patients harboring methylated *e-cadherin* alleles, suggesting a correlation between the silencing of *e-cadherin* expression and an enhanced migration of hematopoietic cells. The e-cadherin protein has been implicated in the interactions between hematopoietic progenitor cells and bone marrow stromal cells, and recent studies have demonstrated that low expression of e-cadherin can decrease adhesion of progenitors to stromal cells, thereby providing a growth advantage, enhancing colony formation, and increasing the likelihood of cell transformation (Rao et al. 2010).

Several reports now exist suggesting a hypermethylator phenotype in AML, in which a subset of tumor-type specific genes including *e-cadherin*, are concurrently silenced via DNA hypermethylation (Ekmekci et al. 2004; Melki et al. 1999; Toyota et al. 2001). Furthermore, clinical studies have determined that acute myeloid leukemia in pediatric patients, young adults, and older adults are very different diseases. Toyota et al. (2001) have described an inverse correlation between the frequency of hypermethylation of a subset of genes in AML and the age of the patient, while Ekmekci et al. (2004) found a greater number of certain methylated genes in young adults than in children or older patients. Both of these studies suggest the benefit of targeting epigenetic therapies to certain subsets of patients. The fact that treatment with compound **2d** results in an increase in the activating chromatin mark H3K4me2 and the re-expression of one of these aberrantly silenced genes validates its potential as a therapeutic agent in the treatment of AML.

In AML clinical trials, DNMT inhibitors, including 5-azacitidine, have shown 30–60 % response rates (Issa et al. 2004; Gore et al. 2006; Silverman et al. 2002); however, the promising effects of these compounds are potentially limited by their own cytotoxicity and the possibility of genomic instability due to hypomethylation

(Eden et al. 2003; Gaudet et al. 2003). Pre-clinical studies in solid tumor models employing the polyamine analogue-based LSD1 inhibitors have demonstrated increased efficacy with regard to tumor suppressor gene re-expression and tumor growth inhibition when used in combination with other inhibitors of epigenetic modifications, including DNMT and HDAC inhibitors. It is likely that an investigation of these combinations in hematopoietic malignancies may have a similar outcome, thus enabling the use of less toxic doses, potentially stimulating the expression of additional aberrantly silenced tumor suppressor genes, and ultimately suppressing tumor growth. Furthermore, LSD1 inhibition by **2d** has recently been shown to reactivate the all-*trans*-retinoic acid (ATRA) differentiation pathway in non-APL AML, potentially allowing for effective ATRA-treatment for patients not in the APL subtype (Schenk et al. 2012).

In summary, through the inhibition of LSD1 and the associated remodeling of local chromatin, the polyamine analogue **2d** induces the re-expression of the aberrantly silenced *e-cadherin* tumor suppressor gene, thereby validating LSD1 as a potential therapeutic target in the treatment of AML.

Acknowledgments This work was funded by NIH Grants CA51085, CA98454, CA149095, and the Samuel Waxman Cancer Research Foundation.

References

Bi X, Lopez C, Bacchi CJ, Rattendi D, Woster PM (2006) Novel alkylpolyaminoguanidines and alkylpolyaminobiguanides with potent antitrypanosomal activity. Bioorg Med Chem Lett 16(12):3229–3232

Bianchi M, Polticelli F, Ascenzi P, Botta M, Federico R, Mariottini P, Cona A (2006) Inhibition of polyamine and spermine oxidases by polyamine analogues. FEBS J 273(6):1115–1123

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Corn PG, Smith BD, Ruckdeschel ES, Douglas D, Baylin SB, Herman JG (2000) E-cadherin expression is silenced by 5′ CpG island methylation in acute leukemia. Clin Cancer Res 6(11):4243–4248

Daniel JA, Pray-Grant MG, Grant PA (2005) Effector proteins for methylated histones: an expanding family. Cell Cycle 4(7):919–926

Deschler B, Lubbert M (2006) Acute myeloid leukemia: epidemiology and etiology. Cancer 107(9):2099–2107

Di Croce L, Raker VA, Corsaro M, Fazi F, Fanelli M, Faretta M, Fuks F, Lo Coco F, Kouzarides T, Nervi C, Minucci S, Pelicci PG (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science 295(5557):1079–1082

Eden A, Gaudet F, Waghmare A, Jaenisch R (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. Science 300(5618):455

Ekmekci CG, Gutierrez MI, Siraj AK, Ozbek U, Bhatia K (2004) Aberrant methylation of multiple tumor suppressor genes in acute myeloid leukemia. Am J Hematol 77(3):233–240

Federico R, Leone L, Botta M, Binda C, Angelini R, Venturini G, Ascenzi P (2001) Inhibition of pig liver and Zea mays L. polyamine oxidase: a comparative study. J Enzyme Inhib 16(2):147–155

Garcia-Bassets I, Kwon YS, Telese F, Prefontaine GG, Hutt KR, Cheng CS, Ju BG, Ohgi KA, Wang J, Escoubet-Lozach L, Rose DW, Glass CK, Fu XD, Rosenfeld MG (2007) Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. Cell 128(3):505–518

Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, Leonhardt H, Jaenisch R (2003) Induction of tumors in mice by genomic hypomethylation. Science 300(5618):489–492

Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhao M, Smith BD, Manning J, Jiemjit A, Dover G, Mays A, Zwiebel J, Murgo A, Weng LJ, Herman JG (2006) Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 66(12):6361–6369

Hakimi MA, Bochar DA, Chenoweth J, Lane WS, Mandel G, Shiekhattar R (2002) A core-BRAF35 complex containing histone deacetylase mediates repression of neuronal-specific genes. Proc Natl Acad Sci U S A 99(11):7420–7425

Heibert SW, Lutterbach B, Durst K, Wang L, Linggi B, Wu S, Wood L, Amann J, King D, Hou Y (2001) Mechanisms of transcriptional repression by the t(8;21)-, t(12;21)-, and inv(16)-encoded fusion proteins. Cancer Chemother Pharmacol 48(Suppl 1):S31–S34

Huang Y, Greene E, Murray Stewart T, Goodwin AC, Baylin SB, Woster PM, Casero RA Jr (2007) Inhibition of lysine-specific demethylase 1 by polyamine analogues results in reexpression of aberrantly silenced genes. Proc Natl Acad Sci U S A 104(19):8023–8028

Huang Y, Stewart TM, Wu Y, Baylin SB, Marton LJ, Perkins B, Jones RJ, Woster PM, Casero RA Jr (2009) Novel oligoamine analogues inhibit lysine-specific demethylase 1 and induce reexpression of epigenetically silenced genes. Clin Cancer Res 15(23):7217–7228

Huang Y, Vasilatos SN, Boric L, Shaw PG, Davidson NE (2012) Inhibitors of histone demethylation and histone deacetylation cooperate in regulating gene expression and inhibiting growth in human breast cancer cells. Breast Cancer Res Treat 131(3):777–789. doi:10.1007/s10549-011-1480-8

Humphrey GW, Wang Y, Russanova VR, Hirai T, Qin J, Nakatani Y, Howard BH (2001) Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. J Biol Chem 276(9):6817–6824

Issa JP, Garcia-Manero G, Giles FJ, Mannari R, Thomas D, Faderl S, Bayar E, Lyons J, Rosenfeld CS, Cortes J, Kantarjian HM (2004) Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 103(5):1635–1640

Jenuwein T, Allis CD (2001) Translating the histone code. Science 293(5532):1074–1080

Kabra PM, Lee HK, Lubich WP, Marton LJ (1986) Solid-phase extraction and determination of dansyl derivatives of unconjugated and acetylated polyamines by reversed-phase liquid chromatography: improved separation systems for polyamines in cerebrospinal fluid, urine and tissue. J Chromatogr 380(1):19–32

Karayiannakis AJ, Syrigos KN, Chatzigianni E, Papanikolaou S, Alexiou D, Kalahanis N, Rosenberg T, Bastounis E (1998) Aberrant E-cadherin expression associated with loss of

differentiation and advanced stage in human pancreatic cancer. Anticancer Res 18(6A):4177–4180
Lachner M, O'Sullivan RJ, Jenuwein T (2003) An epigenetic road map for histone lysine methylation. J Cell Sci 116(Pt 11):2117–2124
Lan F, Collins RE, De Cegli R, Alpatov R, Horton JR, Shi X, Gozani O, Cheng X, Shi Y (2007) Recognition of unmethylated histone H3 lysine 4 links BHC80 to LSD1-mediated gene repression. Nature 448(7154):718–722
Lee MG, Wynder C, Cooch N, Shiekhattar R (2005) An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature 437(7057):432–435
Li LC, Okino ST, Zhao H, Pookot D, Place RF, Urakami S, Enokida H, Dahiya R (2006) Small dsRNAs induce transcriptional activation in human cells. Proc Natl Acad Sci U S A 103(46):17337–17342
Liang G, Lin JC, Wei V, Yoo C, Cheng JC, Nguyen CT, Weisenberger DJ, Egger G, Takai D, Gonzales FA, Jones PA (2004) Distinct localization of histone H3 acetylation and H3–K4 methylation to the transcription start sites in the human genome. Proc Natl Acad Sci USA 101(19):7357–7362
Lim S, Janzer A, Becker A, Zimmer A, Schule R, Buettner R, Kirfel J (2010) Lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancers and a biomarker predicting aggressive biology. Carcinogenesis 31(3):512–520. doi:10.1093/carcin/bgp324
McGarvey KM, Fahrner JA, Greene E, Martens J, Jenuwein T, Baylin SB (2006) Silenced tumor suppressor genes reactivated by DNA demethylation do not return to a fully euchromatic chromatin state. Cancer Res 66(7):3541–3549
Melki JR, Vincent PC, Clark SJ (1999) Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. Cancer Res 59(15):3730–3740
Melki JR, Vincent PC, Brown RD, Clark SJ (2000) Hypermethylation of E-cadherin in leukemia. Blood 95(10):3208–3213
Metzger E, Wissmann M, Yin N, Muller JM, Schneider R, Peters AH, Gunther T, Buettner R, Schule R (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature 437(7057):436–439
Rao Q, Wang JY, Meng J, Tang K, Wang Y, Wang M, Xing H, Tian Z, Wang J (2010) Low-expression of E-cadherin in leukaemia cells causes loss of homophilic adhesion and promotes cell growth. Cell Biol Int 35(9):945–951. doi:10.1042/CBI20100456
Schenk T, Chen WC, Gollner S, Howell L, Jin L, Hebestreit K, Klein HU, Popescu AC, Burnett A, Mills K, Casero RA Jr, Marton L, Woster P, Minden MD, Dugas M, Wang JC, Dick JE, Muller-Tidow C, Petrie K, Zelent A (2012) Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia. Nat Med 18(4):605–611. doi:10.1038/nm.2661
Schneider R, Bannister AJ, Myers FA, Thorne AW, Crane-Robinson C, Kouzarides T (2004) Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. Nat Cell Biol 6(1):73–77
Schulte JH, Lim S, Schramm A, Friedrichs N, Koster J, Versteeg R, Ora I, Pajtler K, Klein-Hitpass L, Kuhfittig-Kulle S, Metzger E, Schule R, Eggert A, Buettner R, Kirfel J (2009) Lysine-specific demethylase 1 is strongly expressed in poorly differentiated neuroblastoma: implications for therapy. Cancer Res 69(5):2065–2071. doi:10.1158/0008-5472.CAN-08-1735
Seely JE, Pegg AE (1983) Ornithine decarboxylase (mouse kidney). Methods Enzymol 94:158–161
Sharma SK, Wu Y, Steinbergs N, Crowley ML, Hanson AS, Casero RA, Woster PM (2010) (Bis)urea and (bis)thiourea inhibitors of lysine-specific demethylase 1 as epigenetic modulators. J Med Chem 53(14):5197–5212. doi:10.1021/jm100217a
Shi Y, Sawada J, Sui G, el Affar B, Whetstine JR, Lan F, Ogawa H, Luke MP, Nakatani Y, Shi Y (2003) Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature 422(6933):735–738
Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119(7):941–953
Shi YJ, Matson C, Lan F, Iwase S, Baba T, Shi Y (2005) Regulation of LSD1 histone demethylase activity by its associated factors. Mol Cell 19(6):857–864
Shimamoto T, Ohyashiki JH, Ohyashiki K (2005) Methylation of p15(INK4b) and E-cadherin genes is independently correlated with poor prognosis in acute myeloid leukemia. Leuk Res 29(6):653–659
Silverman LR, Demakos EP, Peterson BL, Kornblith AB, Holland JC, Odchimar-Reissig R, Stone RM, Nelson D, Powell BL, DeCastro CM, Ellerton J, Larson RA, Schiffer CA, Holland JF (2002) Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol 20(10):2429–2440
Sulzer MA, Leers MP, van Noord JA, Bollen EC, Theunissen PH (1998) Reduced E-cadherin expression is associated with increased lymph node metastasis and unfavorable prognosis in non-small cell lung cancer. Am J Respir Crit Care Med 157(4 Pt 1):1319–1323
Ting AH, Schuebel KE, Herman JG, Baylin SB (2005) Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. Nat Genet 37(8):906–910
Tong JK, Hassig CA, Schnitzler GR, Kingston RE, Schreiber SL (1998) Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature 395(6705):917–921
Toyota M, Kopecky KJ, Toyota MO, Jair KW, Willman CL, Issa JP (2001) Methylation profiling in acute myeloid leukemia. Blood 97(9):2823–2829
Wang J, Hoshino T, Redner RL, Kajigaya S, Liu JM (1998) ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc Natl Acad Sci USA 95(18):10860–10865
Wang Y, Devereux W, Woster PM, Stewart TM, Hacker A, Casero RA Jr (2001) Cloning and characterization of a human polyamine oxidase that is inducible by polyamine analogue exposure. Cancer Res 61(14):5370–5373
Wang J, Scully K, Zhu X, Cai L, Zhang J, Prefontaine GG, Krones A, Ohgi KA, Zhu P, Garcia-Bassets I, Liu F, Taylor H, Lozach J, Jayes FL, Korach KS, Glass CK, Fu XD, Rosenfeld MG (2007) Opposing LSD1 complexes function in developmental gene activation and repression programmes. Nature 446(7138):882–887
Wissmann M, Yin N, Muller JM, Greschik H, Fodor BD, Jenuwein T, Vogler C, Schneider R, Gunther T, Buettner R, Metzger E, Schule R (2007) Cooperative demethylation by JMJD2C and LSD1 promotes androgen receptor-dependent gene expression. Nat Cell Biol 9(3):347–353
You A, Tong JK, Grozinger CM, Schreiber SL (2001) CoREST is an integral component of the CoREST- human histone deacetylase complex. Proc Natl Acad Sci USA 98(4):1454–1458
Zheng Z, Pan J, Chu B, Wong YC, Cheung AL, Tsao SW (1999) Downregulation and abnormal expression of E-cadherin and beta-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. Hum Pathol 30(4):458–466
Zhu Q, Huang Y, Marton LJ, Woster PM, Davidson NE, Casero RA, Jr. (2012) Polyamine analogues modulate gene expression by inhibiting LSD1/KDM1 and altering chromatin structure in human breast cancer cells. Amino Acids in press