Inhibition of Histone Deacetylation Does Not Block Resilencing of p16 after 5-Aza-2′-Deoxycytidine Treatment

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Abstract

Epigenetic drugs are in use in clinical trials of various human cancers and are potent at reactivating genes silenced by DNA methylation and chromatin modifications. We report here the analysis of a set of normal fibroblast and cancer cell lines after combination treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-CdR) and the histone deacetylase inhibitor 4-phenylbutyric acid (PBA). Low doses of the drug combination caused cell cycle arrest, whereas high doses induced apoptosis in T24 bladder carcinoma cells. Both p16 (CDKN2A/INK4) and p21 (CIP1/SDI1/WAF1) expression were induced to similar levels in normal and cancer cells in a dose-dependent fashion after combination treatments. We detected a distinct increase of histone H3 acetylation at lysine 9/14 near the transcription start sites, in both LD419 normal fibroblasts and T24 bladder carcinoma cells, whereas the acetylation changes in the p21 locus were less apparent. Interestingly, the levels of trimethylation of histone H3 on lysine 9, which usually marks inactive chromatin regions and was associated with the p16 promoter in silenced T24 cells, did not change after drug treatments. Furthermore, we provide evidence that the remethylation of the p16 promoter CpG island in T24 cells after 5-aza-CdR treatment cannot be halted by subsequent continuous PBA treatment. The p16 gene is resilient with kinetics similar to 5-aza-CdR only–treated cells, which is also marked by a localized loss of histone acetylation at the transcription start site. Altogether, our data provide new insights into the mechanism of epigenetic drugs and have important implications for epigenetic therapy.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Introduction

DNA methylation occurs at the C-5 position of cytosine residues in CpG dinucleotides, which are found at a lower than expected frequency in mammalian DNA but are clustered in CpG-rich areas called "CpG islands" (1, 2). These islands are often found close to promoters and are usually unmethylated in normal somatic tissues, whether the gene is transcriptionally active. This is in contrast to tumor cells, in which aberrant region-specific hypermethylation of CpG islands located in or near gene promoters is associated with transcriptional silencing. The DNA methyltransferase (DNMT) inhibitors 5-azacytidine and 5-aza-2′-deoxycytidine (5-aza-CdR) can cause global DNA demethylation and induce the reexpression of silent genes in addition to inhibiting the growth of numerous tumor cell lines in vitro and in vivo (3).

Covalent modifications of histone NH2-terminal tails affect chromatin structure and the interaction of DNA with transcriptional regulatory proteins, thereby modulating gene expression. Histone deacetylases (HDAC) remove acetyl groups from conserved lysines within the histone tails resulting in compaction of chromatin structure and thus the repression of gene transcription. Numerous HDAC inhibitors have been shown to deregulate gene expression and inhibit tumor growth both in vitro and in animal models of various cancers by causing hyperacetylation of nucleosomal histones of different target genes (4). Many of these inhibitors are in clinical trials at this time and have already shown promising antitumor activity (5). Most studies have used trichostatin A (TSA), a potent HDAC inhibitor, in combination treatments; however, we chose to study 4-phenylbutyric acid (PBA), which is less cytotoxic and already established in clinical practice.

DNA methylation and histone modifications are dynamically linked in the epigenetic control of gene expression and play an important role in tumorigenesis (3). Various in vitro experiments have shown that DNMT inhibitors, such as 5-aza-CdR, and HDAC inhibitors, such as PBA, are highly synergistic in the reinduction of silenced genes, in the inhibition of cell growth, and in the promotion of apoptosis (6–10). In mice treated with a carcinogen, low doses of 5-aza-CdR and PBA were synergistic in the prevention of lung tumor development (11).

Two well-studied targets of epigenetic drugs are the cell cycle regulators p16 and p21. The promoter CpG island of the p16 gene is frequently silenced by DNA hypermethylation in cancer (12, 13), which can be removed by demethylating agents. In addition, p21 contains a CpG island in its promoter, which has been reported to be methylated in different tumors, although less frequently than p16 (14). Furthermore, p53, a direct upstream inducer of p21, is often mutated in tumors and thereby limits p21 expression. HDAC inhibitors are able to directly induce p21 in a p53-independent manner by causing hyperacetylation of the p21 locus (15).

We determined the doses of 5-aza-CdR and PBA, which led to either cell cycle arrest or to apoptosis in cancer cells and we compared the induction and chromatin states of the cell cycle regulators p16 and p21 in various cancer and normal cell lines at these doses. We analyzed the effects of the drug combination on normal fibroblasts, which are actively dividing, to learn more about the possible consequences of epigenetic therapy on normal tissues. One potential problem with epigenetic therapy is that demethylated promoters often undergo a slow remethylation and resilencing after treatment with DNA methylation inhibitors (16). A main focus of our work therefore was to study the long-term effects of combination treatment of cancer cells. Intriguingly, inhibition of HDAC after demethylation did not...
slow the remethylation and resiliencing of the p16 gene and caused a reversion to a repressive chromatin conformation.

Materials and Methods

**Tissue culture.** T24 (bladder transitional carcinoma cells), CFPAC-1 (pancreatic carcinoma cells), CALU-1 (lung carcinoma cells), NCCIT (embryonal carcinoma cells), and CCD-1070SK (human normal fibroblasts) were obtained from the American Type Culture Collection (Manassas, VA). LD98, LD419, and T1 (human normal fibroblasts) were established in our laboratory and PC3 (prostate carcinoma) was kindly provided by Dr. Gerry Coetzee (Department of Urology, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA). LD98, LD419, T1, and T24 cells were cultured in McCoy’s 5A medium supplemented with 10% heat-inactivated FCS. CFPAC-1 cells were propagated in Iveco’s modified Dulbecco’s medium containing 10% FCS and 1× glutamine (Life Technologies, Inc., Palo Alto, CA). CALU-1 cells were kept in McCoy’s 5A supplemented with 10% FCS and 1× glutamine. PC3 cells were cultured in RPMI 1640 with 5% FCS. CCD-1070SK cells were cultured in MEM containing 10% FCS, 1× sodium pyruvate (Life Technologies), and 1× MEM nonessential amino acids. NCCIT cells were grown in RPMI 1640 with 10% FCS. All cultures were kept in a humidified incubator at 37°C in 5% CO2.

**Drug treatment.** To determine the optimal drug doses, T24 cells were treated with increasing doses of 3-aza-CdR (Sigma, St. Louis, MO) for 24 h, the medium was changed the following day, and cells were harvested on day 3. PBA was given in the indicated doses for 3 days continuously. For long-term treatment, PBA treatment was continued, and the drug was replaced every 24 h. For reverse transcription analyses and chromatin immunoprecipitation (ChIP) assays, cells were treated with 1 μmol/L 5-aza-CdR and 3 mmol/L PBA (high dose) or 3 μmol/L 5-aza-CdR and 3 mmol/L PBA (high dose) concurrently, 24 h after seeding. 5-Aza-CdR was omitted after 24 h of treatment, PBA treatment was continued, and the drug was replaced every 24 h.

**Quantitation of DNA methylation levels.** Genomic DNA was bisulfite converted as described previously (17). After amplification of bisulfite-converted DNA with primers specific for converted DNA, methylation levels were measured by Ms-SNuPE (18). Sequences for primers for bisulfite-PCR were found in Supplementary Table S1.

**Histone preparation and Western blots.** Total histones were prepared by acidic extraction and resolved on 18% SDS-polyacrylamide gels as described previously (19). Antibodies used for Western blotting were directed against acetylated histone H3 lysine 9/14, acetylated histone H4 lysine 3.3, 5-aza-CdR and a maximal effect (∼50%) was seen after addition of 3.3 μmol/L of the drug (Fig. 1A). As expected, PBA treatment did not affect the hypermethylation of the p16 gene (Fig. 1A). Histone acetylation after drug treatment was examined by probing a Western blot of total histone preparations with an antibody recognizing acetylated histone H4 (lysine 5, 8, 12, and 16; Fig. 1B). Whereas 5-aza-CdR treatment had no effect on global histone H4 acetylation, increasing amounts of PBA caused a dose-dependent hyperacetylation of H4. Because 1 mmol/L PBA led to detectable changes and 3 mmol/L to a maximal change in acetylation, we chose 1 μmol/L 5-aza-CdR and 1 mmol/L PBA as low-dose regimens and 3 μmol/L 5-aza-CdR and 3 mmol/L PBA as high-dose regimens for our experiments.

Results

**Determination of drug doses.** Optimal doses of PBA and 5-aza-CdR were determined by monitoring the effects of increasing doses of 5-aza-CdR on DNA demethylation and PBA on histone hyperacetylation in T24 bladder cancer cells (Fig. 1). "Low dose" was defined as the dose of either drug leading to detectable changes and "high dose" as the dose leading to a plateau in change in DNA demethylation or histone acetylation. Substantial demethylation of p16 exon 1 was observed after 24 h of treatment with 1 μmol/L 5-aza-CdR and a maximal effect (∼50%) was seen after addition of 3.3 μmol/L of the drug (Fig. 1A). As expected, PBA treatment did not affect the hypermethylation of the p16 gene (Fig. 1A). Histone acetylation after drug treatment was examined by probing a Western blot of total histone preparations with an antibody recognizing acetylated histone H4 (lysine 5, 8, 12, and 16; Fig. 1B).

**Figure 1.** Determination of drug doses. A, quantitative Ms-SNuPE analysis of the methylation level of the p16 exon 1 CpG island in T24 bladder cancer cells before and after 5-aza-CdR or PBA treatment. Cells were treated with indicated doses of 5-aza-CdR for 24 h or PBA continuously and analyzed on day 3 of treatment. X axis, the different treatments with increasing concentrations; Y axis, the percentage of methylation as analyzed by three independent CpG sites. B, Western blots of total histone preparations of T24 cells treated as in A, sequentially probed with an antibody against acetylated histone H4 (top) or global histone H4 as a loading control (bottom).
Effects of DNMT and HDAC inhibition on growth of T24 cells. Inhibitors of HDACs and DNMTs are potent cell cycle inhibitors and inducers of apoptosis (8, 9). We first investigated the effects of high and low doses of 24 h of exposure to 5-aza-CdR followed by continuous PBA treatment on T24 bladder cancer cells on a long-term basis. Treatment with low doses of 5-aza-CdR or PBA by themselves resulted in minor effects on cell population doublings, whereas a more pronounced decrease of cell numbers was observed when 5-aza-CdR and PBA were used sequentially (Fig. 2A). For high-dose experiments, 3 mmol/L PBA and 3 μmol/L 5-aza-CdR showed remarkable inhibitions of proliferation in single treatments. Combination treatment with high doses of both drugs reduced cell counts to a great extent, such that the experiment could not be continued after day 6 (Fig. 2B). Previous studies have used sequential scheduling for combination treatments (8, 9, 21). We also used concurrent administration but did not observe differences in the effects on proliferation or apoptosis (data not shown).

Effects of combination treatment on cell cycle distribution and viability of T24 cells. Fluorescence-activated cell sorting (FACS) analyses and apoptotic assays were done to test whether the reduction in cell numbers was due to reduced cell proliferation or increased cell death (Fig. 3). Low or high doses of 5-aza-CdR had little effects on the overall cell cycle distribution; only a slight broadening of the G1 peak was observed. Single treatment with PBA led to a dose-dependent reduction of S and G2-M phase cells. Low doses of combination treatment had little effect on the overall FACS profile, whereas high doses of both PBA and 5-aza-CdR together showed a loss of S and G2-M phase cells and a substantial fraction of cells at the sub-G1 level, indicating that high doses of the drugs impaired proper cell cycle regulation (Fig. 3A). Combined Annexin V and propidium iodide staining was done to test whether the increased sub-G1 fraction in the high-dose combination-treated population represented apoptotic cells (Fig. 3B). Control cells and cells treated with 3 mmol/L PBA only showed similar distributions within the bottom left quadrant of the diagram, representing viable cells, with ~10% apoptotic cells, most probably caused by trypsinization of cells before staining. High doses of 5-aza-CdR caused a slight shift of cells to the bottom right quadrant, representing early apoptotic cells. Therefore, high doses of combination

Figure 2. Population doublings. A, T24 cells were seeded on day 0 at a density of 2,500/cm² and treated with 5-aza-CdR (1 μmol/L) for 24 h on day 1. Medium was changed on day 2 and PBA (1 mmol/L) was given on day 3 and added freshly every day. Cells were counted every 6 d and reseeded at the initial density. Note that cell numbers are depicted on a logarithmic scale, determined as population doublings = [log (total number of cells / number of cells seeded)] / log2. B, T24 cells were seeded at a density of 2,500/cm² and treated with 5-aza-CdR (3 mmol/L) and PBA (3 mmol/L) as in (A). Cells were counted every 2 d. Population doublings were calculated as in (A). Note the differences in scale in the long-term (A) and short-term (B) illustrations.

Figure 3. Cell cycle and apoptotic analyses. A, T24 cells were seeded and drug treated as described above (Fig. 2A). Cells were analyzed by flow cytometry on day 6. The two peaks in the FACS diagrams indicate G0-G1 and G2-M cells with S-phase cells between peaks. Sub-G1 fractions represent cells with fragmented DNA, or apoptotic cells. B, determination of apoptosis of T24 cells by flow cytometry using Annexin V (FL-1H) and propidium iodide (FL-2H) staining after indicated drug treatments. 5-Aza-CdR and PBA were given sequentially and cells were assayed on day 6 after seeding. Bottom left quadrant, viable cells; bottom right quadrant, early apoptotic cells; top right quadrant, late apoptotic and necrotic cells.
treatment were synergistic in inducing apoptosis, as observed previously (9), probably due to a global epigenetic destabilization of chromatin.

Effects of combination treatment on the expression of cell cycle regulators. The cell cycle inhibitors p16 and p21 are two well-characterized targets of DNMT and/or HDAC inhibitors (15, 16). We analyzed the expression of these genes by quantitative real-time RT-PCR in a set of normal fibroblast and cancer cell lines untreated or treated with low- and high-dose combinations of 5-aza-CdR and PBA to determine the generality of the findings (Fig. 4). All four fibroblast cell lines examined have an unmethylated p16 promoter (22) and showed basal levels of p16 expression in untreated cells, which were induced in all cases by low- and high-dose combinations of 5-aza-CdR and PBA. In cancer cell lines, p16 was silenced by DNA methylation, except for NCCIT cells (ref. 22; data not shown), and expressed at very low levels but induced after combination treatment to levels comparable with wild-type cell lines and mostly in a dose-dependent fashion (Fig. 4A). On the other hand, p21 expression and induction was markedly different in normal and tumor cell lines. Normal cells expressed higher basal levels of p21, which was up-regulated to a greater extent after combination treatment when compared with cancer cell lines. The extent of p21 expression after high-dose combination treatment in cancer cells was in most cases lower than basal expression levels in normal fibroblasts (Fig. 4B). Taken together, our results suggest that both normal and cancer cell lines respond to 5-aza-CdR and PBA treatment by up-regulation of the cell cycle inhibitors p16 and p21 in both DNA methylation-dependent and DNA methylation-independent fashion. Normal cells generally seem to be less affected by epigenetic drugs (22–24), most likely because of their reduced proliferation rates compared with cancer cells. However, the drug combination we used seemed to be equal or even more potent in inducing cell cycle inhibition in normal fibroblasts, which might have implications for therapy.

Changes in chromatin modifications. To determine covalent chromatin changes after treatments at the p16 and p21 loci, we did ChIP analyses with antibodies against acetylated histone H3, a marker for active chromatin (Fig. 5). LD419 normal fibroblasts, which have an unmethylated p16 promoter (22), showed basal levels of acetylated H3 in untreated and PBA- or 5-aza-CdR only–treated cells at the p16 locus, with slightly elevated levels surrounding the transcription start site. After combination treatment, we detected an increase in H3 acetylation spanning the whole p16 locus, which was especially high around the transcription start site and indicative of activation (Fig. 5A, top). In contrast, we could not detect H3 acetylation at the p16 locus in untreated or PBA only–treated T24 cells, which did not express p16. After 5-aza-CdR or combination treatment, an increase in H3 acetylation was apparent with a prominent peak after the transcription start site, similar to what we observed in LD419 cells (Fig. 5A, bottom). Therefore, the levels of p16 expression correlated with the amount of H3 acetylation present at the transcription start site in both normal fibroblasts and cancer cells. Comparing the levels of the repressive histone mark H3K9 trimethyl (H3K9trim), we found that the transcription start site in nonexpressing T24 cells was associated with higher levels of this modification than LD419 cells (Fig. 5B). Intriguingly, the methylation mark was not lost or decreased after drug treatment and induction of p16 but was maintained at similar or slightly higher levels in T24 cells, indicating that the repressive histone conformation cannot be completely reversed after drug treatments.

The cell cycle inhibitor p21 can be induced in a p53-dependent or p53-independent manner following treatment with several HDAC inhibitors (25) and by 5-aza-CdR treatment (26, 27). ChIP assays upstream and downstream of the p21 transcription start site showed high levels of histone H3 acetylation downstream of the transcription start site in untreated LD419 and T24 cells, which increased slightly on 5-aza-CdR and/or PBA treatment (Fig. 5C). Although we had observed significant differences in the expression levels of p21 in normal fibroblast and cancer cell lines, with a much higher induction level in normal cells (Fig. 4B), we could not detect drastic differences in histone H3 acetylation at the p21 locus between LD419 and T24 cells. Furthermore, we did not find any remarkable levels of H3K9trim at the four regions analyzed in neither cell line before or after treatments (data not shown).

In summary, we detected changes in acetylated histone H3 after 5-aza-CdR and/or PBA treatment, which were more obvious at the p16 locus after combination treatment and less apparent in the p21 regions tested and were similar in normal and tumor cells. Interestingly, H3K9trim, which was higher in cancer cells, was not altered by the various treatments.

Long-term effects of the combination treatment. A major caveat of 5-aza-CdR treatment is the remethylation and resilencing of genes, such as p16, after several rounds of DNA replication (16). Because HDACs and DNMTs work together in the same complexes
(28), we tested whether continuous treatment with PBA after 5-aza-CdR treatment would inhibit or retard this remethylation, thereby prolonging the expression of aberrantly silenced genes (Fig. 6). We chose low doses of both regimens for this experiment to keep cells proliferating at similar levels as untreated cells, thereby avoiding potential artifacts. Treatment of cells with PBA did not change the methylation level of p16 exon 1, whereas 24 h of exposure to 5-aza-CdR reduced the methylation to ~50% (Fig. 6A). No difference in the rate of remethylation was observed between cells treated with 5-aza-CdR only and those subsequently exposed to PBA. The reacquisition of DNA methylation is accompanied by resilingencing of the p16 gene (Fig. 6B). Low doses of 5-aza-CdR or

Figure 5. p16 and p21 ChIP analyses. A, ChIP analysis of the p16 locus after 5-aza-CdR and PBA treatment in LD419 (top) and T24 (bottom) cells. Top, p16 locus, with ChIP PCR amplicons (boxes below graph; 1-8). E1, exon 1; E2, exon 2; shaded boxes, CpG islands; black boxes, repetitive elements; arrow, transcription start site. Bottom, ChIP results obtained with an antibody directed against acetylated histone H3 lysine 9/14, normalized to global histone H3. Treatments were done concurrently and cells were harvested on day 5 of the experiment. 0, untreated; A3, 3 μmol/L 5-aza-CdR; P3, 3 mmol/L PBA; AP3, 3 μmol/L 5-aza-CdR + 3 mmol/L PBA. B, p16 promoter ChIP analysis using an antibody against H3K9 trim with the same chromatin preparations as in (A) normalized to total H3. Top, LD419 cells; bottom, T24 cells. C, p21 ChIP analysis. Top, p21 locus with ChIP PCR amplicons (boxes 1-4). E1, exon 1; shaded box, CpG island; black arrow, transcription start site. The same immunoprecipitated DNA as in (A) was used for real-time PCR analysis.
5-aza-CdR plus PBA induced the expression of p16 to maximal levels between days 3 and 6 of treatment. Thereafter, the expression decreased and was almost completely silenced between days 25 and 35 of treatment. Interestingly, the combination-treated cells maintained higher levels of p16 expression throughout the experiment, although the expression was silenced with comparable kinetics to the 5-aza-CdR-treated cells. We next analyzed the active chromatin marks at the p16 promoter after 31 days of treatments, at the time of p16 resilencing. The peak of histone H3 acetylation, which we observed on day 5 after treatment at the transcription start site (Fig. 5A), was completely ablated after long-term treatment and had returned to the levels of untreated or PBA-treated cells (Fig. 6C). This loss of acetylation was specific for the p16 locus because global histone H4 acetylation levels were still higher in PBA- or combination-treated cells (Fig. 6D). Therefore, DNA methylation seems to be the stronger epigenetic mark not only in terms of reexpression of genes after treatment with different drugs (8) but also in setting up the repressive state.

Discussion

DNMT inhibitors, such as 5-aza-CdR, and HDAC inhibitors, such as PBA, are synergistic in their antitumorigenic effects (3). In the current study, we observed a major decrease in cell growth in T24 cells in the high-dose group (3 μmol/L 5-aza-CdR for 24 h and 3 mmol/L PBA continuous), which was clearly due to the significant apoptosis caused by the drug combination. This occurred within 6 days of treatment, after which very few cells survived. 5-Aza-CdR is a nucleoside analogue, which at high doses causes DNA damage and inhibits chromosome condensation resulting in cytotoxicity (29). It is a potent inducer of heterochromatin decondensation, which can lead to complex alterations in chromosome compaction during mitosis resulting in chromosome instability (30–32). The HDAC inhibitor could contribute to this process by causing the decondensation of chromatin, thereby increasing genomic instability. It has been shown that, in the presence of TSA, cells enter mitosis with hyperacetylated histones, which is associated with marked decondensation of the chromosomes and with segregation defects leading to aneuploidy and defects in chromosome structure (33, 34). Likewise, suberoylanilide hydroxamic acid induced polyploidy and senescence in colon and breast cancer cell lines (35). Therefore, high-dose combination of PBA and 5-aza-CdR might induce an “epigenetic catastrophe” by greatly changing chromosome architecture, which will not allow for controlled DNA replication or mitosis and finally initiate apoptosis. Several different apoptotic pathways have been associated with cell death after HDAC inhibitor treatments, such as caspase-dependent or caspase-independent mechanisms or the tumor necrosis factor–related apoptosis-inducing ligand and FAS pathways (36).

Figure 6. Remethylation patterns of p16 exon 1. A, methylation analysis of p16 exon 1 in T24 cells either untreated or treated for 35 d with 1 μmol/L 5-aza-CdR and 1 mmol/L PBA only or in combination by Ms-SnuPE (18). Cells were seeded in the same density and treated with the same scheduling as described above (Fig. 2). B, p16 reverse transcription analysis of long-term treated T24 cells, treated with 1 μmol/L 5-aza-CdR and/or PBA. Columns, p16/GAPDH (three independent RT-PCRs); bars, SD. C, ChIP analysis of the p16 locus with an antibody against acetylated histone H3 on day 31 of combination or single drug treatments. 0, untreated; A1, 1 μmol/L 5-aza-CdR; P1, 1 mmol/L PBA; AP1, 1 μmol/L 5-aza-CdR + 1 mmol/L PBA. D, Western blot of ChIP input samples from sequentially probed with antibodies against hyperacetylated H4 global H4 (C) on day 31 of treatment.
Low doses of the drugs caused a decrease in population doublings, which was maintained during long-term culture. Because we do not observe an increase in apoptosis in the cells treated in this manner, this is more likely to be a consequence of reexpression of genes involved in tumor suppression and cell cycle regulation, such as p16 and p21. We observed an increase in the expression of p16 with both agents, as has been reported previously with the combination of HDAC inhibitors and 5-aza-CdR (8, 37). Interestingly, both normal and cancer cells showed very similar induction levels after combination treatment. In most of the cancer cell lines, p16 is silent because of DNA hypermethylation, whereas normal cells express basal levels of p16 and are unmethylated (22). Intriguingly, the combination treatments led to a very distinct peak of histone H3 acetylation around the transcription start sites in both normal and cancer cells (Fig. 5). Interestingly, treatment with 5-aza-CdR only also induced elevated levels of H3 acetylation at the p16 start sites in both cell lines, which was not due to an increase in global histone acetylation in T24 cells. Therefore, the induction of the p16 gene was well reflected in changes in active chromatin modifications at the p16 start sites, which suggests that the induction of p16 was caused by direct effects of 5-aza-CdR and PBA on chromatin structure in normal fibroblasts and cancer cells.

It has been shown before that dimethylation of H3K9 (H3K9dim) is decreased after 5-aza-CdR treatment at various promoters (38, 39). H3K9trim can be seen as a stronger repressive mark than H3K9dim, and it is more defined and usually associated with heterochromatic regions and gene silencing. Therefore, the association of H3K9trim spanning the p16 region even after drug treatments in T24 cells was a very interesting although unexpected observation. This has also been observed recently in HCT116 colon cancer cells, where DNA demethylation by 5-aza-CdR did not cause a loss of H3K9trim or H3K27 methylation at several promoters of tumor suppressor genes (40). Therefore, the remaining of H3K9trim at the promoter might attract DNA methylation and cause remethylation of p16 in T24 cells.

Although some authors have reported that the p21 promoter can be methylated (41–43), we did not find this to be the case in T24 bladder cancer cells or LD419 normal fibroblasts. In fact, we saw an even higher induction of p21 in normal compared with cancer cell lines. Our ChIP experiments showed only minor changes in chromatin modifications before and after treatment at the p21 locus. This may suggest that the p21 promoter is already poised for activation in the cell lines tested and that the effects on transcription are primarily indirect and through activation of different transcription factors and not by effects on chromatin structure at the p21 promoter.

Given the fact that HDACs are found in the same complexes with DNMTs (28), we hypothesized that the inhibition of HDAC activity by PBA would prevent the remethylation of the p16 promoter, which is known to occur gradually after withdrawal of 5-aza-CdR (16). However, this was not the case, and addition of PBA-pretreated cells to 5-aza-CdR-pretreated cells did not inhibit or retard the DNA remethylation seen in 5-aza-CdR–treated cells. This confirms again the stronger effect of DNA methylation on gene silencing in both initiating and maintaining stable epigenetic changes. Therefore, only a complete reversal from the “malignant” to a “normal” chromatin state might be effective in stably reversing epigenetic silencing.

Two aspects of our work are of special clinical relevance. First, we found that normal fibroblasts are also affected by the combination drug treatment and show up-regulation of the cell cycle regulators p16 and p21. Therefore, it will be of importance to investigate more normal tissues, in particular proliferating ones, which are predicted to uptake more of the drugs and be more sensitive to treatments. Second, long-term treatments with PBA could not inhibit DNA remethylation and reversal of repressive chromatin at the p16 locus. This problem might be circumvented by a prolonged treatment with less cytotoxic DNMT inhibitors, such as zebularine (44), which might be more potent in stably reverting tumor cells to a more normal epigenetic profile.

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