

Novel Oligoamine Analogues Inhibit Lysine-Specific Demethylase 1 and Induce Reexpression of Epigenetically Silenced Genes

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Abstract Purpose: Abnormal DNA CpG island hypermethylation and transcriptionally repressive histone modifications are associated with the aberrant silencing of tumor suppressor genes. Lysine methylation is a dynamic, enzymatically controlled process. Lysine-specific demethylase 1 (LSD1) has recently been identified as a histone lysine demethylase. LSD1 specifically catalyzes demethylation of mono- and dimethyl-lysine 4 of histone 3 (H3K4), key positive chromatin marks associated with transcriptional activation. We hypothesized that a novel class of oligoamine analogues would effectively inhibit LSD1 and thus cause the reexpression of aberrantly silenced genes.

Experimental Design: Human colorectal cancer cells were treated with the oligoamines and changes in mono- and dimethyl-H3K4 and other chromatin marks were monitored. In addition, treated cells were evaluated for the reexpression of the aberrantly silenced secreted frizzled-related proteins (*SFRP*) Wnt signaling pathway antagonist genes. Finally, the effects of the LSD1 inhibitors were evaluated in an *in vivo* xenograft model.

Results: Treatment of HCT116 human colon adenocarcinoma cells *in vitro* resulted in increased H3K4 methylation and reexpression of silenced *SFRP* genes. This reexpression is also accompanied by a decrease in H3K9me2 repressive mark. Importantly, cotreatment with low doses of oligoamines and a DNA methyltransferase inhibitor highly induces the reexpression of the aberrantly silenced *SFRP2* gene and results in significant inhibition of the growth of established tumors in a human colon tumor model *in vivo*.

Conclusions: The use of LSD1-inhibiting oligoamine analogues in combination with DNA methyltransferase inhibitors represents a highly promising and novel approach for epigenetic therapy of cancer. (Clin Cancer Res 2009;15(23):7217–28)

Epigenetics refers to heritable changes in gene expression patterns that are not regulated by changes in the primary DNA sequence. In cancer, epigenetic silencing of gene expression, including of tumor suppressor genes, is a common occurrence (1) that is associated with abnormal DNA methylation patterns and changes in covalent histone modifications (2). The amino-terminal tails of histones are subject to several

posttranslational modifications, including acetylation, phosphorylation, and methylation, which are closely tied to transcriptional regulation, DNA replication, and DNA repair (2). As shown by the regulation of histone acetylation by histone acetyltransferases and histone deacetylases (HDAC), the addition and removal of these posttranslational modifications is a dynamic process. A similar dynamic regulation occurs for histone methylation with histone methyltransferases for the addition of methyl groups, and we recently discovered families of enzymes for specific histone demethylation. The first of these demethylating enzymes identified was the lysine-specific demethylase (LSD1/KDM1; ref. 3), a flavin adenine dinucleotide (FAD)-dependent amine oxidase, which interacts directly with CoREST and HDAC1/2 proteins, forming a module found in several multiprotein corepressor complexes and is known to act on intact chromatin as part of these complexes (3, 4). LSD1 demethylates H3K4me2/me1 through an oxidative reaction that leads to the reduction of the protein-bound FAD cofactor and the production of H₂O₂ and formaldehyde. More recently, a number of Jumonji (JmjC) domain-containing histone demethylases have been identified and shown to play important roles in concert with other histone-modifying enzymes related to the control of transcriptional regulation, cellular differentiation, and animal development (5, 6).

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Translational Relevance

Aberrant epigenetic silencing of important tumor suppressor genes has been shown in essentially all human cancers. Efforts are ongoing to target this aberrant epigenetic regulation for therapeutic benefit and clinical trials combining agents that inhibit DNA CpG methylation with agents that inhibit histone deacetylase have yielded promising results. The discovery of lysine-specific demethylase 1 (LSD1) and the dynamic nature of histone methylation suggested the potential for inhibiting this transcriptional repressor as a means of reexpressing aberrantly silenced genes. The results of the initial *in vitro* studies from our laboratory and others were consistent with the hypothesis that LSD1 was a rational target for inducing the reexpression of aberrantly silenced genes. Here, we present critical *in vitro* and *in vivo* data that firmly establish LSD1 as a target for therapy and show that inhibitors of this enzyme have considerable promise, both when used alone and in combination with other agents that target epigenetic regulation.

As emerging data suggest that LSD1 has the capacity to broadly repress gene expression, and as dysregulation of epigenetic gene silencing is a common feature in all cancers, LSD1 is being actively pursued as an attractive drug target. In both normal cells and colorectal cancer cells, increasing the promoter region H3K4me2 is particularly tightly tied to increasing levels of active gene transcription (7, 8). Additionally, emerging data indicate that a high expression of LSD1 may be a feature of specific human cancers (9). Several studies, including those from our laboratory, have reported the inhibitory effect of various small molecules on LSD1 (10–12). Structural analysis shows that LSD1 is highly conserved across species and consists of an NH₂-terminal SWIRM domain, a central protruding tower domain, and a COOH-terminal amine oxidase-like domain that contains a FAD-binding subdomain. The FAD-binding oxidase domain is highly homologous, with 20% similarity, to that of FAD-dependent monoamine oxidases and polyamine oxidases, such as spermine oxidase (SMO) and N¹-acetylpolyamine oxidase (6, 13–15). Furthermore, the catalytic domains of LSD1 and SMO possess more than 60% similarity in amino acid sequences.

Although the natural polyamines are not substrates of LSD1 (3), the above structural and catalytic similarities of FAD-dependent oxidases, the strong association of polyamines with chromatin, and the structural similarity between the polyamines and the lysine tail of histones, led us to test the possibility that polyamine analogues might be potent inhibitors of LSD1 (10). Indeed, LSD1 inhibition by these unique compounds resulted in reexpression of multiple aberrantly silenced tumor suppressor genes in colorectal cancer cells, further validating LSD1 as a potential target for therapeutic intervention. Furthermore, the results suggested that other polyamine-like molecules might function as effective inhibitors of LSD1 and would thus be effective epigenetic targeting agents.

We previously found that specific members of a novel class of long-chain polyamine analogues known as oligoamines (16) were not substrates of either N¹-acetylpolyamine oxidase or SMO but were potent inhibitors of these purified polyamine oxidases (17). Because these enzymes are highly homologous to the FAD-dependent LSD1, we hypothesized that specific members of the oligoamines would be effective inhibitors of LSD1. Additionally, because of their multivalent (+10) cationic structure, we postulated that they would be targeted to chromatin (18), the site of action for LSD1, at least as efficiently as the biguanide or bisguanidine analogues we previously examined. We now report that treatment of tumor cells with the oligoamines results in reexpression of specific aberrantly silenced genes *in vitro*. Furthermore, we show for the first time the use of LSD1 inhibitors in combination with a DNA methyltransferase (DNMT) inhibitor, a combination that is not only more efficacious in reactivating specific aberrantly silenced genes but also leads to profound inhibition of the growth of established human colon cancer xenografts in a nude mouse model.

Materials and Methods

Compounds, peptides, histones, and culture conditions. Polyamine analogues were synthesized as previously reported (17). Stock solutions (10 mmol/L in double-distilled H₂O) of each compound were diluted with the medium to the desired concentrations for specific experiments. Synthetic H3K4me2 peptides were purchased from Millipore. The DNMT inhibitors, 5-aza-2'-deoxycytidine (DAC) and 5-azacytidine (5-Aza), and bulk histones were purchased from Sigma. HCT116 colorectal carcinoma cells were maintained in McCoy's 5A medium and RKO cells were maintained in MEM medium, each supplemented with 9% fetal bovine serum (Atlanta Biologicals) and 1% penicillin/streptomycin (Mediatech) and grown at 37°C in 5% CO₂ atmosphere.

MTT growth inhibition assay, Annexin V staining, and poly(ADP-ribose) polymerase cleavage. Cells were seeded at 5,000 per well on a 96-well plate and allowed to attach overnight. Medium was aspirated and replaced with a fresh medium containing the appropriate concentrations of LSD1 inhibitor. Following incubation for 24 or 48 h, 200 μ L of a 1 mg/mL MTT solution (Sigma Chemical Co.), diluted in serum-free culture medium, were added to each well. The plates were incubated at 37°C in 5% CO₂ atmosphere for 4 h. At the end of the 4-h incubation, the MTT solution was removed and 200 μ L of 1:1 (v/v) solution of DMSO/ethanol were added to each well to dissolve the formazan crystals. The absorbance in individual wells was determined at A_{540 nm}.

Evidence for apoptotic cell death was determined by both poly(ADP-ribose) polymerase (PARP) cleavage and Annexin V staining as we previously published (19, 20).

Expression, purification, and demethylase activity of recombinant proteins. Human cDNAs coding either the full-length LSD1 protein or that with a deletion of the NH₂-terminal 184 (Δ 184 LSD1) amino acids were subcloned into the pET15b bacterial expression vector (Novagen) in frame with an NH₂-terminal 6 \times His-tag and transformed into the BL₂₁(DE₃) strain of *Escherichia coli*. Following selection, expression and purification of recombinant LSD1 protein were done as previously described (10). Bound protein was eluted by imidazole and the eluate was dialyzed in PBS at 4°C. The enzymatic activity of LSD1 was examined using both a luminol-dependent chemiluminescence method to measure the production of H₂O₂ and a quantitative Western blot method that measures the amount of H3K4me2 after incubation of purified protein with bulk histones as previously described (15).

Western blotting. Nuclear fractions of treated cells were prepared for Western blot analysis using NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce). Equal amounts (30 μ g/lane) of nuclear protein were fractionated on SDS-PAGE gels and transferred onto

polyvinylidene difluoride membranes. Primary antibodies against H3K4me2, H3K4me3, and H3K4me1 were from Millipore. The H3 polyclonal antibody used for normalization was purchased from Abcam. Dye-conjugated secondary antibodies were used to quantify Western blot results with the Odyssey Infrared Detection system and software (LI-COR Biosciences).

RNA isolation, reverse transcriptase-PCR, quantitative PCR, and bisulfite sequencing. RNA for reverse transcriptase-PCR (RT-PCR) was extracted using the TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase with an oligo(dT) primer (Invitrogen). PCR was done using the following custom primers: *SFRP1* sense, 5'-GGCCCATCTACCCGTGTGC; *SFRP1* antisense, 5'-GATGGCCTCAGATTCAACTCGT (annealing at 60°C); *SFRP2* sense, 5'-AAGCCTGC AAAAATAAAAATGATG; *SFRP2* antisense, 5'-TGTAATGGTCTTGTCTTGGTCT (annealing at 53°C); *SFRP4* sense, 5'-TCTATGACCGTGGCGTGTGC; *SFRP4* antisense, 5'-ACCGATCGGGCTTAGGCGTTAC (annealing at 56°C); *GATA5* sense, 5'-CCTGCGGCTCTACCACAA; *GATA5* antisense, 5'-GGCGGGC-GGGACGAGGAC (annealing at 58°C). A total of 35 cycles of amplification was done and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. Amplified products were analyzed on 2% agarose gels with GelStar staining (Cambrex).

Quantitative PCR (qPCR) of *SFRP1* and *SFRP2* was done as previously described (7). The same forward and reverse primers that were used for RT-PCR were used for qPCR in a MyiQ single color real-time PCR machine (Bio-Rad) with GAPDH as an internal control. Amplification conditions for *SFRP1* consisted of a 15-min denaturation step followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Identical conditions for *SFRP2* were used except the annealing temperature was 53°C. To quantify relative expression, the comparative cycle threshold (C_t) method was used, normalizing the C_t values for the gene of interest to the C_t values of GAPDH relative to untreated control.

Bisulfite sequencing of the *SFRP2* gene promoter was done as we have previously published (10) using the following primers: sense, 5'-GGTAATTTAGTAGAAATTCGGATTG; antisense, 5'-ACTAATCAC-TACTTTCTAAATCTAATT.

Chromatin immunoprecipitation. Control and analogue-treated cells were exposed to 1% formaldehyde to cross-link proteins, and 2×10^6 cells were used for each chromatin immunoprecipitation (ChIP) assay performed as previously described (10). Antibody against H3 was purchased from Abcam. Primary antibodies against LSD1, H3K4me2, H3K4me1, H3K4me3, H3K9me2, acetyl-H3K9, or acetyl-H4K16 were from Millipore. PCR primer sets used for amplification of precipitated fragments were as follows: *SFRP1* sense, 5'-GGCCCATC-TACCCGTGTGC; antisense, 5'-GATGGCCTCAGATTCAACTCGT; *SFRP2* (fragment A) sense, 5'-CTCCCTCCAGCCTGCCATCTT; antisense, 5'-ACTGCCACCATTTCCCGTTTTC; *SFRP2* (fragment B) sense, 5'-CCTGTGTGACTGGTGAAGTGC; antisense, 5'-CGGCGAAGTTCGTTTCCCTC; *SFRP2* (fragment C) sense, 5'-GCTGCTGAACGGTGGCTGGA-GATT; antisense, 5'-CAGGCATGAGTGGGAGAGGGTGTG; *SFRP2* (fragment D) sense, 5'-TGGCAACCCAGCAGAACTTC; antisense, 5'-ACGCGCTTGTGGAAGGGAATTC; *SFRP2* (fragment E) sense, 5'-AGGGGCATTCTCAACGCAAAACC; antisense, 5'-GGGACACCGGGAG-GACAGC; *SFRP2* (fragment F) sense, 5'-GCTTCTCCGCGCCCCAG-CCGCC; antisense, 5'-AGCCCAGGCAGCAGTGCGAGG. Sheared genomic DNA was used as a positive control (input). Quantitative ChIP confirmed changes in histone marks at the promoters of examined genes by using qPCR. qPCR was performed with the same primers used for standard ChIP on the MyiQ single-color real-time PCR machine (Bio-Rad). Conditions used were 40 cycles of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s. DNA immunoprecipitated by H3 antibody was used for normalization.

Animal studies. BALB c *nu/nu* athymic nude mice (Harlan Bioproducts for Science, Inc.) were implanted with the human colorectal cancer HCT-116 cells. By 20 d, the postimplantation average tumor

size was $>350 \text{ mm}^3$ and mice were randomized into treatment ($n = 5$) and control ($n = 5$) groups. The single-agent treatment groups were as follows: vehicle (saline control at daily $\times 5$), PG-11144 (10 mg/kg, twice per week), 2d (10 mg/kg, daily $\times 5$), and 5-Aza (2 mg/kg, daily $\times 5$). The combination treatment groups were as follows: PG-11144 (10 mg/kg, twice per week)/5-Aza (2 mg/kg, daily $\times 5$) and 2d (10 mg/kg, daily $\times 5$)/5-Aza (2 mg/kg, daily $\times 5$). Test agents were administered by i.p. injection for 3 wk. The drugs administered on a twice per week schedule (PG-11144) were given on days 20, 23, 27, 30, 34, and 37 after implantation, and drugs given on the daily $\times 5$ schedule (2d, 5-Aza) were injected on days 20 to 24, 27 to 31, and 34 to 38. The control group was treated with vehicle (sterile saline) using the same daily $\times 5$ treatment schedule. The animals were observed for adverse clinical signs after each dose and once daily thereafter throughout the course of the study. Morbidity and mortality was monitored daily following the initial dose and continued throughout the course of the study. The experiment was terminated on day 38 after tumor implantation and the tumors were harvested for Western blotting analysis of H3K4me2. It should be noted that 5-Aza was chosen over DAC for *in vivo* experiments because 5-Aza has been the drug of choice for ongoing clinical trials in our institution (21); 5-Aza has been shown to have efficacy in the preleukemic disease myelodysplasia and is approved by the Food and Drug Administration for use in patients with this disorder (22).

Results

Inhibition of recombinant LSD1 activity by oligoamines. We tested a series of conformationally restricted and saturated polyamine and oligoamine analogues, including pentamines, hexamines, octamines, and decamines (Fig. 1A), for their effect on recombinant LSD1 activity. The conformationally restricted analogues tested incorporate molecular alterations that restrict the free rotation of specific bonds in otherwise flexible molecules and are longer than the natural polyamines. Most of those tested here have four methylene (CH_2) residues between each imine nitrogen (16). Whereas the pentamine and hexamine analogues marginally affect LSD1 activity, octamine and decamine analogues inhibited this activity by $>50\%$ at 10 $\mu\text{mol/L}$ using 5 $\mu\text{mol/L}$ H3K4me2 peptide (1-21 aa) as a substrate (B). The two most potent inhibitors, the decamine analogues PG-11144 and PG-11150, which are *cis* and *trans* isomers, were chosen for further study.

We confirmed that PG-11144 and PG-11150 inhibit LSD1 demethylase activity by Western blot using bulk histone as a substrate (Fig. 2A). Both analogues inhibit recombinant LSD1 activity in a concentration-dependent manner with IC_{50} values around 5 $\mu\text{mol/L}$ using 5 $\mu\text{mol/L}$ H3K4me2 peptide (1-21 aa) as substrate (Fig. 2B). Using purified recombinant LSD1, PG-11144 exhibited competitive inhibition kinetics at concentrations $<10 \mu\text{mol/L}$ (Fig. 2C), suggesting that PG-11144 may compete with H3K4me2 at the LSD1 active site. Similar kinetics were observed for PG-11150 (data not shown).

Inhibition of growth by oligoamines. The sensitivity of the HCT-116 and RKO cell lines to the oligoamines PG-11144 and PG-11150 was assessed with the MTT cell proliferation assay. Both cell lines exhibited time- and concentration-dependent growth inhibition by oligoamines (Supplementary Fig. S1). The IC_{50} values for both cell lines are ~ 2.5 to 5 $\mu\text{mol/L}$ for 48-hour treatment.

To determine the cause of the observed decrease in proliferation, PG-11144 (5 $\mu\text{mol/L}$)-treated cells were assessed for apoptotic cell death by Annexin V staining and poly(ADP-ribose) polymerase (PARP) cleavage. Annexin V staining

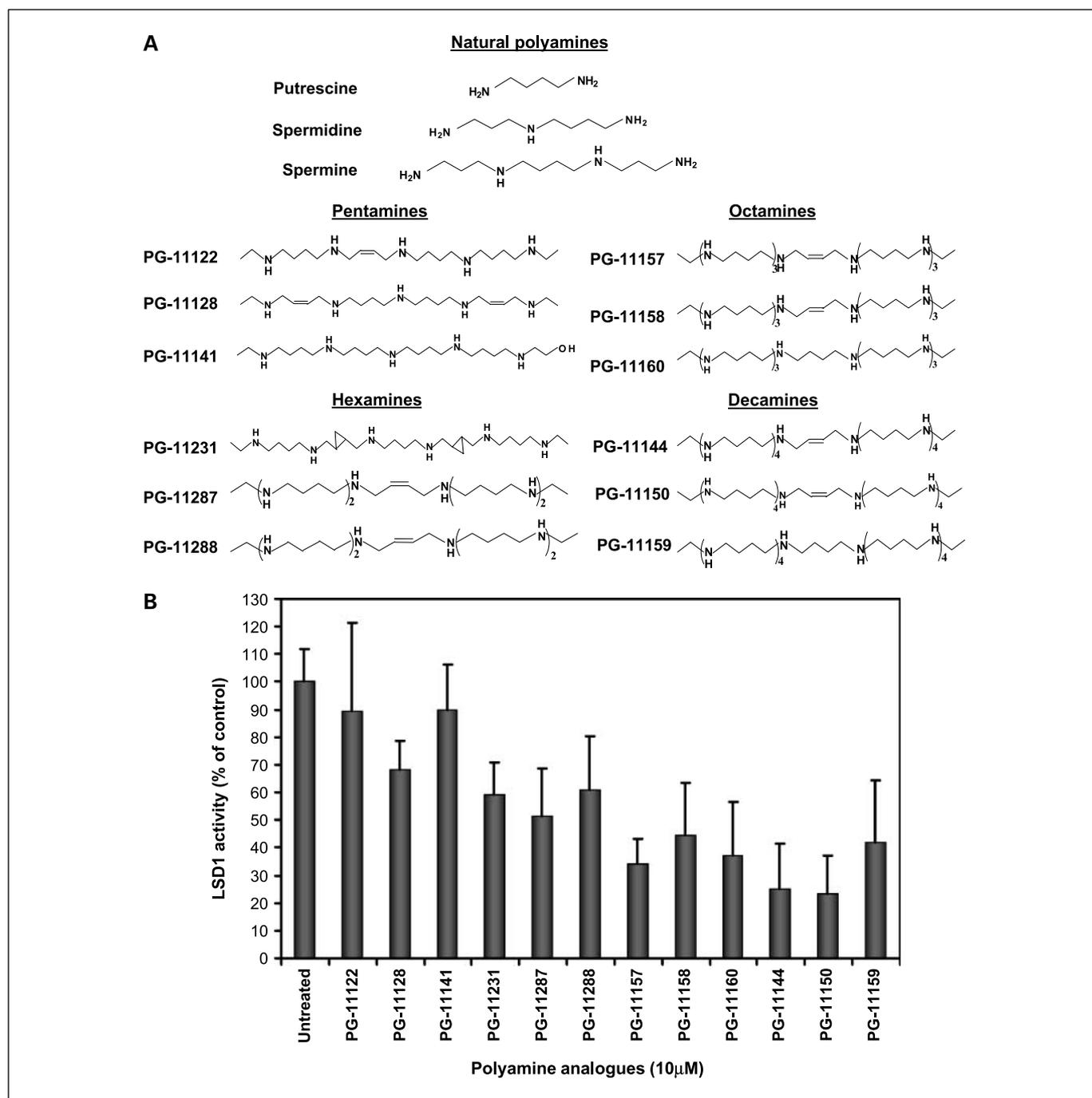


Fig. 1. Inhibition of LSD1 by polyamine analogues. *A*, structures of natural polyamines and members of the oligoamine family of polyamine analogues. *B*, 0.038 μg/μL of purified Δ184 LSD1 protein was incubated with 5 μmol/L H3K4me2 (1-21 aa) as substrate in the presence of 10 μmol/L of the indicated analogue. The effect of oligoamines on enzymatic activity of LSD1 was examined using luminol-dependent chemiluminescence to measure the production of H₂O₂. The integral values were calibrated against standards containing known concentrations of H₂O₂, and the activities were expressed as picomoles of H₂O₂ per milligram of protein per minute. Columns, means of independent experiments done in triplicate; bars, SD.

analysis indicates that 24-hour treatment with PG-11144 induces an increase in apoptotic cell death (19.3% Annexin V-positive cells in treated versus 4% in controls, 10,000 events each), but DAC treatment (100 nmol/L), either alone (5% Annexin V positive) or in combination with PG-11144 (20.4% Annexin V positive), did not significantly increase apoptotic cell death. The PARP cleavage data (Supplementary Fig. S2) are entirely consistent with the Annexin V results indicating that the

observed apoptosis is a result of PG-11144 treatment. It should be noted that the 24-hour treatment time was chosen for analysis as this treatment time was used for the chromatin and gene expression analyses presented below.

Oligoamines increase global H3K4 methylation in colorectal tumor cells. To determine whether the *in vitro* inhibition of LSD1 activity by oligoamines translates into cellular response, the effects on global H3K4 (lysine 4 of histone 3) methylation

were examined after exposure of human colon cancer HCT116 and RKO cells to increasing concentrations of each compound. This exposure did not reduce protein levels of nuclear LSD1 but produced significant global increases of both H3K4me1 and H3K4me2, both of which are substrates of LSD1 (Fig. 3A). H3K4me3 levels were not affected in treat-

ed cells (not shown). These results indicate that the oligoamines effectively inhibit LSD1 enzymatic activity *in situ*. Furthermore, these results suggest that the oligoamines are selective for the FAD-dependent LSD1 and do not inhibit the JmjC histone demethylases, as no changes in H3K4me3 levels were observed.

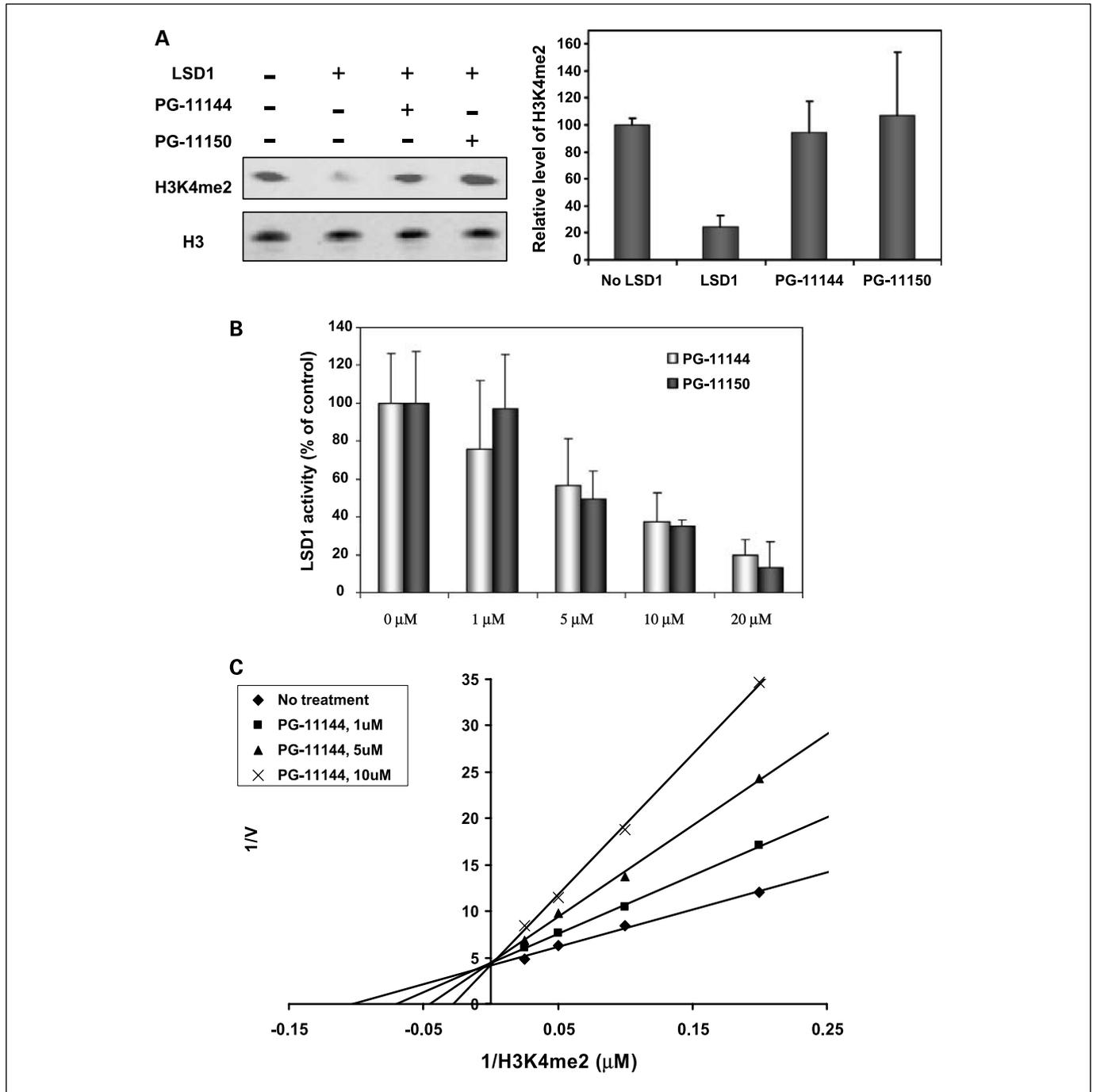


Fig. 2. Inhibition of recombinant human LSD1 by oligoamines. **A**, 0.05 μg/μL of purified bulk histones was incubated with or without 0.01 μg/μL purified full-length LSD1 for 3 h at 37°C. The effects of LSD1 enzymatic activity were analyzed by Western blotting using antibodies that specifically recognize the dimethyl group of H3K4. Relative levels were determined by quantitative Western blot analysis using the Odyssey IR detection system. Total H3 was used as a loading control. Columns, means of three determinations; bars, SD. **B**, 0.038 μg/μL of purified Δ184 LSD1 protein was incubated with 5 μmol/L H3K4me2 (1-21 aa) as substrate in the presence of increasing concentrations of PG-11144 and PG-11150. The effect of oligoamines on enzymatic activity of LSD1 was examined using luminol-dependent chemiluminescence to measure the production of H₂O₂. Columns, means; bars, SD. **C**, the effects of increasing concentrations of PG-11144 on LSD1 activity in the presence of increasing substrate concentrations. Double reciprocal plots indicate inhibition of LSD1 by PG-11144 to be competitive. Points, means of independent experiments done in triplicate; bars, SD.

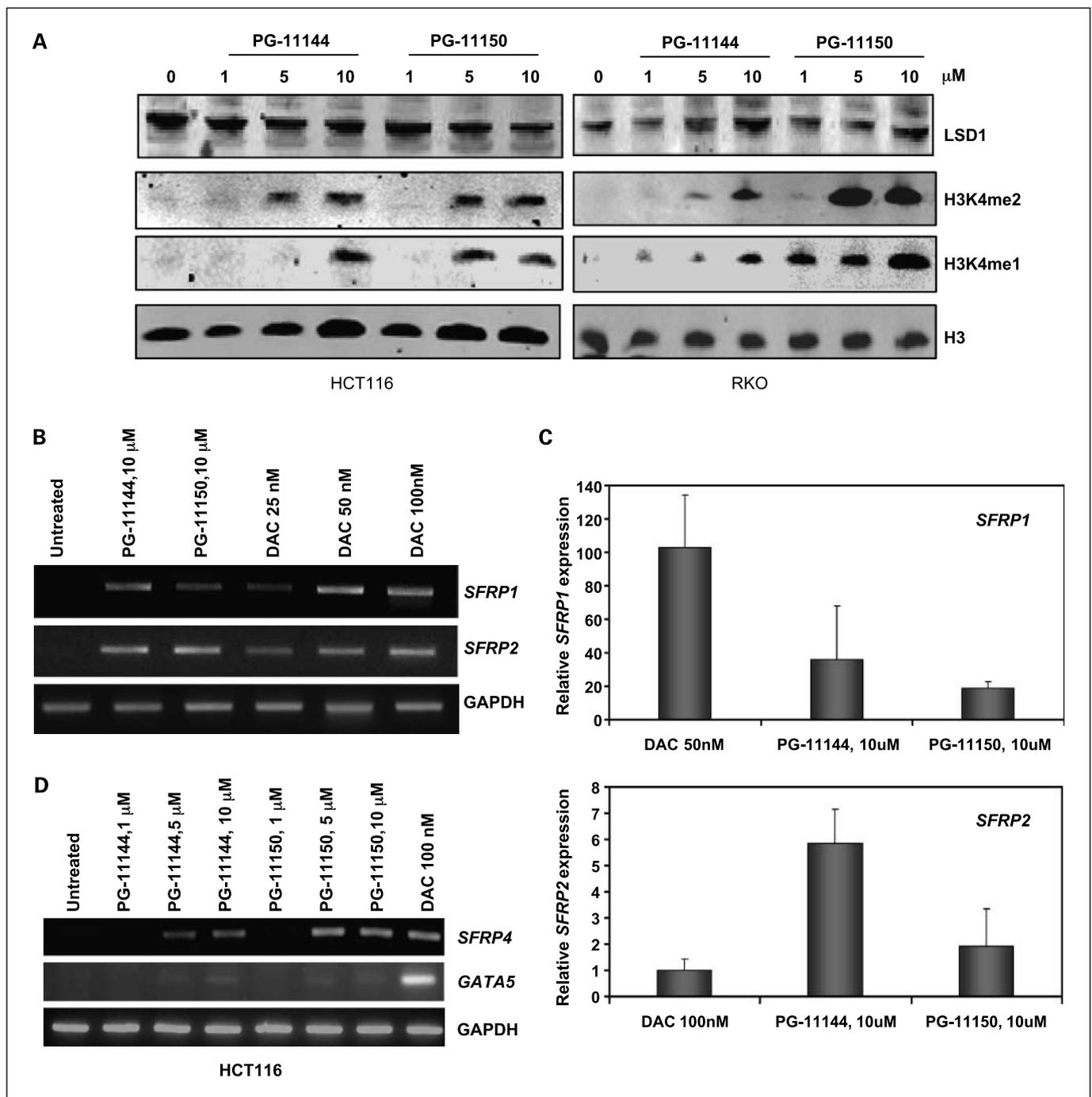


Fig. 3. Inhibition of LSD1 by oligoamines increases global H3K4me2, resulting in reexpression of aberrantly silenced genes in treated human colon cancer cells. **A**, HCT116 and RKO cells were exposed to increasing concentrations of PG-11144 or PG-11150 for 24 or 48 h, and 30 μg of nuclear protein per lane were analyzed for expression of LSD1, H3K4me1, H3K4me2, and H3 as a loading control. **B**, HCT116 cells were treated for 24 h with the indicated concentrations of oligoamines and DAC. Total RNA was extracted for RT-PCR analysis of *SFRP1* and *SFRP2* expression. GAPDH is included as an internal control. **C**, the levels of reexpression of *SFRP1* and *SFRP2* induced by oligoamines in HCT116 cells were compared with levels resulting from DAC treatment using SYBR green/qPCR with GAPDH as an internal control. **D**, HCT116 cells were treated for 24 h with the indicated concentrations of oligoamines or DAC. Total RNA was extracted for RT-PCR analysis of *SFRP4* and *GATA5* expression. GAPDH is included as an internal control.

Oligoamines reactivate the expression of multiple aberrantly silenced genes. In colorectal cancer cells, the promoter region H3K4me2 is usually associated with open chromatin and active transcription (7, 8). The occupancy of H3K4me2 is typically found to be at a low level in the promoters of some frequently DNA hypermethylated and epigenetically silenced genes important in tumorigenesis (1, 8). Multiple such silenced genes are

present in HCT116 cells as well as in primary human colon carcinomas. These genes include members of the Wnt signaling pathway antagonists, the secreted frizzled-related protein family (*SFRP*), and the *GATA* family of transcription factors (1, 23–25). Therefore, we examined whether such genes could be reexpressed following the exposure of colorectal cancer cells to oligoamine treatment. In HCT116 cells, treatment with 10 μmol/L

PG-11144 or PG-11150 for 24 hours led to substantial reexpression of aberrantly silenced *SFRP1* and *SFRP2* genes (Fig. 3B). The gene reexpression achieved with oligoamine treatment was compared with that achieved with the DNMT inhibitor DAC, as determined by quantitative real-time PCR. Treatment of cells with oligoamines resulted in robust reexpression of *SFRP2* (≈ 150 - 550% that achieved by 100 nmol/L DAC treatment) and modest reexpression of *SFRP1* (≈ 20 - 40% that achieved by 50 nmol/L DAC treatment; Fig. 3C). Oligoamine treatment also led to the detectable reexpression of the *SFRP4* gene and modest reexpression of the *GATA5* gene in HCT116 cells (Fig. 3D). These results show that oligoamines are effective in producing significant reexpression of multiple epigenetically silenced genes in human colorectal cancer cells.

Oligoamine treatment increases activating H3K4 methylation and decreases repressive H3K9 methylation marks at the promoters of reexpressed genes. To investigate whether oligoamine-induced gene reexpression was accompanied by changes in regulatory chromatin marks at specific gene promoters, quantitative ChIP analysis was done on HCT116 cells exposed to 10 $\mu\text{mol/L}$ PG-11144 for 24 hours. Results revealed that treatment-induced gene reexpression was accompanied by increased enrichment of H3K4me1 and H3K4me2 at the promoters of both *SFRP1* (upstream proximal region to transcription start site) and *SFRP2* (spanning transcription start site; Fig. 4A) genes. Consistent with the results observed above for global methylation, no change in promoter H3K4me3 levels was detected, implying that oligoamines do not affect the activity of the JmjC domain-containing histone demethylases that act on H3K4me3. The increased H3K4 methylation was accompanied by decreases in the repressive mark H3K9me2 in both genes and a modest increase in the active mark acetyl-H3K9 in *SFRP2*. The level of LSD1 occupancy remains unchanged after PG-11144 treatment, suggesting that the oligoamine actually inhibits LSD1 activity without decreasing its presence in the promoter of specific genes. Although increases in H3K4 methylation were consistently observed after exposure to the oligoamines, changes in the activating histone acetylation marks H3K9 and H4K16 were more variable. Treatment with PG-11144 led to a decrease of both acetyl-H3K9 and acetyl-H4K16 at the promoter of *SFRP1*, whereas the level of acetyl-H3K9 was slightly increased by PG-11144 and acetyl-H4K16 remained unchanged at the promoter of *SFRP2*.

To more precisely determine the effect of oligoamines on the promoter histone marks, we extended the ChIP analyses of the *SFRP2* gene to cover the proximal promoter region from approximately -1,000 bp to +300 bp relative to the transcriptional start site (TSS). *SFRP2* was selected because it is the gene that shows the most significant induction by the oligoamines in HCT116 cells. Oligoamine treatment led to significant increase of H3K4me2 enrichment at promoter regions B, C, and E (TSS site), and modest increase of H3K4me2 enrichment at region F downstream of the TSS site (Fig. 4B). This analysis more precisely defines the effects of oligoamines on the histone marks in the critical proximal promoter regions that are likely responsible for the oligoamine-induced reexpression of *SFRP2*.

Combination treatment with oligoamines and a DNMT inhibitor results in greater reexpression of specific aberrantly silenced genes than when either agent is used alone. Promoter CpG island DNA hypermethylation typically collaborates with specific histone marks in the transcriptional silencing of specific genes in cancer cells. Previous studies have shown synergy between

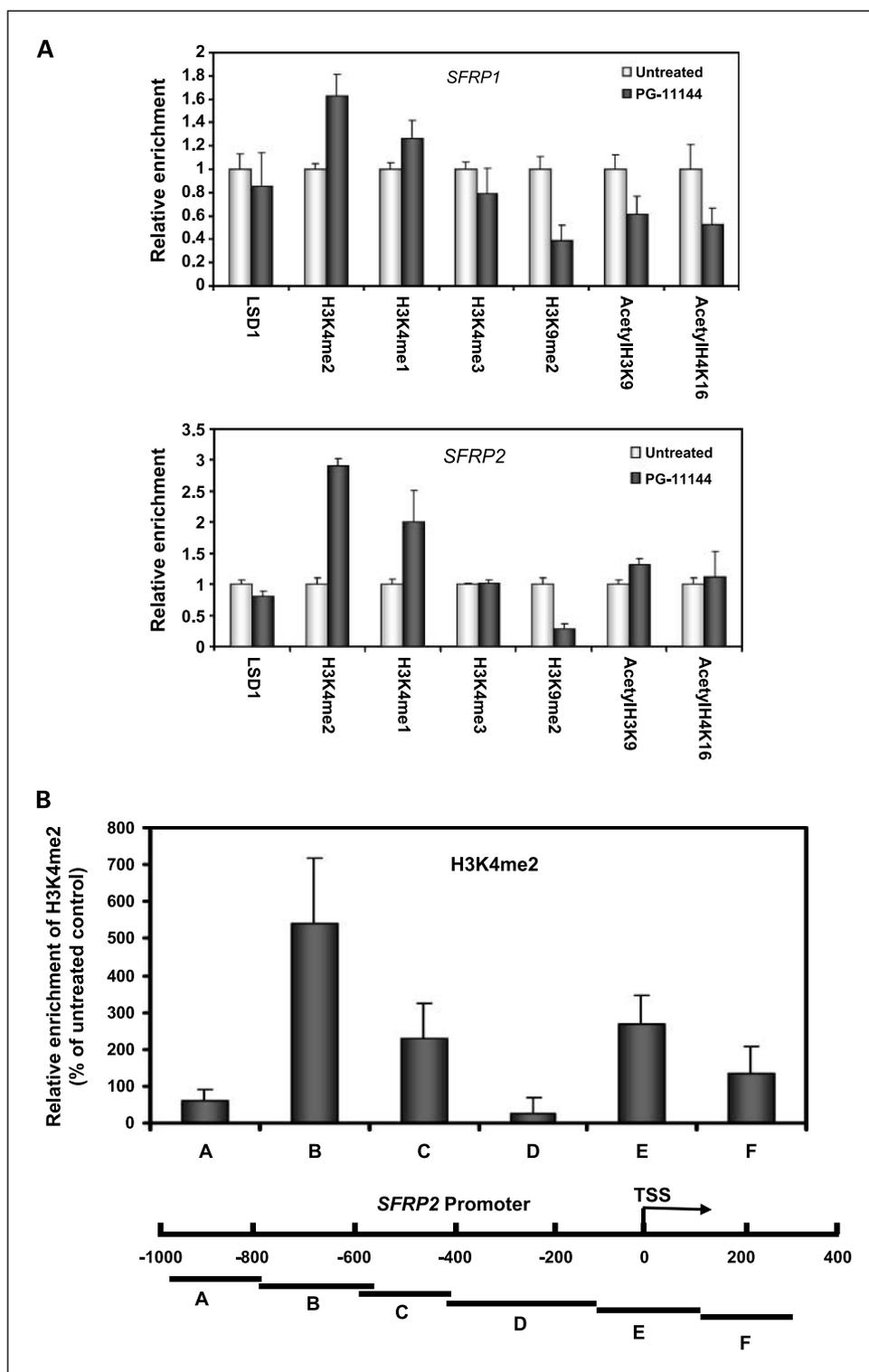
DNA methylation inhibition and histone deacetylase inhibition in the reexpression of silenced genes in colorectal cancer cells (26). To determine whether the combination of LSD1-inhibiting oligoamines with DNMT inhibitors can lead to enhanced expression of epigenetically silenced genes in cancer cells, we investigated the expression status of the *SFRP1* and *SFRP2* genes in HCT116 cells following a 24-hour treatment with low doses of oligoamines and the DNMT inhibitor DAC, alone and in combination. The combination resulted in a striking synergistic increase in expression of *SFRP2* (Fig. 5) but not *SFRP1* (data not shown). This selectivity suggests that similar to other agents targeting epigenetic regulation, the genes that are reexpressed may be tumor type and agent specific (27). However, a broader-based analysis of genome-wide effects of the combination treatments will be necessary to understand the underlying mechanism resulting in selective reexpression of genes.

To determine if the synergistic response of *SFRP2* expression to the combination treatment with PG-11144 and DAC was a result of a greater reduction of promoter CpG methylation, bisulfite sequencing was done on cells treated with either PG-11144 or DAC alone or in combination. The results indicate that PG-11144 treatment has no effect on CpG methylation and that the combination treatment did not result in greater demethylation of the *SFRP2* promoter than treatment with DAC alone (Supplementary Fig. S3).

PG-11144 combined with a DNMT inhibitor increases H3K4 methylation and profoundly inhibits growth of established tumors in vivo. The preceding *in vitro* results demonstrating apparent synergy between the LSD1-inhibiting polyamine analogues and a DNMT inhibitor for gene reexpression raised the important question of whether such results might also translate to therapeutic efficacy. As previously stated, the concept of combination epigenetic therapies is an emerging theme for ongoing clinical trials. Therefore, we tested the *in vivo* therapeutic effect of polyamine analogues as LSD1 inhibitors alone or in combination with the DNMT inhibitor 5-Aza, using human colorectal cancer cell HCT116 xenografts in athymic nude mice. As a comparison, the *in vivo* effect of PG-11144 was compared with the effects of a previously identified potent LSD1 inhibitor, the biguanide polyamine analogue **2d** (10). Increasing doses of either PG-11144 or **2d** alone, or in combination with the DNMT inhibitor, were administered *i.p.* as described in Materials and Methods. Western blot analyses show that both PG-11144 and **2d** increase H3K4me2 in treated HCT116 tumors (Fig. 6A). Treatment with either PG-11144 or 5-Aza alone each generally displayed significant antitumor effects against growth of HCT116 xenografts, whereas treatment with **2d** alone did not. However, for both polyamine analogues, a marked increase in the inhibition of tumor growth was noted when combined with the DNMT inhibitor (Fig. 6B). Importantly, for future therapeutic translation of these data, there was no significant overall toxicity seen in the treatment groups as indicated by animal weight. These results indicate that the polyamine analogues effectively inhibit LSD1 and exhibit significantly increased growth inhibition when used in combination with a DNMT inhibitor.

Discussion

In this study, we merged the rapidly increasing interest in epigenetic abnormalities in cancer and the possibilities for reversing these changes as a cancer therapy target, with the latest



discoveries of enzymes that reverse key histone lysine modifications. The discovery of the amine oxidase homologue LSD1 as the first of these enzymes created an exciting new epigenetic target for which there has been an increasing effort to identify and design effective inhibitors (9–12). Our initial strategy for identifying effective inhibitors of LSD1 (3, 10, 14, 15, 28) was based on the structural similarity between LSD1 and the FAD-dependent

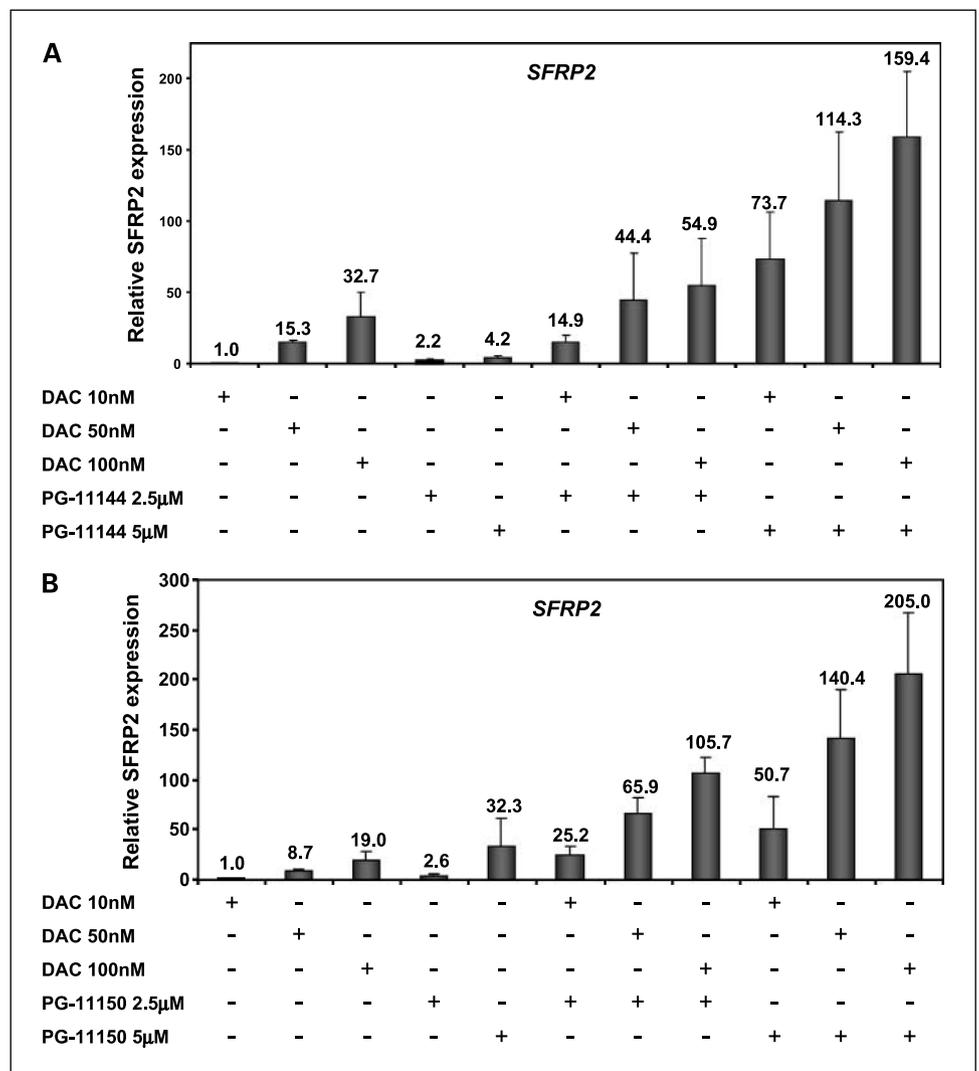
SMO. This strategy proved to be effective and resulted in the successful identification of biguanide and bisguanidine polyamine analogues as LSD1 inhibitors (10). In the present study, we explored the oligoamines, another class of polyamine analogues that inhibit polyamine oxidases and that have high affinity for DNA based on the increased number of positively charged nitrogens compared with the biguanide and bisguanidine analogues

(10, 15, 16). The oligoamines have shown efficacy in inhibiting growth and inducing apoptosis in multiple types of cancer cells *in vitro* and *in vivo* (29, 30). In human prostate carcinoma cells, oligoamines are efficacious in the 10 to 100 nmol/L dose range and are markedly more cytotoxic against tumor cells in culture than any previously described polyamine analogue (16). In human breast cancer cell lines, oligoamines can effectively inhibit growth in culture and in nude mouse xenografts without inducing significant weight loss (17).

We now show, in context with the above, that the oligoamine analogues are competitive inhibitors of recombinant LSD1, suggesting that the oligoamines may directly compete with the substrate at the active site. Although the precise mechanism of LSD1 inhibition by oligoamines remains unclear, our findings suggest that an understanding of the structural and functional similarities between amine oxidases can assist in identifying mechanism-based molecules that can specifically target LSD1. It should also be noted that it seems that the inhibitory activity of the oligoamines increases somewhat proportionally to increased charge and chain length. More rigorous structure/activity relationship studies will be required to determine whether this observation holds true over a wide range of analogues.

Abnormal epigenetic silencing of tumor suppressor and growth-regulatory genes due to promoter CpG hypermethylation is usually associated with the initiation and progression of multiple human cancers (1). CpG island hypermethylation frequently acts in concert with abnormal histone mark activities in silencing these genes. The active histone mark H3K4me2 is associated with open chromatin and increases track uniformly with increasing levels of active gene transcription (7, 8, 23, 31). Importantly, occupancy of H3K4me2 is globally found at low levels in the promoters of DNA hypermethylated and epigenetically silenced genes important in tumorigenesis (8, 32). Critically, such depressed levels of this histone mark have been found to provide a suitable milieu for recruitment of DNMTs to nucleosomes (33–35). Studies of the important role that these epigenetic changes play in tumorigenesis, and of how DNA methylation and the H3K4me2 mark may be tightly related, thus fuel active efforts to develop anticancer drugs that can target these epigenetic mechanisms and restore expression of important growth regulatory genes. The data presented here show that treatment of colorectal cancer cells with oligoamines indeed increases both global and gene promoter levels of H3K4me2 and H3K4me1. This increase in histone

Fig. 5. Synergy of oligoamines and DNMT inhibitor in the reexpression of an aberrantly silenced gene. HCT116 cells were treated for 24 h with the indicated doses of DAC and oligoamines (A) PG-11144 and (B) PG-11150 alone or simultaneously. RNA was extracted and cDNA was synthesized and subjected to quantitative real-time PCR for the *SFRP2* gene using the DNA-intercalating SYBR green reagent. GAPDH expression was used as an internal standard. Columns, means of three independent experiments done in triplicate in the MyiQ single-color real-time PCR machine (Bio-Rad); bars, SD.



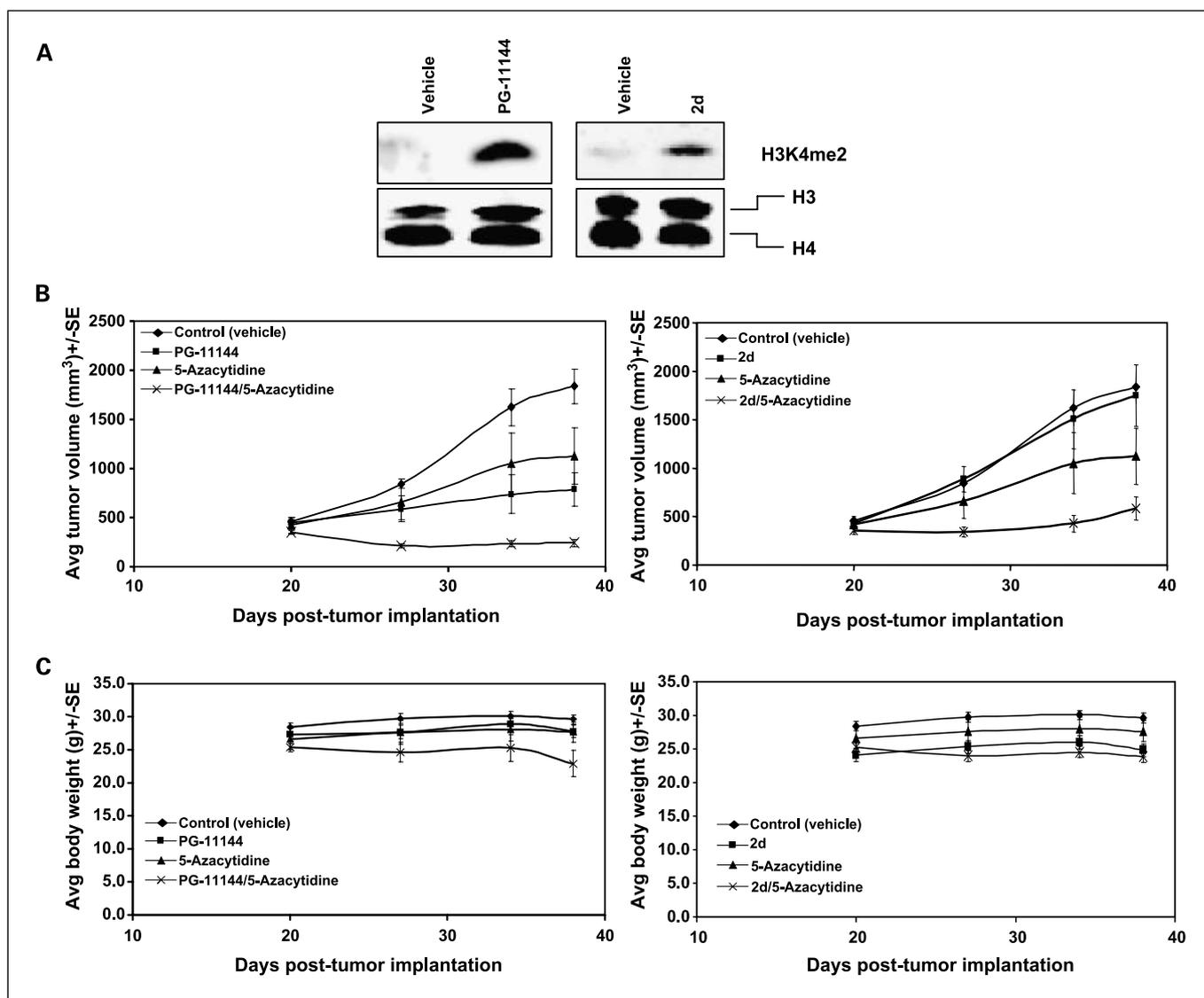


Fig. 6. Effects of polyamine analogues on LSD1 and tumor growth in nude mice bearing HCT116 xenografts. Mice were randomized into treatment ($n = 5$) and control ($n = 5$) groups by 20 d postimplantation of HCT116 xenograft. The treatment groups given as single agents were as follows: vehicle (saline control, daily $\times 5$), PG-11144 (10 mg/kg, twice per week), 2d (10 mg/kg, daily $\times 5$), and 5-Aza (2 mg/kg, daily $\times 5$). The combination treatment groups were as follows: PG-11144 (10 mg/kg, twice per week)/5-Aza (2 mg/kg, daily $\times 5$), and 2d (10 mg/kg, daily $\times 5$)/5-Aza (2 mg/kg, daily $\times 5$). A, tumor sample of HCT116 xenografts treated with vehicle (saline control, daily $\times 5$), PG-11144 (10 mg/kg, 2 \times wk), or 2d (10 mg/kg, daily $\times 5$) for 37 d. Thirty micrograms of nuclear protein per lane were analyzed for expression of H3K4me2 and H3/H4 as a loading control. B, tumor volumes of mice measured twice weekly. Points, mean tumor size (in mm³); bars, SEM. C, weights of mice were measured weekly. Points, mean mouse weight (g); bars, SEM.

methylation is accompanied by a substantial reexpression of multiple important genes that are aberrantly silenced in tumorigenesis, including the Wnt signaling pathway antagonist family members; secreted frizzled-related proteins *SFRP1*, *SFRP2*, and *SFRP4*; and the *GATA* family transcription factor *GATA5*. Importantly, ChIP analysis not only confirms that LSD1 is present at the promoter of each gene examined but also that treatment of cells with oligoamines has no obvious effect on this promoter occupancy. This latter finding suggests that the effects seen for the oligoamines result from inhibition of LSD1 enzymatic activity rather than displacement of the protein from its target locations. Another key finding is that levels of another histone modification associated with active genes, H3K4me3, remain unchanged with oligoamine treatment, suggesting that these compounds are selective for

LSD1 inhibition and do not affect the JmjC domain demethylases that target H3K4me3 (36–38). Interestingly, increased H3K4me2 that results from oligoamine treatment was accompanied by a decrease in the repressive mark H3K9me2 in both genes examined. These results are similar to the downregulation of H3K9me2 that is observed with gene reactivation in colon cancer cells treated with DNMT inhibitors or biguanide and bisguanidine polyamine analogues, as well as in colon cancer cells in which two key DNMTs have been genetically disrupted (8, 10, 23, 39). This suggests that reduced levels of H3K9me2 might be a common mechanism contributing to re-expressing genes silenced in colorectal cancer cells treated with various epigenetic reagents. However, it should be noted that in contrast to results with the biguanide and bisguanidine LSD1 inhibitors, no consistent increase in H3K9 acetylation

was observed after oligoamine treatment (10), demonstrating that oligoamine-induced expression of previously silenced genes occurs in the absence of substantial increases in this activating mark. These differences suggest that the classes of LSD1 inhibitors may have varied off-target effects that contribute to reactivation of aberrantly silenced genes, including their ability to alter chromatin structure based on their affinity for DNA (16, 40, 41).

It is notable that the local pattern of H3K4me2 observed in the promoter of the *SFRP2* gene after treatment with PG-11144 mirrors what has been reported for other silenced genes whose expression has been induced by DAC, suggesting that this local pattern is a common occurrence when previously silenced genes are reexpressed (8). Additionally, this pattern of a biphasic H3K4me2 peak proximal to the transcriptional start site is commonly seen in actively transcribed genes and is likely the result of a combination of nucleosomal positioning and the positioning of transcriptional repressing or activating complexes (42–44).

The combination of DNMT and HDAC inhibitors has shown synergistic effect in reexpressing epigenetically silenced genes in cultured cancer cells and resulted in clinical response in patients with leukemias (21, 26). Therefore, we hypothesized that the combination of the LSD1-inhibiting oligoamines would be more effective when combined with DNMT inhibitors in reexpression of silenced genes and in tumor growth inhibition. Here, we report the first attempt to combine LSD1 inhibitors with agents that target DNA CpG island methylation. The results of these experiments show that the *in vitro* combination of low-dose oligoamine and DNMT inhibitor also results in ro-

bust reexpression of the *SFRP2* gene. *In vivo*, treatment with either PG-11144 or the previously identified LSD1 inhibitor 2d (10) produced increases in tumor H3K4me2 levels. Treatment with PG-11144 alone also significantly inhibited the growth of HCT116 xenograft tumors in nude mice. Importantly, the most efficacious inhibition of tumor growth was observed when either of the LSD1-inhibiting analogues was used in combination with the DNMT inhibitor 5-Aza. These results indicate that, similar to what has been observed with the HDAC inhibitors, LSD1 inhibitors may be most effective when used in combination with agents that target other epigenetic regulatory mechanisms and provide the basis for clinical trial combining agents targeting these two epigenetic regulatory pathways.

In summary, we show that a novel class of polyamine analogues, the oligoamines, inhibits LSD1 activity and results in the reactivation of epigenetically silenced genes important in tumorigenesis. The results of these studies are consistent with the hypothesis that LSD1 represents a rational and important epigenetic target for drug development. Additionally, these studies show the promise of using LSD1 inhibitors in combination with other chromatin-modifying agents as a novel approach to cancer treatment with considerable clinical potential.

Disclosure of Potential Conflicts of Interest

R.A. Casero, Jr., and P.M. Woster received commercial research support from and are consultants for Progen Pharmaceuticals; L.J. Marton, Chief Scientific Officer, Progen Pharmaceuticals; S.B. Baylin, consultant, Oncomethylome Sciences; R.A. Casero, Jr., P.M. Woster, and S.B. Baylin, scientific advisors to Progen Pharmaceuticals; R.A. Casero, Jr., P.M. Woster, L.J. Marton, and S.B. Baylin are inventors with patents/patent applications related to this article.

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Novel Oligoamine Analogues Inhibit Lysine-Specific Demethylase 1 and Induce Reexpression of Epigenetically Silenced Genes

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