


General Thoracic Surgery

Depletion of DNA methyltransferase 1 and/or DNA methyltransferase 3b mediates growth arrest and apoptosis in lung and esophageal cancer and malignant pleural mesothelioma cells

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 Supplemental material is available online.

Objective: DNA methyltransferase (DNMT)1, DNMT3b, or both, facilitate malignant transformation through chromatin remodeling mechanisms. The present study was undertaken to examine the effects of antisense-mediated inhibition of DNMT expression in cultured thoracic malignancies.

Methods: CALU-6 and A549 lung cancer, SKGT5 and BIC esophageal adenocarcinoma, and H2373 and H2052 malignant pleural mesothelioma (MPM) cells, as well as normal human bronchial epithelial (NHBE) cells, were transfected with phosphorothioate-modified antisense oligos targeting DNMT1, DNMT3b, or both, or mismatch oligos. Quantitative reverse transcription-polymerase chain reaction, Western blotting, trypan blue exclusion, and ApoBrdU techniques were used to evaluate DNMT expression, proliferation, and apoptosis after antisense oligo transfections. Gene expression profiles were assessed by using long-oligo array techniques.

Results: Antisense oligos mediated specific and dose-dependent depletion of DNMT1 and DNMT3b, resulting in pronounced inhibition of proliferation of all thoracic cancer lines, but not NHBE cells. Depletion of DNMT1 or DNMT3b coincided with dramatic, caspase-dependent, p53-independent apoptosis in 4 of the 6 thoracic cancer lines. The antiproliferative effects of the antisense oligos were not attributable to induction of RASSF1A, p16, or p21 tumor suppressor genes, and did not coincide with demethylation of genes encoding cancer-testis antigens. DNA methyltransferase knockdown mediated induction of numerous genes regulating response to genotoxic stress. Gene expression profiles after DNMT1, DNMT3b, or combined DNMT1/3b depletion were remarkably similar, yet distinctly different from expression profiles mediated by 5-aza-2'-deoxycytidine.

Conclusions: Antisense oligos targeting DNMT1 and DNMT3b induce genomic stress, and mediate potent growth inhibition in lung and esophageal cancer and MPM cells. These findings support further evaluation of DNMT knockdown strategies for cancer therapy.

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Dynamic patterns of gene expression during embryonic development and cellular homeostasis, as well as stable repression of gene expression associated with X chromosome inactivation and imprinting, are mediated by epigenetic mechanisms involving DNA methylation in conjunction with acetylation,

Abbreviations and Acronyms

ASO	= antisense oligo
DAC	= 5 aza 2' deoxycytidine
DNMT	= DNA methyltransferase
HDAC	= histone deacetylase
MM	= mismatch
MPM	= malignant pleural mesothelioma
NHBE cell	= Normal human bronchial epithelial cell
RT-PCR	= reverse transcription–polymerase chain reaction

phosphorylation, and methylation of core histone proteins.^{1,2} In mammalian cells methylation of DNA occurs exclusively at the 5' position of cytosine in the context of CG dinucleotides.³ Methylation of cytosines within CpG islands of promoter and proximal coding regions facilitates recruitment of methyl-binding proteins, such as MeCP2 and MBD2, as well as histone deacetylases (HDACs), which inhibit gene expression through complex mechanisms that render promoters inaccessible to the transcription machinery.⁴ Reversible posttranslational modifications of core histone proteins (histones H2a, H2b, H3, and H4) form a histone code that determines the specificity of DNA-protein and protein-protein interactions within the nucleosome, thus modulating activation status of chromatin.⁵ Recent studies indicate that DNA methylation is mechanistically linked to the histone code⁶⁻⁸ and that DNA methylation is the predominant epigenetic mechanism regulating gene expression in healthy, as well as cancer, cells.⁹

DNA methylation is mediated by opposing actions of DNA methyltransferases (DNMTs) and DNA demethylases.¹⁰ To date, 4 DNMTs have been identified (DNMT1, DNMT2, DNMT3a, and DNMT3b), all of which mediate transfer of a methyl group from S-adenosyl methionine to the 5' position of cytosine.³ Recent studies in which individual DNMTs have been knocked out in cultured cells have revealed unique functions and considerable overlapping activities of these isozymes. In normal somatic cells DNMT1 is the predominant methyltransferase, functioning primarily to maintain DNA methylation patterns after DNA replication. DNMT3a and DNMT3b are also expressed in healthy tissues, albeit at levels considerably lower than DNMT1, and function primarily in de novo methylation. DNMT2 appears to have minimal activity, and its role in regulating DNA methylation in mammalian cells is presently unknown. DNMT1, DNMT3a, or DNMT3b knockout mice die in utero or shortly after birth, indicating that despite redundant functions, all of these DNMTs are essential for normal development.^{10,11}

Aberrant DNMT expression-activity facilitates malignant transformation in part by mediating site-specific promoter methylation of tumor suppressor genes, such as p16,

FHIT, and RASSF1A. Robertson and colleagues¹² observed a 3- to 7-fold increase in DNMT1, DNMT3a, and DNMT3b expression in primary tumor specimens (including lung carcinomas) relative to adjacent normal tissues. More recently, Yakushiji and associates¹³ observed overexpression of DNMT1, DNMT3a, and DNMT3b in 72%, 56%, and 64%, respectively, of oropharyngeal cancers relative to corresponding normal epithelia, suggesting that increased DNMT expression might be a common theme of tobacco-induced aerodigestive tract carcinogenesis. Additional studies have demonstrated that DNMT1 overexpression induces DNA methylation and malignant transformation of NIH-3T3 cells,¹⁴ and that increased DNMT activity in type II pneumocytes coincides with progression to malignancy in mice exposed to tobacco carcinogens.¹⁵ Furthermore, DNMT3b enhances malignant transformation of SV40 T antigen-transformed human bronchial epithelial cells expressing activated ras and telomerase¹⁶ and cooperates with DNMT1 to silence tumor suppressor genes in cancer cells.¹⁷ These observations, as well as data reported by Robert and coworkers¹⁸ and Beaulieu and colleagues¹⁹ demonstrating that specific inhibition of DNMT1 or DNMT3b (but not DNMT3a) expression promotes growth arrest in cancer cells attest to the relevance of DNMT expression-activity during malignant transformation. The present study was undertaken to examine the effects of antisense-mediated inhibition of DNMT expression in cultured thoracic malignancies as a prelude to possible evaluation of DNMT knockdown in patients with these neoplasms.

Materials and Methods**Cell Lines**

A549 and CALU-6 NSCLC cells, SKGT-5 and BIC esophageal adenocarcinoma cells, and H2373 and H2052 MPM cells were available in our laboratory. All cancer lines were maintained in RPMI supplemented with 10% fetal calf serum and antibiotics (herein referred to as normal media). Normal human bronchial epithelial (NHBE) cells were purchased from Clonetics and cultured per the vendor's recommendations. All cell lines were confirmed to be free of mycoplasma contamination.

Oligo Transfections

Cancer cells and NHBE cells were seeded into 10-cm dishes at concentrations predetermined to yield 40% to 50% confluency within 24 hours. The following day, media was aspirated, and cells were transfected with 2'-O-methylphosphorothioate-modified antisense oligos (ASOs) targeting DNMT1, DNMT3B, or both or mismatch (MM) oligo sequences in Opti-MEM (Gibco, Inc) in the presence of 2.5 $\mu\text{g}/\text{mL}$ lipofectin at 37°C \times 4 hours, as described by Beaulieu and colleagues.¹⁹ Thereafter, transfection medium was aspirated and replaced with normal medium. This process was repeated every 24 hours for 2 additional transfections. Additional cells were treated with lipofectin alone to rule out carrier toxicity. All transfections were performed in triplicate. Seventy-two hours after commencement of the initial transfections, cells were har-

TABLE 1. Quantitative RT-PCR analysis of DNMT1 and DNMT3b expression in cancer cells after ASO treatment [(copy no./ β -actin) $\times 10^4$]

	CALU-6	A549	BIC	SKGT5	H2373	H2052
DNMT1 quantitative RT-PCR						
NM	31,869	8226	3277	1850	11,766	7952
Lipofectin	33,121	11,866	5584	2001	11,056	11,270
DNMT1 ASO 75 nmol/L	11,799	863	685	817	4220	1735
DNMT3b ASO 75 nmol/L	30,217	6753	4327	2095	10,865	10,401
DNMT1/DNMT3b ASO 75/75 nmol/L	11,901	3076	1272	1222	5042	1999
Mismatch	34,568	11,735	4434	2290	9885	11,938
DNMT3b quantitative RT-PCR						
NM	26,374	13,645	8165	2761	2802	6203
Lipofectin	25,454	18,273	12,727	4339	2304	6503
DNMT1 ASO 75 nmol/L	17,767	10,378	10,998	3053	1891	4825
DNMT3b ASO 75 nmol/L	759	1483	1278	696	394	1210
DNMT1/3b ASO 75/75 nmol/L	928	1762	1879	970	556	1142
Mismatch	24,521	46,211	13,447	3876	1894	6284

RT-PCR, Reverse transcription–polymerase chain reaction; DNMT, DNA methyltransferase; ASO, antisense oligos; NM, normal media.

vested for simultaneous analysis of proliferation and apoptosis by using standard trypan blue exclusion techniques and protocols—reagents contained in the APO-BrdU Kit (BD PharMingen), respectively, as well as isolation of RNA and protein for assays described below, by using triplicate samples for all respective analyses.

Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from cell lines by using the Stratagene Absolutely RNA RT-PCR Miniprep Kit. Synthesis of cDNA was performed with 1 μ g of total RNA by using the Reverse Transcription System (Promega) and oligo (dT)₁₅ primers. Real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously described²⁰ using an ABI Prism 7700 Sequence Detection System (Perkin Elmer). Primer-probe sequences are listed in Appendix E1. Samples were quantitated by dividing the copy number of the respective gene of interest by that of β -actin.

Caspase Inhibition Assay

A549 cells were transfected with DNMT ASOs or MM oligos as described above. After each 4-hour transfection, the cells were washed with Hanks' balanced salt solution and incubated in normal media containing 80 μ mol/L of the pancaspase inhibitor Z-VAD-FMK or the specific caspase 3 inhibitor Z-DEVD-FMK (R&D Systems). At the 72-hour time point, cells were harvested, and apoptosis was evaluated by using ApoBrdU techniques described above.

Long-oligo Array

Microarray techniques were used to examine gene expression profiles of A549 cells exposed to normal media; lipofectin; ASOs for DNMT1, DNMT3b, or both; MM oligos; or the DNA-demethylating agent 5-aza-2'-deoxycytidine (DAC). Techniques for hybridization and analysis of the arrays are discussed in Appendix E2.

Results

Preliminary experiments were performed to optimize transfection conditions and define appropriate time points for reproducible molecular analysis of oligo-transfected cells. Real-time quantitative RT-PCR analysis demonstrated considerable variation in basal expression of DNMT1, as well as DNMT3b, in cultured cancer cells, which did not appear to coincide with histology or p53 expression status (Table 1). Subsequent studies revealed that ASOs targeting DNMT1 and DNMT3b mediated pronounced and specific dose-dependent reduction of DNMT1 and DNMT3b transcription in all of the cancer lines (data pertaining to 75 nmol/L oligo transfections are depicted in Table 1). In general, the DNMT3b ASO appeared to more efficiently inhibit target transcription compared with the ASO recognizing DNMT1. Inhibition of DNMT transcription by the ASOs coincided with depletion of DNMT protein levels in cancer cells (representative results pertaining to DNMT1 knockdown in A549 cells are shown in Figure E1).

Additional experiments were performed to ascertain the growth-inhibitory effects of the ASOs in cultured cells. In general, the individual ASOs mediated dose-dependent growth inhibition within 72 hours after initiation of the transfection experiments (Figure 1, A). The effects of combined DNMT1/DNMT3b ASO transfections were somewhat variable, ranging from a rather limited combination effect, as seen in BIC esophageal adenocarcinoma cells, to an apparent supra-additive effect noted in A549 lung cancer cells. Overall, the growth-inhibitory effects of the DNMT ASOs alone or in combination appeared to coincide with their ability to diminish DNMT expression in cancer cells (Figure 1, A, and Table 1). No growth inhibition was observed in cancer cells transfected with

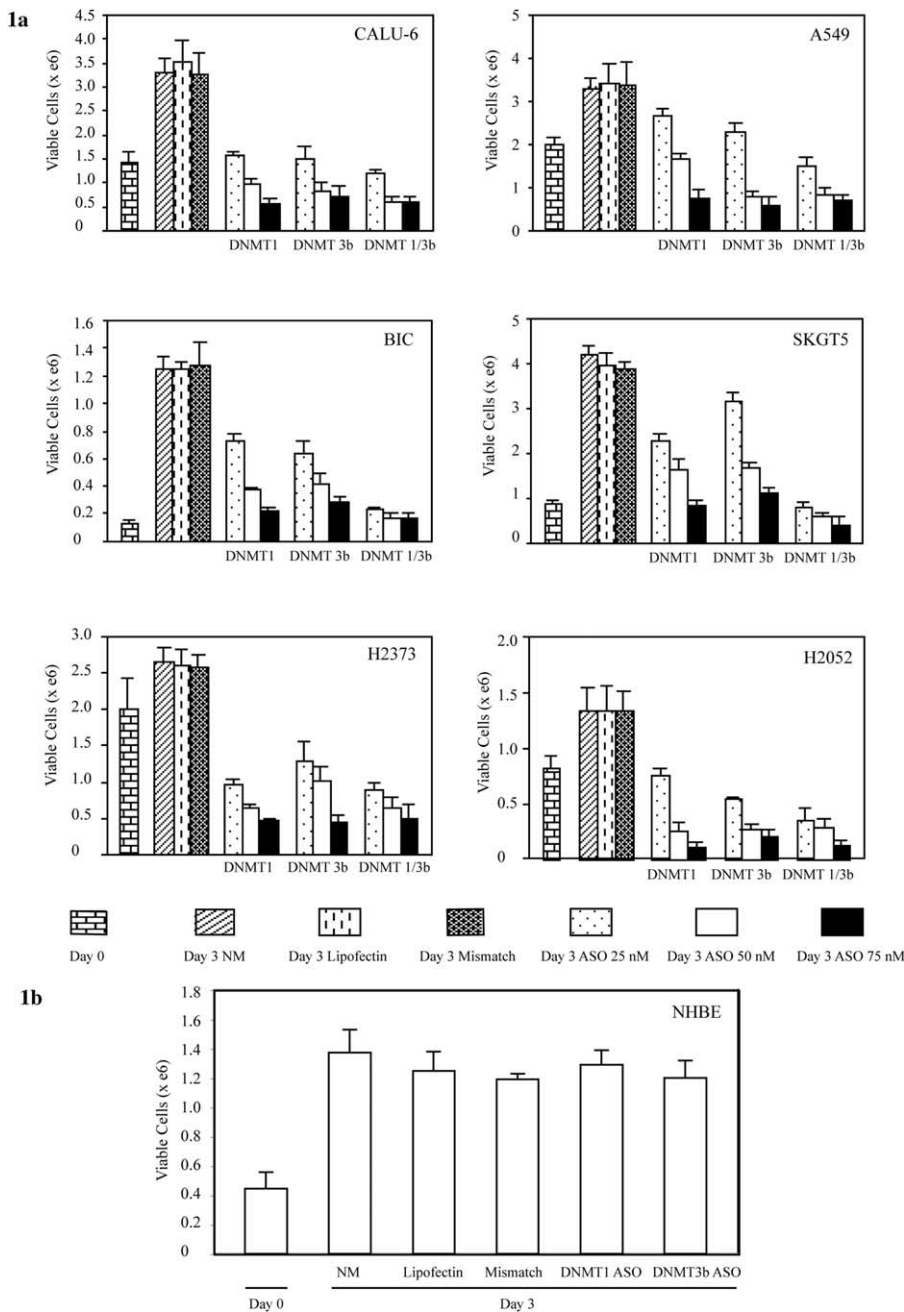


Figure 1. Trypan blue exclusion analysis of proliferation of CALU-6, A549, BIC, SKGT5, H2373, and H2052 (A) and normal human bronchial epithelial cells (NHBE; B) after antisense oligo (ASO) transfection. Day 0 counts represent the number of viable cells immediately before initial exposure to DNA methyltransferase (DNMT) ASOs, mismatch (MM) oligos, or lipofectin. Day 3 counts represent cell numbers at the 72-hour time point for normal media (NM), lipofectin (lipofectin alone), MM oligos, DNMT1 ASOs (25, 50, and 75 nmol/L), DNMT3b ASOs (25, 50, and 75 nmol/L), and combination DNMT1/DNMT3b ASOs (25, 50, 75 nmol/L). NHBE cells were exposed only to 75 nmol/L DNMT 1 or DNMT 3b.

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MM oligos or cells exposed to lipofectin alone. Interestingly, DNMT ASO transfections had minimal inhibitory effects on proliferating NHBE cells (Figure 1, B), which exhibited DNMT1 and DNMT3b mRNA levels 2- to 12-fold lower than those seen in cultured cancer cells (data not shown).

Additional studies were performed to further define the mechanisms by which the DNMT ASOs mediate growth arrest in cultured cancer cells. Whereas the combination of DNMT1 and DNMT3b ASOs appeared to have relatively

modest effects when assessed by trypan blue exclusion techniques, parallel terminal deoxynucleotidyl transferase experiments with the same cells revealed that knockdown of DNMT3b markedly augmented apoptosis in CALU-6 (p53 null) and A549 (p53 wt) lung cancer cells, as well as SKGT5 esophageal adenocarcinoma cells (p53 mt) mediated by the DNMT1 ASO (Figure 2). The combination effect was less readily discernable in H2373 MPM (p53 wt) cells because of their exquisite sensitivity to the DNMT3b oligo. Interestingly, 2 cell lines (BIC esophageal adenocar-

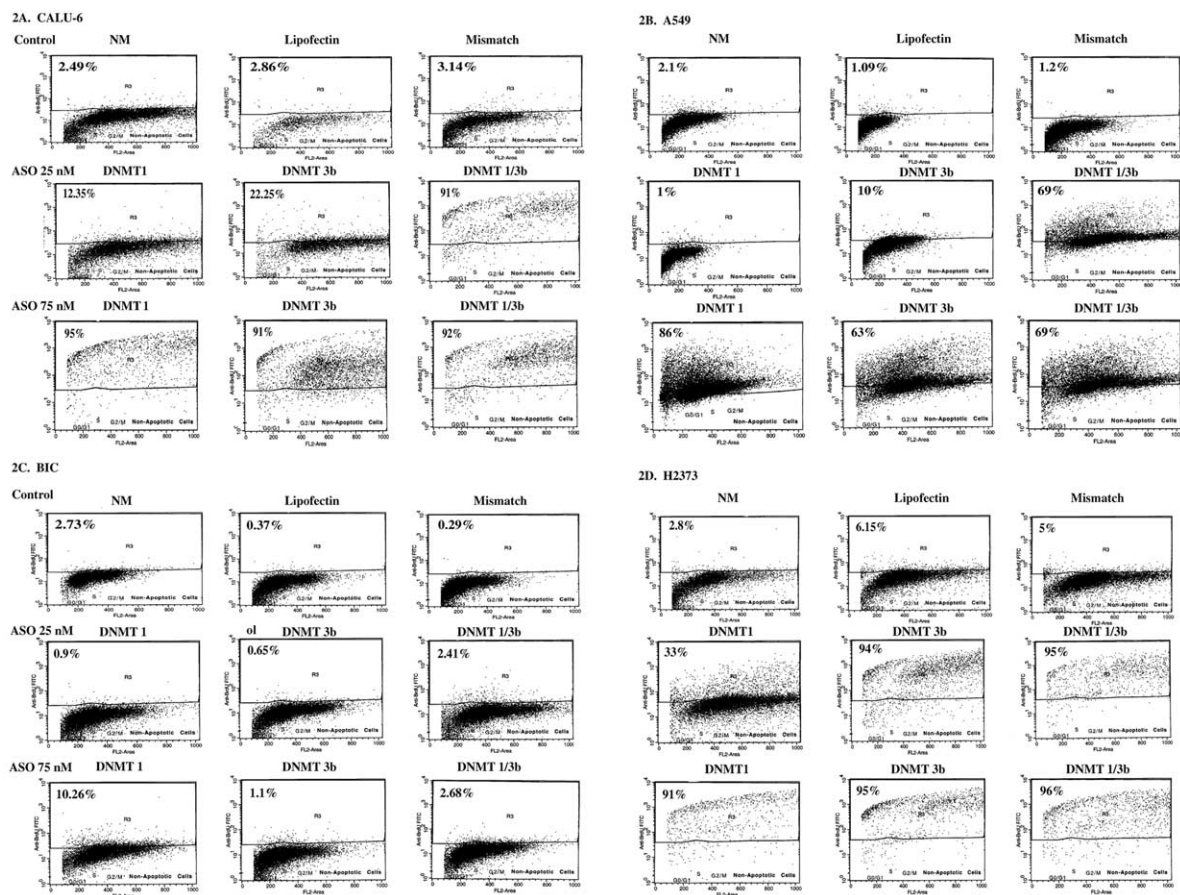


Figure 2. ApoBrdU analysis of CALU-6 (A), A549 (B), BIC (C), and H2373 (D) cell lines exposed to normal media (NM), lipofectin alone, mismatch oligonucleotides, or DNA methyltransferase (DNMT) 1, DNMT3b, or both antisense oligos (ASOs; 25 or 75 nmol/L). The percentage of apoptotic cells for each exposure is indicated in the corresponding box. SKGT5 cells exhibited a pattern of apoptosis similar to that observed for CALU-6 and A549 cells, whereas results pertaining to H2052 cells were similar to those observed for BIC cells (data not shown).

cinoma and H2052 MPM cells) appeared to exhibit minimal apoptosis after exposure to the ASOs, despite comparable knockdown of target transcripts. Additional experiments revealed that apoptosis mediated by DNMT1 or DNMT3b ASOs was partially abrogated by the pancaspase inhibitor Z-VAD, as well as the specific caspase 3 inhibitor DEVD (Figure 3). Collectively, these data indicated that apoptosis mediated by DNMT1 knockdown, DNMT3b knockdown, or both, was mediated through p53-independent, caspase-dependent mechanisms.

Additional real-time quantitative RT-PCR experiments were performed to ascertain whether growth inhibition and apoptosis coincided with induction of tumor suppressor genes or genomic demethylation in cancer cells after DNMT knockdown. Representative results are depicted in Table 2. Overall, knockdown of DNMT1, DNMT3b, or combined DNMT1/DNMT3b depletion resulted in rela-

tively modest effects regarding RASSF1A, p16, p21, and TFPI-2 tumor suppressor gene expression in cancer cells, irrespective of their propensity to undergo apoptosis after ASO exposure. Furthermore, no consistent derepression of NY-ESO-1 or MAGE-3 cancer testis antigens was observed. In light of the fact that the genes encoding these tumor suppressors and cancer testis antigens are robustly induced in cancer cells by the DNA-demethylating agent DAC,²⁰⁻²² these data strongly suggested that the ASOs targeting DNMT1 and DNMT3b mediated their growth-inhibitory effects through mechanisms more subtle than restoration of tumor suppressor gene expression or global DNA demethylation. Long-oligo array techniques were used to evaluate gene expression profiles in A549 lung cancer cells after DNMT knockdown to further examine this issue. This analysis revealed that depletion of DNMT expression resulted in a dramatic induction of a variety of

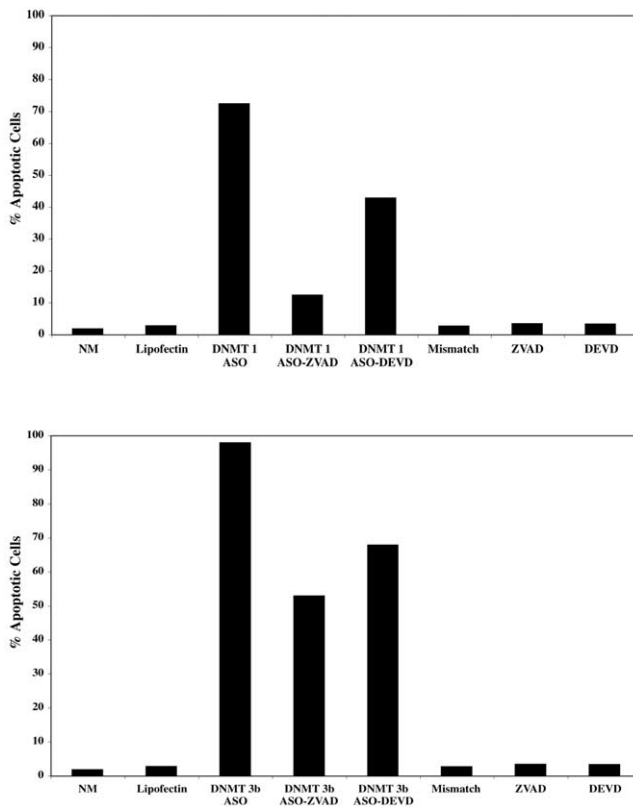


Figure 3. ApoBrdU analysis of apoptosis in A549 cells exposed to DNA methyltransferase (DNMT) 1 antisense oligo (ASO) alone, DNMT1 ASO plus Z-VAD, or DNMT1 ASO plus DEVD (A) or A549 cells exposed to DNMT3b ASO alone, DNMT3b ASO plus Z-VAD, or DNMT3b ASO plus DEVD (B). Cells were also treated with control exposure to normal media (NM), lipofectin alone, and mismatch (MM) oligos. The x axis indicates the percentage of apoptotic cells. Both caspase inhibitors partially abrogated the apoptotic effects of DNMT1 and DNMT3b ASOs.

genes known to modulate cellular response to genomic stress through p53-dependent, as well as p53-independent, mechanisms (a partial list of these genes is included in Table 3). Interestingly, the patterns of genes induced by the DNMT1 and DNMT3b ASOs were remarkably similar, and there was little evidence of a cumulative effect of DNMT1/DNMT3b knockdown in terms of the genes that were most profoundly induced under these treatment conditions. Furthermore, none of the genes induced by ASO transfections were upregulated to a significant extent after 72-hour exposure to DAC at maximal concentrations achievable in clinical settings (0.1 $\mu\text{mol/L}$).¹¹

Discussion

The emerging relationships between chromatin structure, gene regulation, and malignant transformation provide a

compelling rationale for evaluation of DNMT inhibitors for the treatment and prevention of cancer.^{11,23} Clinical studies conducted recently in the Thoracic Oncology Section, Surgery Branch, National Cancer Institute, indicate that when administered via 72-hour continuous intravenous infusion, DAC mediates induction of tumor suppressor and cancer-testis gene expression in primary thoracic malignancies. Despite these encouraging results, dose-limiting myelosuppression and drug instability in vivo (half-life, approximately 5 minutes) limit chronic administration of DAC in patients with thoracic malignancies, or individuals at high risk for development of these neoplasms.¹¹

In light of the cooperativity of DNMT1 and DNMT3b regarding inhibition of tumor suppressor gene expression¹⁷ and the requirement of DNMT3b for cancer cell survival,¹⁹ we sought to ascertain the potential utility of ASO-mediated depletion of these DNMTs for the treatment of thoracic malignancies. At concentrations 200-fold less than those achieved in phase I studies examining 2-hour DNMT1 ASO infusions in patients with cancer,²⁴ DNMT1 and DNMT3b ASOs mediated potent and dose-related depletion of target transcripts, markedly inhibiting proliferation of all thoracic cancer lines. Interestingly, 2 cancer lines (BIC and H2052) appeared refractory to the proapoptotic effects of DNMT ASOs, despite depletion of target transcripts comparable with cell lines that readily underwent apoptosis after ASO transfection. Although the basis for this has not been elucidated, these findings might be related in part to the kinetics of DNMT knockdown, differential modulation of multiprotein chromatin-remodeling complexes, and the status of apoptotic pathways in these cancer cells. Of note, the DNMT ASOs mediated minimal toxicity in proliferating NHBE cells. Although these observations could be attributable, at least in part, to relative transfection efficiencies, the data suggest that cancer cells are more susceptible to antisense-mediated inhibition of DNMTs; these findings are consistent with previously published data from our laboratory indicating that DNA-demethylating agents and HDAC inhibitors mediate apoptosis preferentially in cancer cells.^{22,25}

Several studies have been conducted to examine mechanisms by which DNMT knockdown inhibits cancer cell proliferation in cancer cells. Beaulieu and colleagues¹⁹ observed that apoptosis mediated by DNMT knockdown in A549 lung cancer cells coincided with inhibition of proliferating cell nuclear antigen expression and induction of RASSF1A and p21, as well as caspases 9 and 10; in our study we did not observe significant induction of these genes in these cells. Robert and coworkers¹⁸ reported that DNMT1 knockdown, but not depletion of DNMT3b or DNMT3a, markedly augmented the ability of DAC to reactivate silenced tumor suppressor genes in cancer cells. More recently, Milutinovic and associates²⁶ ob-

TABLE 2. Quantitative RT-PCR analysis of tumor suppressor and cancer-testis gene expression in cancer cells after ASO treatment ([copy no./ β -actin] $\times 10^4$)

	RASSF1A	p16	p21	TFPI-2	NY-ESO-1	MAGE-3
A549						
NM	6	0	4692	273,290	0	1214
DNMT1 ASO 75 nmol/L	6	0	21,889	459,952	1	1237
DNMT3 ASO 75 nmol/L	7	0	39,197	429,845	1	1051
DNMT1/3 ASO 75/75 nmol/L	11	0	23,359	399,188	1	1151
MM 75/75 nmol/L	6	0	6632	268,718	1	1482
CALU-6						
NM	102,836	0	278	8	1	3011
DNMT1 ASO 75 nmol/L	48,469	7	458	9	2	4828
DNMT3 ASO 75 nmol/L	49,566	2	270	12	2	5285
DNMT1/3 ASO 75/75 nmol/L	39,028	6	253	8	2	4076
MM 75/75 nmol/L	97,657	1	190	8	0	3921
BIC						
NM	11,466	0	288	1	35	1
DNMT1 ASO 75 nmol/L	8745	1	375	1	65	2
DNMT3 ASO 75 nmol/L	14,984	1	664	1	29	2
DNMT1/3 ASO 75/75 nmol/L	14,077	1	644	2	35	1
MM 75/75 nmol/L	10,687	0	394	1	33	1

RT-PCR, Reverse transcription–polymerase chain reaction; ASO, antisense oligos; NM, normal media; DNMT, DNA methyltransferase; MM, mismatch.

served that knockdown of DNMT1 mediated intra-S-phase cell-cycle arrest and genotoxic stress in A549 cells, which was due specifically to depletion of DNMT1, rather than global DNA demethylation. Interestingly, these effects were not noted in cells treated with DAC, which inhibits DNA demethylation by covalently trapping DNMTs at the replication fork, without inhibiting their transcription.^{27,28} Suzuki and colleagues²⁹ reported that short interfering RNA (siRNA)–mediated knockdown of DNMT1 induced expression of a variety of tumor suppressor genes, including p16, RASSF1A, and E-cadherin, in H1299 lung cancer cells. Modulation of gene expression was transient because genes that had been induced were repressed within 17 days after exposure to the siRNA, indicating a selective pressure to silence these genes in cancer cells. Collectively, these data

indicate that the antiproliferative effects of DNMT inhibitors in cancer cells are contingent on the mechanisms by which these agents abrogate DNMT expression-activity, as well as the specificity, extent, and duration of DNMT depletion.

Results of our studies clearly indicate that DNMT1 and DNMT3b contribute to the malignant phenotype of thoracic malignancies. Interestingly, our data suggest that apoptosis mediated by DNMT knockdown is not readily attributable to genomic DNA demethylation, as evidenced by the lack of induction of tumor suppressor genes or derepression of cancer testis genes, which are regulated through DNA methylation mechanisms.^{11,23} Instead, DNMT knockdown mediates a genomic stress response, which appears remarkably similar, irrespective of whether DNMT1, DNMT3b, or both

TABLE 3. Long-oligo array analysis of gene expression in A549 lung cancer cells after ASO treatment (approximate fold induction relative to normal media)

Gene	Mismatch	DNMT1	DNMT3b	DNMT1/DNMT3b	DAC 0.1 μ mol/L
GADD45A	1	12	18	19	2
TNFAIP3	1	11	9	14	1
SESN2	1	9	8	14	1
EGR1	1	6	24	26	1
EPLIN	1	5	5	6	1
GADD45B	1	5	6	6	1
AXUD1	1	5	8	7	1
HISTONE 2, Hbe	1	5	10	10	1

ASO, Antisense oligos; DNMT, DNA methyltransferase; DAC, 5-aza-2'-deoxycytidine.

were depleted in cancer cells. These observations support the notion that knockdown of DNMTs initially (and perhaps primarily) disrupts cellular processes other than DNA methylation, resulting in growth arrest and apoptosis in cancer cells.³⁰ For example, DNMTs participate in multiprotein complexes involving methyl-binding proteins, HDACs, p21, and PCNA, which regulate chromatin structure and cell-cycle progression.^{10,11} As such, depletion of DNMTs might alter the stability of a variety of critical protein complexes, thereby inducing lethal genotoxic stress. The fact that DAC did not induce similar genotoxic stress is consistent with the notion that depletion of DNMT protein levels rather than covalent trapping of these methyltransferases onto DNA directly contributes to the cytotoxicity of DNMT ASOs. The mechanisms by which DNMT depletion mediates growth arrest in cancer cells are a focus of ongoing investigation in our laboratory.

Our current study is the first comprehensive analysis of combined DNMT depletion in a broad panel of well-characterized lines established from thoracic malignancies of diverse histologies. Although the mechanisms have yet to be fully defined, data presented in this article clearly indicate that DNMT knockdown represents a novel strategy for the treatment of thoracic neoplasms. These data warrant further analysis of DNMT expression in aerodigestive tract malignancies and the development of more potent and efficacious DNMT inhibitors for cancer therapy.

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Discussion

Dr David R. Jones (*Charlottesville, Va*). Dr Kassis, you and your colleagues are to be congratulated for a nice piece of scholarly work that was well presented. You have shown that the role of aberrant methylation in thoracic malignancies, particularly with DNA methyltransferase (DNMT) 1 and DNMT3b, promotes apoptosis in this model system.

I have 3 questions for you. First, most of the trials looking at antisense oligonucleotides to DNMT require very long treatment periods. The patients have to receive their infusion over 2 hours or even longer. They also have to receive multiple cycles of this therapy, and it is limited to an intravenous formulation. Given the potential limitations of this, do you believe antisense oligo (ASO) therapy is even a reasonable treatment strategy, or do you know perhaps whether there is a pending oral formulation of this type of treatment?

Dr Kassis. There are now 2 published phase I trials looking at antisense therapy. As you mentioned, they involved prolonged infusions, and several months of treatment were necessary before cancer regressions were noted. What we aimed to do here was to establish that depletion of DNMT1 or DNMT3b is cytotoxic in thoracic malignancies. These findings support the development of small molecular compounds that can be orally administered, which can efficiently inhibit DNMT expression in cancer cells. Conceivably, the ASOs used in this study could be formulated for aerosolized delivery to the respiratory tract or used for local treatment of dysplastic Barretts epithelium.

Dr Jones. Have you or others looked at combining these DNMT inhibitors with other kinds of genotoxic stressors, such as chemotherapy or radiation, to see whether the tumor cell death or apoptosis could be even further enhanced?

Dr Kassis. To my knowledge, that has not yet been done, although Dr Schrupp and I have discussed combining DNMT ASOs with deoxyazacytidine. There have now been several studies demonstrating that DNMT ASOs significantly increase the deoxyazacytidine-mediated induction of silenced tumor suppressor genes. So that might be a model that we could pursue in the future.

Dr Jones. Finally, perhaps for me at least, from a mechanistic standpoint, the most interesting observation of the study is that the apoptosis that was mediated by the DNMT knockdowns did not appear to be attributable to genomic demethylation but appeared to be more likely the result of just a global cellular stress phenomenon. Can you postulate why this is occurring and why DNMT depletion appears to induce the growth arrest seen in your study?

Dr Kassis. Thank you for those questions.

Deoxyazacytidine is a potent DNA-demethylating agent that mediates re-expression of a variety of silenced tumor suppressor genes. DNMTs have various interactions with methyl-binding proteins, p21, PCNA, and histone deacetylases. Deoxyazacytidine covalently traps the DNMTs at the replication fork, promoting growth arrest and DNA demethylation, with upregulation of genes that have been silenced by epigenetic mechanisms. In contrast, the

ASOs inhibit expression of the DNMTs, thereby disrupting the stoichiometry of critical protein complexes involved in chromatin remodeling and cell-cycle regulation. We speculate that disruption of these protein-protein interactions results in profound genotoxic stress, as indicated by the genes that we saw induced in our microarray experiments. This genotoxic stress response is not seen after treatment with deoxyazacytidine.

Dr Chi Ming Wei (*Baltimore, Md*). I have 3 questions. One, you tested many different cell lines. Did you find that there is a different effect for this antisense in different cell lines, or is it a pretty similar effect?

Dr Kassis. Well, in terms of the growth-inhibitory effect, it was across the board. In all of the cancer cell lines that we studied, we saw comparable dose-dependent depletion of the DNMTs. Where they diverged was in terms of the induction of apoptosis. CALU-6, A549, 2373, and SKGT5 exhibited significant apoptosis after DNMT knockdown. In contrast, BIC and H2052 exhibited growth arrest without significant apoptosis after ASO exposure. This difference could be related to the kinetics of DNMT knockdown and the status of the apoptotic machinery in these cells. Experiments are underway to examine this issue.

Dr Wei. Second, you found that there is no difference in the p21 gene expression. Did you see the upstream p53 genes?

Dr Kassis. In one cell line, A549, we did see a modest induction of p21, but we did not study the expression of p53 in this study.

Dr Wei. Third, in the future, do you think it is possible to do an *in vivo* study, to do this antisense in animal studies?

Dr Kassis. There have been some *in vivo* studies now looking at antisense oligonucleotides. As I mentioned, there are actually 2 phase I trials already published, and there is a phase II trial in patients with oropharyngeal cancer underway in Toronto. Therefore yes, I think that further *in vivo* studies are definitely feasible with this technique.

Dr Wei. Thank you very much.

Dr Ching Tzao (*Taipei, Taiwan*). I think this is a successful approach to study the possible mechanism for methylation. Did you really look at the methylation status and methyltransferase changes in parallel to see whether it could provide direct evidence that your findings were indeed involved in methylation? And another part of this story is histone deacetylation. Are you trying to find out whether histone deacetylation is related to methylation of some of the tumor suppressor genes? Is there any cross-talk between these potential mechanisms?

Dr Kassis. Well, in terms of looking at methylation-specific PCR to identify the methylation status of some of these promoters, that was going to be our next step had we identified significant re-expression of known silenced genes. Therefore no, we did not do that. And, as you mentioned, there are known interactions between DNMTs and histone deacetylases. This is an area of ongoing investigation, but we have not done that yet.

Dr Tzao. Thank you.

Appendix E1. Primer-probe sequences for real-time quantitative RT-PCR

DNMT1

Forward 5'-GTTCTTCCTCCTGGAGAATGTCA-3'

Reverse 5'-GGGCCACGCCGTAAGT-3'

Probe 5'-TTGTCTCCTTCAAGCGCTCCATGGTC-3'

DNMT3b

Forward 5'-GACTCGAAGACGCACAGCTG-3'

Reverse 5'-CTCGGTCTTTGCCGTTGTTATAG-3'

Probe 5'-AGCCACCTCTGACTACTGCCCCGC-3'

RASSF1A

Forward 5'-ACG CAC ACG TGG TGC-3'

Reverse 5'-AGT GGC AGG TGA ACT TGC A-3'

Probe 5'-TCG TGC GCA AAG GCC TGC AGT G-3'

p16

Forward 5'-TGCCCAACGCACCGA-3'

Reverse 5'-CGCTGCCCATCATCATGA-3'

Probe 5'-AGTTACGGTCGGAGGCCGATCCA-3'

p21

Forward 5'-CTGGAGACTCTCAGGGTCGAA-3'

Reverse 5'-GGCGTTTGGAGTGGTAGAAATCT-3'

Probe 5'-ACGGCGGCAGACCAGCATGA-3'

TFPI-2

Forward 5'-CAGGAAATAACGCGGAGATCTG-3'

Reverse 5'-ACGGAGAAGTAGGGCCCG-3'

Probe 5'-CTCCTGCCCTAGACTACGGACCCTG-3'

NY-ESO-1

Forward 5'-TGCTTGAGTTCTACCTCGCCAT-3'

Reverse 5'-GCTCCTGCGGGCCAG-3'

Probe 5'-TTTCGCGACACCCATGGAAGCAG-3'

MAGE-3

Forward 5'-TCCTGTGATCTTCAGCAAAGCTT-3'

Reverse 5'-GGGTCCAATTCCATCAGTCT-3'

Probe 5'-CAGTTCCTTGCAGCTGGTCTTTGGCAT-3'

The primers and probes labeled at the 5' end with the reporter molecule FAM (6-carboxyfluorocein) and at the 3' end with the quencher molecule TAMRA (6-carboxytetramethylrodamine) were purchased from PE Biosystems.

Appendix E2. Microarray analysis of gene expression in cancer cells after DNMT knockdown

Gene expression profiles in A549 lung cancer cells exposed to normal media, lipofectin, mismatch oligos, and DNMT1, DNMT3b, or DNMT1/DNMT3b ASOs were examined by using long-oligo array techniques. Briefly, after 2-round RNA amplification, amino-allyl uridine triphosphate-incorporated microarray probes were synthesized and labeled from 1 μ g of total RNA by use of the Ambion aRNA kit. Cy3- and Cy5-labeled probes were combined and purified in the kit columns and denatured at 100°C for 1 minute with 10 μ g of COT-1 DNA (Invitrogen) and 10 μ g of poly(A) (Amersham Biosciences). NCI glass slide microarrays representing 23K genes/features were prehybridized at 42°C for 2 hours in 5 \times standard saline citrate (SSC), 0.1% sodium dodecylsulfate (SDS), and 1% bovine serum albumin. The arrays were then hybridized with a probe in 50% formamide, 10 \times SSC, and 0.2% SDS at 50°C overnight (10-16 hours). The slides were successively washed for 2 minutes in 2 \times SSC, 0.1% SDS, 1 \times SSC, and 0.2 \times SSC and spun dry at 100g for 10 minutes. Arrays were analyzed with a Genepix 4000B scanner (Axon Instruments) and software developed at the Center for Information Technology, National Institute of Health. All arrays were repeated by using reciprocal fluorescence alteration to establish reproducibility and minimize the effects of labeling bias. A significant expression was defined as a relative ratio of 3.0 or 0.33 in the experimental sample relative to reference RNA in 2 reciprocal microarray analyses.

DNMT1 Western Blot of A549 Cell Line

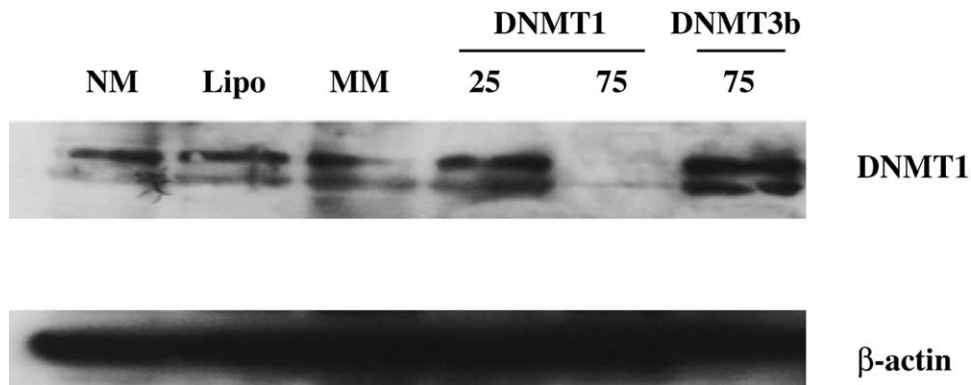


Figure E1. Western blot analysis of DNA methyltransferase (DNMT) 1 expression in A549 lung cancer cells after DNMT antisense oligo (ASO) transfection by using protocols and reagents described by Beaulieu and colleagues.¹⁹ Shown are treatment groups NM (normal media), lipofectin, ASO to DNMT1 (25 and 75 nmol/L), and ASO to DNMT 3b (75 nmol/L). A dose-dependent and specific inhibitor of DNMT1 was observed after transfection with the DNMT1 ASO.