Halogenated Thymidine Analogues Restore the Expression of Silenced Genes without Demethylation

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Abstract

Transcriptional silencing of tumor suppressor genes by aberrant DNA methylation is a characteristic frequently observed in cancer cells. Therefore, reversing this process is a therapeutic target against cancer. In this study, we established a screening system for silencing inhibitors with cell lines transfected by a retroviral vector containing a luciferase gene. More than 100 nucleosides were tested for antisilencing activity with a selected clone in which the silenced expression of luciferase could be recovered by 5-aza-2'-deoxycytidine. A group of halogenated thymidine analogues was found to reactivate transcription of not only the reporter retrovirus vector but also endogenous glutathione-S-transferase 1 gene, without influence to DNA hypermethylation. Gel mobility shift assay showed that 5-bromo-2'-deoxyuridine (BrdUrd) or 5-iodo-2'-deoxyuridine incorporation did not affect the binding of the methyl-CpG binding protein motif to methylated DNA. Finally, in the retroviral promoter, BrdUrd treatment increased the acetylated histone H3 level and decreased methylation of histone H3 Lys9 in accordance with recovered transcription. This study shows that halogenated thymidines have an antisilencing effect without changing DNA methylation status by interfering with step(s) between DNA methylation and histone acetylation. (Cancer Res 2005; 65(15): 6927-33)

Introduction

CpG methylation, which is established and maintained by DNA methyltransferases, is a common modification in vertebrate genome and is associated with development, differentiation, and transcriptional suppression (1). Aberrant DNA methylation in the promoter region and the subsequent silencing of tumor suppressor genes is frequently observed in various tumors, indicating that DNA methylation plays an important role in tumorigenesis (2–5). Such epigenetic changes in tumors suggest the idea that reversing aberrant hypermethylation and reactivating abnormally silenced tumor suppressor genes should be effective against tumors; this has now been designated as epigenetic therapy (5). Methylation inhibitors, which demethylate and reactivate silenced tumor suppressor genes, have shown their antitumor effects both experimentally and clinically (6). Evidence from either knockout mice or siRNA-based knockdown experiments have shown that a defect in methyl-CpG binding protein 2 (MBD2), a member of the methyl-CpG binding domain proteins that specifically bind with methylated DNA (1, 7), protected tumor-prone mice from developing tumors (8, 9). This observation implies that processes downstream of DNA methylation toward gene silencing could also be good targets for epigenetic therapy.

5-Aza-2'-deoxycytidine (5-aza-dC), first synthesized in 1964, is one of the best known methylation inhibitors (10). 5-Aza-dC is a potent inhibitor of DNA methyltransferases, and is incorporated into DNA by substituting physiologic deoxycytidine during DNA replication and functions by forming covalent complexes with DNA methyltransferases (5). To date, 5-aza-dC has been shown to be clinically effective in treatment of several human tumors (6, 11). Some other nucleoside analogues with demethylation activity have also been developed, including azacytidine, fazarabine, DHAC, and MG98 (12). More recently, another nucleoside analogue, zebularine, that demethylates and reactivates the silenced genes, was identified. Although the mechanism of zebularine is similar to that of 5-aza-dC (13), a characteristic of zebularine is that it can be p.o. administered because of its stability in an aqueous solution (14). In addition, the sequential treatment with 5-aza-dC followed by zebularine has been shown to prevent remethylation, a common obstacle in antimethylation treatments (15).

In this study, we established a screening system for compounds with antisilencing activity using a Moloney murine leukemia virus (MLV)–based retroviral vector, and identified a group of halogenated thymidine analogues that could recover gene expression without influence on DNA methylation.

Materials and Methods

Retroviral vector construction and viral particle production. The MLV-based retroviral vector, pRCV, was constructed by replacement of the neomycin phosphotransferase gene and the SV40 promoter of pNSX with an oligonucleotide containing a multicloning site. A DNA fragment that has a luciferase gene connected with a neomycin phosphotransferase gene by an internal ribosomal entry site (IRES) was inserted into the blunt HindIII site in pRCV, yielding the retroviral vector pRCV/LIG (Fig. 1A).

The recombinant viral particles were produced by cotransfection of equal amounts of pRCV/LIG plasmids and pcDNA-VSV-G (generous gift from Hiroyuki Miyoshi, RIKEN, Tsukuba, Japan), which encodes vesicular stomatitis virus envelope glycoprotein, into a gag-pol–expressing packaging cell line, GP293 (Clontech, Palo Alto, CA) using Fugene reagent (Roche, Indianapolis, IN). Culture supernatants were harvested, aliquoted 48 hours later, and then stored at –80°C.

Cell culture and compounds. A human myeloid leukemia cell line, K562, was grown in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L l-glutamate at 37°C under a 5% CO2 atmosphere. The human breast cancer cell line MCF7 was grown in DMEM (Sigma) supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L l-glutamate.

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After selection with G418 (~2 weeks), transfected clones were isolated by limiting dilution. Then, each clone was cultured under two conditions; one was cultured in medium with G418 (selection medium) and the other one in medium without G418 (nonselection medium).

 Luciferase assays. Two systems, single-tube luciferase assay and 96-well plate luciferase assay, were used in this study. Single-tube luciferase assay was carried out using Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, 2 × 10^4 cells were collected and lysed, and then luciferase activity was detected by mixing the cell lysate and luciferase assay reagent in a luminometer tube and measured with a LB 9507 luminometer (Berthold, Bad Wildbad, Germany). For the 96-well plate luciferase assay, the Steady-Glo Luciferase Assay System (Promega) was used. Steady-Glo Reagent (100 μL) was added directly to cells growing in 100 μL of medium on a 96-well plate. Luciferase activity was measured using a Wallac 1450 Microbeta Jet Luminometer (Perkin-Elmer, Wellesley, MA). In this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylerazolium bromide (MTT) assay was also done to normalize the results of luciferase assay as described previously (16).

 Synergistic effects of compounds. Dose-effect assays were done as reported (17). Briefly, cells were seeded onto 96-well plates and exposed to serial dilutions of each compound individually and concomitantly to both 5-aza-dC and BrdUrd or CldU at a fixed ratio (a ratio of 5-aza-dC to BrdUrd or CldU is 1:100) of doses for 3 days followed by 96-well plate luciferase assay. Doses used for 5-aza-dC were 0.05, 0.1, 0.2, 0.4, and 0.8 μmol/L; doses used for BrdUrd or CldU were 5, 10, 20, 40, and 80 μmol/L. A combination index (CI) value was calculated by the computer-based software Calcsyn developed by Chou et al. (18).

 Bisulfite genomic sequencing. Sodium bisulfite treatment of genomic DNA was done as described previously (19). DNA regions were amplified using bisulfite-treated genomic DNA by nested PCR. To amplify 5′-long terminal repeat (5′-LTR) promoter region of RCV/LIG, primers used in the first PCR were 5′-TAGGATATTTGTGTTAGTTAGTTTTGTG-3′ and 5′-CATAACATCAATACAAACTCAAATTCC-3′. Primers for the second PCR were 5′-GTTTGGTTTGAAGTAGGTTGTTTGAAGTT-3′ and 5′-CACAATAAAATTACCAATTACCTCC-3′. Primer sets target the promoter region of glutathione-S-transferase 1 (GSTP1) gene as were as follows: For first PCR, the primer set was: 5′-TGGAGTGGAATTGTTAGT-3′ and 5′-TTCTGAAATCCCTTAAACCCC-3′. Primer sets used in second PCR were 5′-TGGTAGGAATTGTTAGT-3′ and 5′-CTAAACCTAAACCCATCC-3′. PCR products were purified, cloned into pGEM-T Easy vector (Promega), and sequenced using the ABI PRISM DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

 Quantitative real-time reverse transcription-PCR. Total RNA was isolated from MCF7 cells using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was treated with DNase I (Invitrogen) to eliminate the genomic DNA. Reverse transcription was done using random hexamer and SuperScript III reverse transcriptase (Invitrogen). cDNA product was analyzed by real-time PCR using the Taqman Universal PCR Master Mix and ABI Prism 7700 (PE Applied Biosystems, Foster City, CA) sequence detector according to the manufacturer’s instruction. Specific primers and Taqman probes for GSTP1 gene and for 18S internal control gene were used as described previously (20). PCRs were carried out in triplicate. Data was analyzed by comparative Ct method according to manufacturer’s protocols (PE Applied Biosystems). The lowest detectable GSTP1 expression that has been induced in MCF7 cells after compound treatment was set arbitrarily as 1.0, as untreated MCF7 cells are null for GSTP1 gene expression.

 Quantitative chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was done according to the protocol recommended by Upstate Biotechnology (Lake Placid, NY). Cells (1.5 × 10^6) were fixed with 1% formaldehyde for 10 minutes at room temperature, washed twice with ice-cold PBS, treated with SDS-lys/m buffer [1% SDS, 50 mmol/L EDTA and 200 mmol/L Tris-HCl] for 10 minutes on ice and then sonicated. Thereafter, the DNA/protein complexes were immunoprecipitated with antibodies specific for acetylated-Histone H3 or anti-dimethyl-Histone H3 (Lys9) antibodies (Upstate Biotechnology) overnight at 4°C. Immune complexes were collected with salmon sperm DNA-protein A and G Sepharose slurry, washed, and eluted with freshly prepared elution buffer.
Halogenated Thymidines as Antisilencing Agents

Individual PCRs were done in triplicate to control for PCR variation and labeled TaqMan probe was \( 5'-D- \) and \( 5'-V \). Vectors that express the GST-tagged methyl-CpG binding domain of assay.

With expression vectors were cultured overnight at 20\(^\circ\)C in borate EDTA buffer. Gel was dried and exposed to Medical X-ray film.

\[ \text{IP} = 2^{(t - t_0)} = 2^{-2^{(t_0 - t_0)}} \]

Finally, the fold difference value from the positive control cell line (K5/s) was arbitrarily set as 1.0 and relative abundance of the analyzed protein from untreated or compound treated K5/s cells was subsequently calculated.

Purification of recombinant proteins used in gel mobility shift assay. Vectors that express the GST-tagged methyl-CpG binding domain of MBD1 (pGEX-2TH-MBD 1-84 amino acids) or only GST (pGEX-2TH) as control were constructed as described previously (22). Escherichia coli BL21 with expression vectors were cultured overnight at 20\(^\circ\)C, and then expression of recombinant proteins was induced by isopropyl-\( \beta \)-D-thiogalactopyranoside (a final concentration of 0.4 mmol/L), following further 6-hour incubation. Cells were harvested and resuspended in ice-cold PBS followed by sonication. Recombinant proteins were purified with ReadiPack GST Purification Modules (Amerham, Buckinghamshire, England), aliquoted into small fractions and stored at –20\(^\circ\)C.

**Gel mobility shift assay.** Probes for mobility shift assay are shown in Fig. 5A. Probes (20 fmol) labeled with \( \gamma^32\)PdATP were mixed with 0.4 \( \mu \)M purified recombinant protein in EMSA binding buffer [10 mmol/L Tris-HCl (pH 8.0), 3 mmol/L MgCl\(_2\), 50 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1% NP40, 2 mmol/L DTT, 5% glycerol, and 0.4 ng/mL bovine serum albumin; ref. 23] at room temperature for 30 minutes. For the competition assay, unlabeled probe DNA was added into the reaction mixture at a concentration of 10,000, or 1,000 fold relative to the \( [\gamma^32\text{P}] \text{dATP}-\text{labeled probe} \). The reaction mixture (3 \( \mu \)L) was loaded on 8% polyacrylamide gel and run in 0.5 \( \times \) Trisborate EDTA buffer. Gel was dried and exposed to Medical X-ray film (Kodak, Rochester, NY) overnight at –80\(^\circ\)C.

**Results** Establishment of a screening system for compounds with antisilencing activity. To establish a screening system for compounds with antisilencing activity, we used an MLV-based retroviral vector to monitor the silencing and recovery of reporter gene expression (24). For this purpose, an MLV-based retroviral vector pRCV/LIG was constructed, which carried a luciferase reporter cassette under the control of the retroviral \( 5’-\text{LTR} \) promoter (Fig. 1A). A neomycin-resistant gene was included in this vector for positive selection of transfected cells. K562 and MCF7 cell lines were infected with recombinant RCV/LIG, subcloned in the presence of G418, and tested for luciferase expression by single-tube luciferase assay. Each clone with detectable luciferase activity was subcloned in the presence (positive) or the absence of G418 (negative). Luciferase activity could be maintained in cells cultured with G418, so these cells could be used as a control when the degree of silencing was judged in “negative” cells.

Time-dependent diminishing of luciferase activity was observed at various rates and degrees among the different clones after the removal of G418. Cells with a luciferase activity lower than 30% relative to their “positive” counterpart were arbitrarily considered as being silenced. Then, the silenced clones were tested about responsiveness to 5-aza-dC and TSA, an inhibitor of histone deacetylases (HDAC; Fig. 1B and C; ref. 25). Among isolated clones, the clone 5 derived from K562 (K5) showed the most prominent responsiveness to 5-aza-dC (Fig. 1B), whereas it did not respond to TSA. The \( 5’-\text{LTR} \) promoter region in this clone was hyper-methylated although it was demethylated after exposure to 5-aza-dC (see below), indicating that promoter hypermethylation was directly associated with silencing in K5 cells. Therefore, we selected this clone for further analyses to identify antisilencing compounds. To establish a screening system with K5 cells, data from 96-well luciferase assay was normalized by values from MTT assay as parameters of the cell number (Fig. 1D).

On the other hand, MCF7 clones with a silenced luciferase gene could not respond to 5-aza-dC, but expressed luciferase after treatment by TSA (Fig. 1C). Among these clones, \( 5’-\text{LTR} \) was not methylated, indicating that silencing of the reporter gene promoter in MCF7 cells was not associated with DNA methylation (data not shown).

Halogenated thymidine analogues exhibit antisilencing activity. With this system, we screened >100 ribonucleoside analogues provided by Yamasa Corporation (Choshi, Japan) or purchased from Sigma, and identified a group of halogenated thymidines that could reactivate the transcription of luciferase gene in K5 cells (Fig. 2A and B). The identified thymidines were similar in their chemical structures (i.e., halogenated at position 5 of the pyrimidine ring; Fig. 2A). Among identified compounds, the effect of BrdUrd and CldU was prominent (Fig. 2B). In addition to thymidine analogues, CldC also showed antisilencing activity. Because CldC showed a weak antisilencing activity, it is possible that CldC is deaminated, resulting in incorporation into DNA as CldU. On the other hand, it was shown that this system successfully evaluated the antisilencing activities of the two control compounds, 5-aza-dC and 5-aza-C (Fig. 2B), indicating that our system was applicable for screening of antisilencing compounds.

By performing single-tube luciferase assay, we confirmed that these compounds reactivated the silenced retroviral promoter in K5 cells in a dose-dependent manner (Fig. 2C). Halogenated deoxycytidines substitute thymidine during incorporating into DNA, whereas 5-aza-dC is incorporated instead of deoxycytidine. Therefore, we concurrently exposed K5 cells to 5-aza-dC and either BrdUrd or CldU in various ratios and found that these compounds significantly enhanced the effect of each other (Fig. 2C). Luciferase activity peaked when a suboptimal dose of 5-aza-dC was paired with a high dose of BrdUrd or CldU (data not shown). Dose effect analysis was done using the method developed by Chou and Talalay (17), showing that combinations of 5-aza-dC and either BrdUrd or CldU exhibited strong synergism with the CI value decreasing dose dependently (Fig. 2D). These findings suggested that halogenated thymidines and 5-aza-dC exerted their antisilencing effects by different mechanisms.

**Effect of 5-bromo-2-deoxyuridine on the expression of silenced endogenous genes.** Because halogenated thymidines showed antisilencing activity on hypermethylated reporter gene, we analyzed the antisilencing activity of BrdUrd on endogenous genes that are silenced through promoter DNA methylation. It has been reported that endogenous GSTP1 gene is silenced in breast

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cancer cells by DNA methylation (26). As shown in Fig. 3, BrdUrd restored GSTP1 gene expression in a dose-dependent manner. The combination of BrdUrd and 5-aza-dC significantly increased the expression level of this gene. These results showed that BrdUrd could reactivate the silenced transcription of not only retroviral promoter in K5 cells, but also endogenous GSTP1 gene in MCF7 cells that has been silenced by DNA methylation.

Antisilencing activity of halogenated thymidines is not associated with DNA demethylation. To clarify the mechanisms of antisilencing activities observed in halogenated thymidines, DNA methylation in the promoter region of reporter gene and GSTP1 gene was examined by bisulfite genomic sequencing. The retroviral 5'-LTR promoter region in K5 cells cultured without G418 (K5/−) was hypermethylated (77%), whereas almost no methylation was detected in K5 cells cultured with G418 (K5/+; Fig. 4A). 5-Aza-dC significantly decreased the numbers of methyl-CpG to 34%, which was directly associated with the up-regulated luciferase activity. In contrast, BrdUrd and CldC did not demethylate hypermethylated retroviral promoter although they restored the silenced luciferase gene transcription (Fig. 4A). Similarly, BrdUrd did not influence the methylation status of the endogenous GSTP1 promoter (Fig. 4B), whereas 5-aza-dC decreased DNA methylation. Our results showed that halogenated thymidines could activate a hypermethylated promoter by targeting processes other than DNA methylation.

Effect of halogenated thymidines on binding of methyl-CpG binding domain to methylated DNA. It is generally thought that promoter DNA methylation is translated to transcriptional silencing by a family of methyl-CpG binding domain proteins, which bind to methylated DNA through the evolutionarily conserved methyl-CpG binding domain (1). It is possible that incorporated halogenated thymidines disturb the binding of MBD to methyl-CpG sites, resulting in the antisilencing effect. The effect of halogenated thymidines on binding of MBD with methylated DNA was analyzed by gel shift assay using an artificially designed 20 bp double-stranded oligonucleotide probe containing two symmetrically methylated CpG sites with all thymidines replaced by BrdUrd (Fig. 5A). BrdUrd incorporation did not affect its binding to a recombinant MBD (Fig. 5B). Similar results were obtained when all thymidines were substituted with IdU in gel shift assay (data not shown). It is unlikely that BrdUrd disturbs MBD binding before incorporation into DNA because addition of the monophosphate or triphosphate form of BrdUrd into the protein-DNA binding reaction mixture did not influence the results in our gel shift experiment (data not shown). Taken together, BrdUrd did not disturb the binding of the methyl-CpG binding domain of MBD1 protein with methylated DNA in vitro.

Effect of 5-bromo-2-deoxyuridine on histone modification. DNA methylation is usually associated with changes in the modifications of histone tails, which establish and maintain an inactive chromatin structure (27, 28). To clarify the antisilencing mechanism of BrdUrd, we did quantitative ChIP assay to detect changes in histone modification after BrdUrd treatment. Because acetylation of histone lysine tail is generally associated with
transcriptional active chromatin (29), histone H3 acetylation of the retroviral promoter was studied. K5 cells maintained in G418 selection medium (K5/+) were used as a control, in which retroviral promoter sustained an active transcription state during long-term culture. As shown in Fig. 6A, K5/+ cells exhibited decreased level of histone H3 acetylation in the retroviral promoter region compared with K5/+ cells, suggesting histone deacetylation in the proviral silencing. By exposing K5/+ cells to BrdUrd for 3 days, histone H3 acetylation of the retroviral promoter recovered to a level comparative to that in K5/+ cells (Fig. 6A). On the other hand, the close linkage between methylation of histone H3 Lys9 (H3K9) and transcriptional silencing has been reported (30, 31). In silenced K5/− cells, H3K9 in the retroviral promoter has been methylated (Fig. 6B). Treatment with 5-aza-dC or BrdUrd diminished this methylation. Thus, BrdUrd increased acetylation of histone H3 and decreased methylation of H3K9 along with intact DNA methylation.

Discussion
In this study, an MLV-based retroviral vector was used to establish a screening system for antisilencing compounds. This system successfully identified compounds with antisilencing activity. Although it has been reported that silenced retroviral vector becomes resistant to antisilencing agents during passage (32), the responsiveness of K5 cells to 5-aza-dC persisted for >6 months (data not shown). It is noteworthy that clones derived from MCF7 cells respond to TSA, but not to 5-aza-dC, which indicates that silencing of the retroviral promoter in these clones is not associated with DNA methylation, but is correlated with histone deacetylation. Indeed, the promoter region of the reporter gene in a MCF7 clone was not methylated at all (data not shown). This clone will be useful to screen compounds with antisilencing activity that is not associated with DNA methylation.

With this system, a group of halogenated thymidine analogues was identified as silencing inhibitors, which could activate the transcription of silenced genes without DNA demethylation. This is in agreement with the previous observation that BrdUrd-containing genome, even at very high substitution level, had no change in the content of 5-methylcytosine (33). In addition, BrdUrd has been shown to induce the expression from silenced endogenous genes.
retrovirus without effect on DNA methylation (34), which was also consistent with our findings. However, DNA methylation has not been studied regarding endogenous genes after treatment with BrdUrd. This study first showed that these halogenated thymidines reactivated the transcription of not only retrovirus, but also a heavily methylated endogenous gene. In addition, halogenated thymidines could not reactivate the transcription from the viral promoter in MCF7 clones (data not shown), which could respond to TSA but not to 5-aza-dC, suggesting that the antisilencing effects of halogenated thymidines are closely linked with DNA methylation. This finding coincides with the observation that BrdUrd could reactivate the transcription of endogenous GSTP1 gene silenced by DNA hypermethylation in MCF7 cells, implicating that halogenated thymidines interfere with cellular protein(s) that function between methylated CpG and HDACs.

Several lines of evidence have indicated that DNA demethylation is not a prerequisite for the reactivation of heavily methylated loci. In helper T cells, GATA3 seems to activate interleukin-4 expression from a hypermethylated promoter by interfering with MBD2 binding to its target CpG sites without altering DNA methylation (35). Similarly, Lembo et al. (36) recently reported that MBDin, a regulatory factor of MBD2, reversed MBD2-mediated transcriptional repression from a methylated promoter by binding with the COOH-terminal region of MBD2. Another example of antisilencing without DNA demethylation has been shown in plants (37). MOM is an Arabidopsis gene that has been found to be important in maintaining gene silencing in a methylation-independent fashion. Suppression of its expression by siRNA reactivated the transcription of the genes, which existed in several densely methylated loci. It has been reported that BrdUrd-substituted DNA has changed its interaction with some chromatin and nonchromatin proteins (38), showing the possibility that incorporated halogenated thymidines activate transcription by interfering with the function of cellular proteins that are associated with DNA methylation–mediated gene silencing.

The synergistic effects of halogenated thymidines with 5-aza-dC also indicate that the mechanism of halogenated thymidines differs from that of 5-aza-dC. In spite of retained DNA methylation, the level of histone H3 acetylation was increased when treated by

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**Figure 5.** Binding of BrdUrd-incorporated DNA with methyl-CpG binding domain. A, oligonucleotides used in gel mobility shift assay. "C" represents 5-methylcytosine. "U" represents bromouracil. B, gel mobility shift assay with GST-tagged recombinant MBD of MBD1 protein (GST-MBD). Triangles indicate an increasing amount of competitors (10-, 100-, and 1,000-fold relative to the probes).

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**Figure 6.** Quantitative ChIP assay for histone acetylation and methylation status of the retroviral promoter in K5 cells. K5 cells cultured in either G418 selection medium (K5+/+) or nonselection medium (K5/−) were subjected to ChIP assay after exposure to 5-aza-dC or BrdUrd for 3 days using antiacetylated histone H3 antiseraum (A) or antidimethyl histone H3 Lys9 (B). The presented diagram shows the fold difference of acetylated or methylated histone H3, where the data from K5+/+ cells was arbitrarily set as 1.0. Three independent ChIP experiments have been done and a representative data is shown.
halogenated thymidines, indicating that these compounds impair the pathway by which DNA methylation is translated into histone deacetylation. Because in vitro experiments in this study showed that halogenated thymidines had no effect on the binding of the methyl-CpG binding domain with methylated DNA, they are considered to interfere with some steps downstream of MBDS. Further studies will be needed to clarify the antisilencing mechanism of these compounds.

The findings that halogenated thymidines have a different antisilencing mechanism from and show remarkable synergistic effects with 5-aza-dC raise possibilities that halogenated thymidines could become attractive lead compounds in treatments of cancers. Furthermore, this study implicates a new mechanism of halogenated thymidines that interfere with steps(s) between DNA methylation and histone acetylation.

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