Comparison of biological effects of non-nucleoside DNA methylation inhibitors versus 5-aza-2′-deoxycytidine

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Abstract

DNA cytosine methylation plays a considerable role in normal development, gene regulation, and carcinogenesis. Hypermethylation of the promoters of some tumor suppressor genes and the associated silencing of these genes often occur in certain cancer types. The reversal of this process by DNA methylation inhibitors is a promising new strategy for cancer therapy. In addition to the four well-characterized nucleoside analogue methylation inhibitors, 5-azacytidine, 5-aza-2′-deoxycytidine (5-Aza-CdR), 5-fluoro-2′-deoxycytidine, and zebularine, there is a growing list of non-nucleoside inhibitors. However, a systemic study comparing these potential demethylating agents has not been done. In this study, we examined three non-nucleoside demethylating agents, (−)-epigallocatechin-3-gallate, hydralazine, and procainamide, and compared their effects and potencies with 5-Aza-CdR, the most potent DNA methylation inhibitor. We found that 5-Aza-CdR is far more effective in DNA methylation inhibition as well as in reactivating genes, compared with non-nucleoside inhibitors. [Mol Cancer Ther 2005;4(10):1515–20]

Introduction

The relationship between epigenetic alterations such as DNA methylation and human carcinogenesis has become increasingly evident (1–3). DNA cytosine methylation is employed in normal cells as a mechanism to silence gene expression, such as in genomic imprinting and X chromosome inactivation (4–6). During cancer development, cells can undergo abnormal hypermethylation of CpG islands in the promoters of tumor suppressor genes, which leads to the silencing of these genes (1–3, 7). Thus, reactivation of tumor suppressor genes by demethylating agents has become a potential and promising area of cancer therapy (8–11). There is a growing list of DNA methylation inhibitors in addition to 5-azacytidine and 5-aza-2′-deoxycytidine (5-Aza-CdR; ref. 12), the first demethylating agents with well-characterized mechanisms of action. The list includes, but is not limited to, 5-fluoro-2′-deoxycytidine, zebularine, antisense oligodeoxynucleotides, mitoxantrone, psammaplin A, procaine, *N*-acetylprocainamide, procainamide, hydralazine, and (−)-epigallocatechin-3-gallate (EGCG; refs. 11, 13–27).

Hydralazine and procainamide were first reported to have DNA methylation–inhibition properties in 1988 (15). Hydralazine is a vasodilator and is used clinically as an antihypertensive drug. It has been found to decrease the expression of DNA methyltransferases (DNMT1 and DNMT3A), and induces autoimmunity (16). Procainamide is used clinically as an antiarrhythmic, and previous studies have shown that it inhibits DNA methyltransferase activity, thus leading to DNA hypomethylation (19, 20). Recently, EGCG, the major polyphenol in green tea that has been reported to have chemopreventive activity (28, 29), has been reported to directly inhibit the DNA methyltransferase enzyme and reactivate methylation-silenced genes such as *RARβ* and *p16* (17).

Despite the identification of an increasing number of DNA methylation inhibitors, there has been no systemic study comparing the DNA-demethylating effects and potencies of these agents. In this study, we compare several potential non-nucleoside DNA methylation inhibitors—EGCG, hydralazine, and procainamide—to the nucleoside analogue methylation inhibitor 5-Aza-CdR (see Fig. 1). We found that 5-Aza-CdR is far more effective both in its DNA methylation inhibition activity and in its ability to reactivate methylation-silenced genes in cancer cells.

Materials and Methods

Cell Lines

T24 cells (urinary bladder transitional cell carcinoma), PC3 cells (prostate adenocarcinoma), and HT29 cells (colorectal adenocarcinoma) were obtained from American Type Culture Collection (Manassas, VA). T24 and HT29 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum. PC3 cells were cultured in RPMI medium plus 10% fetal bovine serum. All cells were grown in a humidified 37°C incubator containing 5% CO_2.

Cell Treatments

Cells were seeded at 2×10^5 cells per 100 mm dish 24 hours prior to treatments. Cells were treated with 1 μmol/L 5-Aza-CdR (Sigma-Aldrich Chemical Company, St. Louis, MO), 20 and 30 μmol/L of EGCG, 10 and 20 μmol/L of hydralazine (Sigma-Aldrich Chemical Company), and 100 and 200 μmol/L of procainamide (Sigma-Aldrich Chemical

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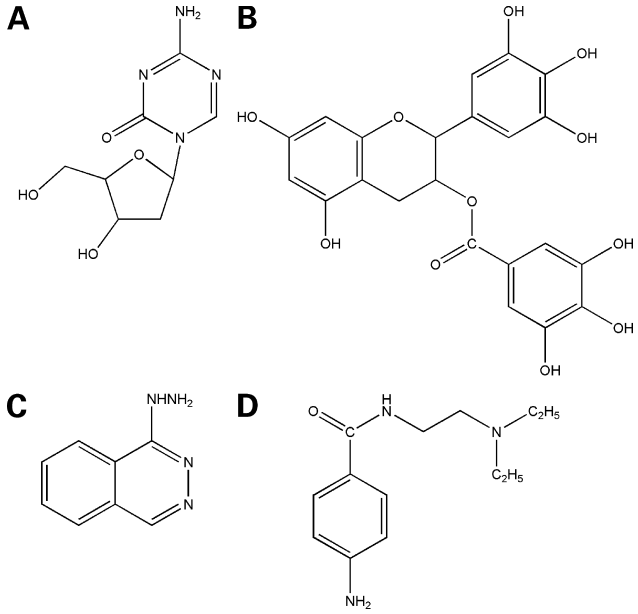

Figure 1. Comparison of chemical structures of DNA methylation inhibitors 5-Aza-CdR (**A**), EGCG (**B**), hydralazine (**C**), and procainamide (**D**).

Company). The EGCG sample was a generous gift from Dr. Chung S. Yang (from Unilever Bestfoods; ref. 17), and a separate sample was obtained from Sigma-Aldrich Chemical Company. 5-Aza-CdR was prepared in PBS and was removed after 24 hours, whereas the other treatments were continuous. EGCG was prepared in DMSO and replaced every 2 days. Hydralazine and procainamide were prepared fresh in PBS and replaced daily with new medium. All treatment regimens have been shown to be effective in inhibiting DNA methylation in previous studies (16, 17, 21). Cells were collected after 6 days of treatment. Genomic DNA and total RNA were extracted for subsequent methylation and expression studies using standard methods.

Quantitative DNA Methylation Analysis by Methylation-Sensitive Single-Nucleotide Primer Extension

Genomic DNA was extracted from cells with the Qiagen DNeasy tissue kit (Valencia, CA). Two micrograms of each DNA sample was converted with sodium bisulfite as previously described (30), and each region of interest was amplified by PCR. The PCR conditions for *MAGE-A1* were as follows: 94°C for 4 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for 1 minute. The PCR conditions for LINE elements were as follows: 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 51°C, and extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR conditions for *p16* were as follows: 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The bisulfite-specific PCR primer sequences are as follows: *MAGE-A1* sense, 5′-GTTTATTTTTATTTTTATTTAGGTAGGATT-3′, *MAGE-A1* antisense, 5′-TTACCTCCTCACAAAACCTAAA-3′; LINE sense, 5′-TTTTTTGAGTTAGGTGTGGG-3′, LINE antisense, 5′-CATCTCACTAAAAAATACCAAACAA-3′; *p16* sense, 5′-GTAGGTGGGGAGGAGTTTAGTT-3′, *p16* antisense, 5′-TCTAATAACCAACCAACCCCTCCT-3′. The methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) conditions for *MAGE-A1* and *p16* were as follows: 95°C for 2 minutes, 50°C for 2 minutes, and 72°C for 1 minute. The Ms-SNuPE conditions for LINE elements were as follows: 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute. The *MAGE-A1* SNuPE primers are as follows: 5′-TTTTATTTTTATTTAGGTAGGATT-3′, 5′-TGGGGTAGAGAGAAG-3′, and 5′-AGGTTTTTATTTTGAGGGA-3′. The LINE SNuPE primers are as follows: 5′-GGGTGGGAGTGATT-3′, 5′-GAAAGGGAATTTTTTGATTTTTTG-3′, and 5′-TTTTTTAGGTGAGGTAATGTTT-3′. The *p16* SNuPE primers are as follows: 5′-TTTTAGGGGTGTTATATT-3′, 5′-TTTTTTTGTTTGGAAAGATAT-3′, and 5′-TTTGAGGGATAGGGT-3′.

The PCR amplicons were extracted with the Qiagen gel extraction kit, and Ms-SNuPE analysis was done to examine the methylation level changes as previously described (31).

Pyrosequencing

Bisulfite-converted DNA was used for pyrosequencing analysis as previously described (32). Pyrosequencing was done for LINE elements, Alu elements, and *MAGE-A1* gene. The primers used are listed as follows: LINE elements sense, 5′-TTTTTTGAGTTAGGTGTGGG-3′; LINE elements antisense, 5′-biotin-TCTCACTAAAAAATACCAAACAA-3′; LINE elements sequencing, 5′-GGGTGGGAGTGAT-3′; Alu elements sense, 5′-biotin-TTTTTATTAAAAATATAAAAATT-3′; Alu elements antisense, 5′-CCCAAACTAAAATACAATAA-3′; Alu elements sequencing, 5′-AATAACTAAAATTACAAAC-3′; *MAGE-A1* sense, 5′-biotin-TATTGTGGGGTAGAGAGAAG-3′; *MAGE-A1* antisense, 5′-AAATCCTCAATCCTCCCTCAA-3′; *MAGE-A1* sequencing, 5′-AACCTAAATCAAATTCCTT-3′.

Reverse Transcription-PCR and Quantitative Real-time Reverse Transcription-PCR

Total RNA was extracted from cells with the Qiagen RNeasy miniprep kit. Reverse transcription was done with Moloney murine leukemia virus reverse transcriptase and random hexamers from Promega (Madison, WI). Reverse transcription-PCR was done for the *p16* gene as previously described (14) using the following primers: *p16* sense, 5′-AGCCTTCGGCTGACTGGCTGG-3′; *p16* antisense, 5′-CTGCCCATCATCATGACCTGGA-3′. PCR conditions for the *p16* gene were as follows: 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 30 seconds, and extension at 72°C for 40 seconds, and a final extension at 72°C for 5 minutes. We also did quantitative real-time reverse transcription-PCR analysis as previously described (33) using DNA Engine Opticon System (MJ Research, Hercules, CA). The primers used are listed below: *MAGE-A1* sense, 5′-GAACCTGACCCAGGCTCTGTG-3′; *MAGE-A1* antisense, 5′-CCACAGGCAGATCTTCTCCTTG-3′; *MAGE-A1* fluorogenic probe,

5′-CAAGGTTTTCAGGGGACAGGCCAAC-3′; *MAGE-B2* sense, 5′-CGGCAGTCAAGCCATCATG-3′; *MAGE-B2* antisense, 5′-TTGCGGCGTTTCTCACG-3′; *MAGE-B2* fluorogenic probe, 5′-TCGTGGTCAGAAGAGTAAGCTCCGTGC-3′; *RARβ* sense, 5′-CCCTTCACTCTGCCAGCTG-3′; *RARβ* antisense, 5′-GCCCAGGTCCAGTCGGA-3′; *RARβ* fluorogenic probe, 5′-AAATACACCACGAATTCCAGTGCTGACCA-3′; *p16* sense, 5′-AGCCTTCGGCTGACTGGCTGG-3′; *p16* antisense, 5′-CTGCCCATCATCATGACCTGGA-3′; *p16* fluorogenic probe, 5′-TGGATCGGCCTCCGACCGTAACT-3′. The real-time reverse transcription-PCR conditions for all four genes were as follows: 95°C for 9 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute.

Results

5-Aza-CdR Is Considerably More Effective in DNA Methylation Inhibition than Non-Nucleoside Agents

The quantitative Ms-SNuPE and pyrosequencing methods were used to compare the methylation status of several

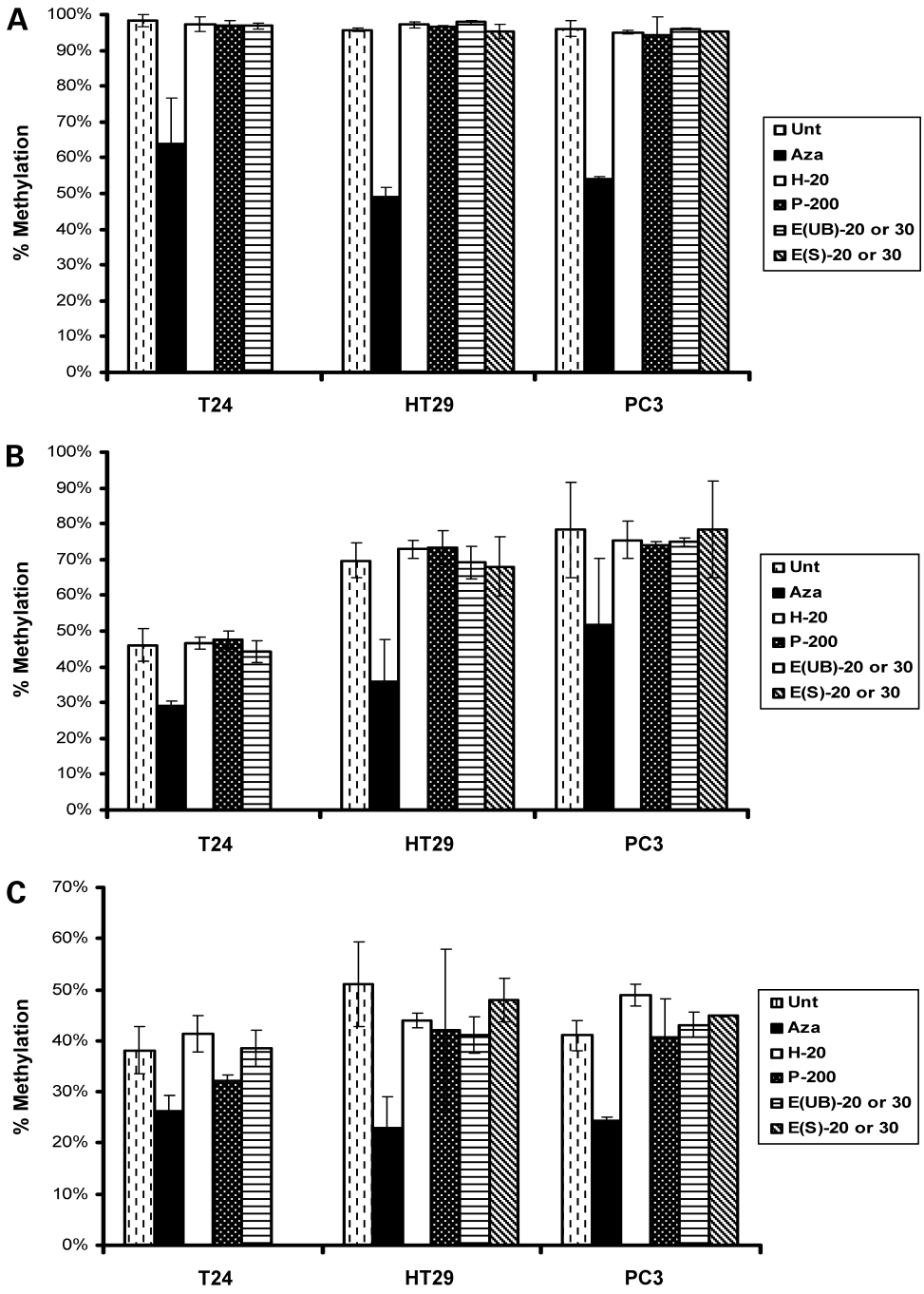

Figure 2. Comparison of the methylation inhibition potencies of the various agents by Ms-SNuPE. Ms-SNuPE results of the methylation levels of *p16* promoter (**A**), *MAGE-A1* (**B**), and LINE elements (**C**). T24, HT29, and PC3 cells were treated with 5-Aza-CdR for 24 h or with hydralazine, procainamide, or EGCG continuously. Cells were collected on day 6. *Columns,* percentage of methylation of two independent experiments; *bars,* ± SD. The percentage of methylation is calculated as the average cytosine / (cytosine + thymine) signal ratio of three separate CpG sites for each region examined. *Unt,* untreated; *Aza,* 1 μmol/L 5-Aza-CdR; *E-20* or *30(S),* 20 or 30 μmol/L of EGCG from Sigma; *E-20* or *30(UB),* 20 or 30 μmol/L of EGCG from Unilever Bestfoods; *H-20,* 20 μmol/L of hydralazine; *P-200,* 200 μmol/L of procainamide.

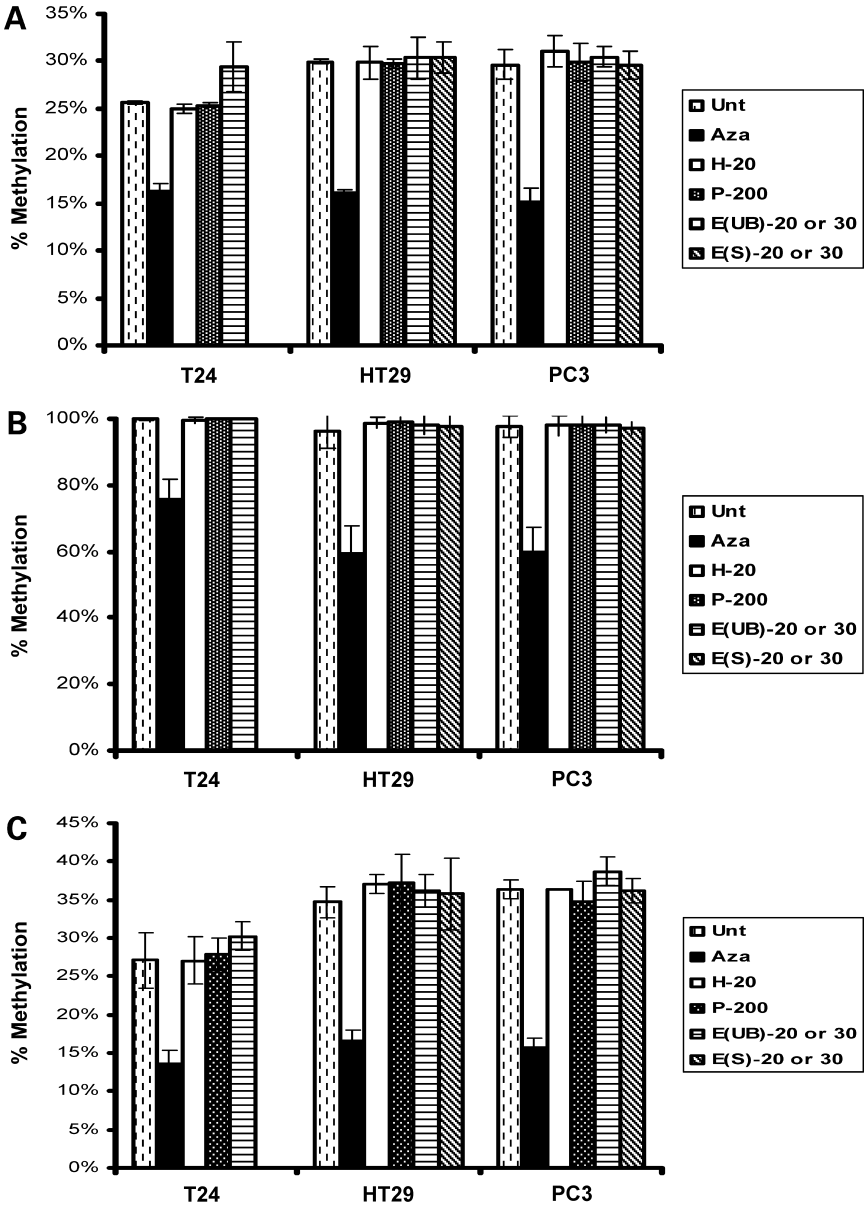

Figure 3. Comparison of the methylation inhibition potencies of the various agents by pyrosequencing. Pyrosequencing results of the methylation levels of Alu repetitive elements (**A**), *MAGE-A1* (**B**), and LINE repetitive elements (**C**). T24, HT29, and PC3 cells were treated with 5-Aza-CdR for 24 h or with hydralazine, procainamide, or EGCG continuously. Cells were collected on day 6. The percentage methylation is calculated as the cytosine / (cytosine + thymine) ratio of the most proximal CpG sites to the primers. *Columns,* average percentage of methylation of two independent experiments; *bars,* ± SD. *Unt,* untreated; *Aza,* 1 μmol/L 5-Aza-CdR; *E-20* or *30(S),* 20 or 30 μmol/L of EGCG from Sigma; *E-20* or *30(UB),* 20 or 30 μmol/L of EGCG from Unilever Bestfoods; *H-20,* 20 μmol/L of hydralazine; *P-200,* 200 μmol/L of procainamide.

loci in the genome before and after treatment with potential inhibitors. Ms-SNuPE analysis was done to examine the methylation levels of the *p16* promoter, *MAGE-A1*, and LINE repetitive elements (Fig. 2). EGCG from Sigma seemed to be more toxic than EGCG from Unilever Bestfoods; the highest doses of EGCG with surviving cells tested are shown in Figs. 2 and 3: for T24 cells, EGCG from Unilever Bestfoods was used at 20 μmol/L. For HT29 cells, 30 μmol/L of EGCG from Unilever Bestfoods and 30 μmol/L of EGCG from Sigma were used. For PC3 cells, 30 μmol/L of EGCG from Unilever Bestfoods and 20 μmol/L of EGCG from Sigma were used.

The data also shows that the three cell lines have different sensitivities to the agents tested. Only 5-Aza-CdR treatment was able to consistently reduce methylation levels in T24, HT29, and PC3 cells. Of the non-nucleoside agents tested, 200 μmol/L of procainamide reduced the methylation level of LINE repetitive elements in T24 cells by roughly 6%. No other non-nucleoside agents tested showed any measurable demethylating activity. Minor reductions in LINE repetitive element methylation levels (5–10%) were observed in HT29 cells treated with EGCG, hydralazine, and procainamide treatments. Pyrosequencing analysis was done for *MAGE-A1*, Alu, and LINE repetitive elements (Fig. 3) to further analyze the methylation level changes after treatment and to confirm our Ms-SNuPE data. Pyrosequencing results also showed that only 5-Aza-CdR was able to reduce methylation levels after treatment. Treatments with 10 μmol/L hydralazine, 100 μmol/L procainamide, and EGCG from both Sigma and Unilever Bestfoods (20 μmol/L; ref. 9)

were also done and did not show any notable decrease in methylation by Ms-SNuPE and pyrosequencing analyses (data not shown).

5-Aza-CdR Is Considerably More Effective in Reactivating Silenced Genes in Cancer Cells

To examine the ability of 5-Aza-CdR and the non-nucleoside agents to reactivate gene expression, we did reverse transcription-PCR for the *p16* gene. Figure 4 shows a representative result of three independent reverse transcription-PCR experiments using *p16* as the indicator gene. Only 5-Aza-CdR was able to activate the expression of the *p16* gene.

Real-time reverse transcription-PCR was also done to check for the expression of *MAGE-A1, MAGE-B2, RARβ*, and *p16* after treatments with 5-Aza-CdR, hydralazine, procainamide, and EGCG (results not shown). The *RARβ* gene was resistant to any of the agents tested. The remaining three genes examined—*MAGE-A1, MAGE-B2*, and *p16*—were all reactivated by 5-Aza-CdR but not by any of the non-nucleoside methylation inhibition agents (data not shown).

Discussion

Previous studies have shown that EGCG, hydralazine, and procainamide are able to reduce DNA methylation and reactivate gene expression in cancer cells (15–21). We examined a total of six different genes/repetitive elements in three separate cell lines for their DNA methylation levels using quantitative Ms-SNuPE and pyrosequencing, and their mRNA expression levels by real-time reverse transcription-PCR and reverse transcription-PCR. Both the Ms-SNuPE and pyrosequencing results show that only the nucleoside analogue 5-Aza-CdR can very reliably reduce methylation in all three cell lines. All three non-nucleoside agents have much weaker, if any, demethylating activities, with procainamide being the only agent able to reduce DNA methylation of LINE elements in T24 cells. The slight differences between the Ms-SNuPE and the pyrosequencing results for the LINE elements may be attributed to the fact that the Ms-SNuPE method examined three separate CpG sites, whereas only one CpG site was assayed in the pyrosequencing method. The expression studies with reverse transcription-PCR also show that only 5-Aza-CdR was able to appreciably reactivate *MAGE-A1, MAGE-B2*, and *p16* genes as shown previously in other studies (34–37). The *RARβ* gene may require the simultaneous administration of 5-Aza-CdR along with a histone deacetylase inhibitor such as trichostatin A for its reactivation in these cell lines (38).

At present, we cannot explain the discrepancy between our data and earlier studies. There are many potential reasons for this, however, these other agents seem unlikely to be robust and reliable inhibitors of DNA methylation. The discrepancies could arise from one or more of the following possibilities: the actions of the non-nucleoside agents could be gene-specific or cell line–specific, the treatment methods might have been ineffective to show efficacy, or the methods of analysis were different from previous studies.

We do not believe that the discrepancies were solely due to the set of genes in our study because we examined some of the genes that have been shown to be responsive to these agents in other studies, such as *p16* and *RARβ* (17, 21). In addition, we examined global methylation level changes with LINE and Alu repetitive elements and did not observe methylation inhibition from the non-nucleoside agents.

Testing with different cell lines could be another source of discrepancy (15, 16, 19, 20). We examined the effect of DNA methylation inhibitors on T24, HT29, and PC3 cells. From our results, it is apparent that different cell lines have different sensitivities to these agents. Other studies with Jurkat (16) and LnCAP (19) cell lines have shown apparent methylation inhibition activities of hydralazine and procainamide, respectively. Perhaps studies with different cell lines and/or a higher dose regimen will show the demethylating effect of these agents. However, we followed the treatment methods that were reported to be effective in previous studies for the non-nucleoside agents, and therefore we do not believe this to be the cause of the discrepancy (16, 17, 21). Nevertheless, longer treatments with these agents might be able to induce noticeable methylation inhibition (19).

Finally, discrepancies could arise from different methods of study. We used Ms-SNuPE and pyrosequencing analyses,

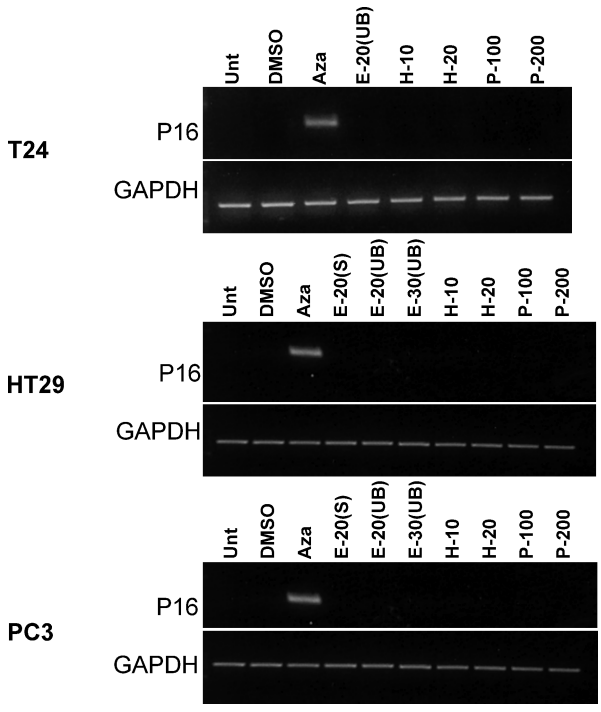

Figure 4. Effects of the various agents on the reactivation of *p16*. Reverse transcription-PCR analysis of *p16* gene expression in T24, HT29, and PC3 cells. The three cell lines were treated with various agents for 6 d. Reaction products were analyzed on ethidium bromide–stained agarose gels. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. *Unt,* untreated; *DMSO,* DMSO control (same amount added as the EGCG 30 μmol/L sample); *Aza,* 1 μmol/L 5-Aza-CdR; *E-20* and *30(S),* 20 and 30 μmol/L of EGCG from Sigma; *E-20* and *30(UB),* 20 and 30 μmol/L of EGCG from Unilever Bestfoods; *H-10* and *20,* 10 and 20 μmol/L of hydralazine; *P-100* and *200,* 100 and 200 μmol/L of procainamide.

two quantitative and reliable methods, to measure methylation levels. It is possible that the differences between the methods we employed and other methods such as methylation-specific PCR could lead to different results.

Green tea, which contains EGCG, is often consumed habitually. Additionally, hydralazine and procainamide are both used for long-term management. The possible weaker demethylating effects of these agents should not be ignored. Although they are considerably weaker in their DNA methylation inhibition activity compared with 5-Aza-CdR, it is feasible that long-term usage of these agents might have small effects. However, one should consider the potential and feasibility of these non-nucleoside agents in chemotherapy regimens. Plasma levels of procainamide >10 μg/mL (~36.8 μmol/L) are associated with toxicity in a patient such as ventricular tachycardia or fibrillation (39). The concentrations we tested in the cell culture were much higher than the toxic plasma level. Taken together, our results do not support the idea that the three non-nucleoside agents tested are likely to be effective as epigenetic therapies with clinical or preventative actions.

References

1. Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K, Herman JG. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet 2001;10:687 – 92.
2. Esteller M, Herman JG. Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. J Pathol 2002;196:1 – 7.
3. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415 – 28.
4. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science 2001;293:1068 – 70.
5. Kaneda M, Okano M, Hata K, et al. Essential role for *de novo* DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 2004;429:900 – 3.
6. Csankovszki G, Nagy A, Jaenisch R. Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J Cell Biol 2001;153:773 – 84.
7. Issa JP. CpG island methylator phenotype in cancer. Nat Rev Cancer 2004;4:988 – 93.
8. Aparicio A, Eads CA, Leong LA, et al. Phase I trial of continuous infusion 5-aza-2′-deoxycytidine. Cancer Chemother Pharmacol 2003;51:231 – 9.
9. Lubbert M. DNA methylation inhibitors in the treatment of leukemias, myelodysplastic syndromes and hemoglobinopathies: clinical results and possible mechanisms of action. Curr Top Microbiol Immunol 2000;249: 135 – 64.
10. Pinto A, Zagonel V. 5-Aza-2′-deoxycytidine (Decitabine) and 5-azacytidine in the treatment of acute myeloid leukemias and myelodysplastic syndromes: past, present and future trends. Leukemia 1993;7 Suppl 1:51 – 60.
11. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004;429:457 – 63.
12. Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. Cell 1980;20:85 – 93.
13. Cheng JC, Matsen CB, Gonzales FA, et al. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst 2003;95:399 – 409.
14. Cheng JC, Yoo CB, Weisenberger DJ, et al. Preferential response of cancer cells to zebularine. Cancer Cell 2004;6:151 – 8.
15. Cornacchia E, Golbus J, Maybaum J, Strahler J, Hanash S, Richardson B. Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. J Immunol 1988;140:2197 – 200.
16. Deng C, Lu Q, Zhang Z, et al. Hydralazine may induce autoimmunity by inhibiting extracellular signal-regulated kinase pathway signaling. Arthritis Rheum 2003;48:746 – 56.
17. Fang MZ, Wang Y, Ai N, et al. Tea polyphenol (−)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res 2003;63:7563 – 70.
18. Gorbunova V, Seluanov A, Mittelman D, Wilson JH. Genome-wide demethylation destabilizes CTG CAG trinucleotide repeats in mammalian cells. Hum Mol Genet 2004;13:2979 – 89.
19. Lin X, Asgari K, Putzi MJ, et al. Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. Cancer Res 2001;61:8611 – 6.
20. Scheinbart LS, Johnson MA, Gross LA, Edelstein SR, Richardson BC. Procainamide inhibits DNA methyltransferase in a human T cell line. J Rheumatol 1991;18:530 – 4.
21. Segura-Pacheco B, Trejo-Becerril C, Perez-Cardenas E, et al. Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. Clin Cancer Res 2003;9:1596 – 603.
22. Villar-Garea A, Fraga MF, Espada J, Esteller M. Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells. Cancer Res 2003;63:4984 – 9.
23. Zhou L, Cheng X, Connolly BA, Dickman MJ, Hurd PJ, Hornby DP. Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases. J Mol Biol 2002;321:591 – 9.
24. Pina IC, Gautschi JT, Wang GY, et al. Psammaplins from the sponge *Pseudoceratina purpurea*: inhibition of both histone deacetylase and DNA methyltransferase. J Org Chem 2003;68:3866 – 73.
25. Parker BS, Cutts SM, Nudelman A, Rephaeli A, Phillips DR, Sukumar S. Mitoxantrone mediates demethylation and reexpression of cyclin d2, estrogen receptor and 14.3.3σ in breast cancer cells. Cancer Biol Ther 2003;2:259 – 63.
26. Richardson B, Cornacchia E, Golbus J, Maybaum J, Strahler J, Hanash S. *N*-acetylprocainamide is a less potent inducer of T cell autoreactivity than procainamide. Arthritis Rheum 1988;31:995 – 9.
27. Saikawa Y, Kubota T, Maeda S, Otani Y, Kumai K, Kitajima M. Inhibition of DNA methyltransferase by antisense oligodeoxynucleotide modifies cell characteristics in gastric cancer cell lines. Oncol Rep 2004;12:527 – 31.
28. Moyers SB, Kumar NB. Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials. Nutr Rev 2004;62:204 – 11.
29. Park OJ, Surh YJ. Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies. Toxicol Lett 2004;150:43 – 56.
30. Frommer M, McDonald LE, Millar DS, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 1992;89:1827 – 31.
31. Gonzalgo ML, Jones PA. Quantitative methylation analysis using methylation-sensitive single-nucleotide primer extension (Ms-SNuPE). Methods 2002;27:128 – 33.
32. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 2004;32:e38.
33. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6:986 – 94.
34. De Smet C, Lurquin C, Lethe B, Martelange V, Boon T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. Mol Cell Biol 1999;19: 7327 – 35.
35. Gonzalgo ML, Hayashida T, Bender CM, et al. The role of DNA methylation in expression of the p19/p16 locus in human bladder cancer cell lines. Cancer Res 1998;58:1245 – 52.
36. Lurquin C, De Smet C, Brasseur F, et al. Two members of the human MAGEB gene family located in Xp21.3 are expressed in tumors of various histological origins. Genomics 1997;46:397 – 408.
37. Weber J, Salgaller M, Samid D, et al. Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2′-deoxycytidine. Cancer Res 1994;54:1766 – 71.
38. Bovenzi V, Momparler RL. Antineoplastic action of 5-aza-2′-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor β and estrogen receptor α genes in breast carcinoma cells. Cancer Chemother Pharmacol 2001;48:71 – 6.
39. Physicians' Desk Reference, 59th ed. Thomson Healthcare; 2004.