Phase I Study of Decitabine-Mediated Gene Expression in Patients with Cancers Involving the Lungs, Esophagus, or Pleura

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Abstract

Purpose: The DNA methylation paradox, manifested as derepression of cancer-testis antigens, and silencing of tumor suppressors during malignant transformation, provides the rationale for the utilization of chromatin remodeling agents for cancer therapy. A phase I trial was done to examine pharmacokinetics, toxicities, and gene expression mediated by 5-aza-2′-deoxycytidine (DAC) in patients with thoracic malignancies.

Experimental Design: Thirty-five patients with cancers refractory to standard therapy received continuous 72-hour DAC infusions using a phase I dose-escalation schema. Each full course of therapy consisted of two identical 35-day cycles. Plasma DAC levels were evaluated by liquid chromatography-mass spectrometry techniques. Quantitative reverse transcription-PCR, methylation-specific PCR, and immunohistochemical techniques were used to evaluate NY-ESO-1, MAGE-3, and p16 expression in tumor biopsies. Long oligonucleotide arrays were used to evaluate gene expression profiles in laser-captured tumor cells before and after DAC exposure.

Results: Thirty-five patients were evaluable for toxicities; 25 were evaluable for treatment response. Myelosuppression constituted dose-limiting toxicity. The maximum tolerated dose of DAC was 60 to 75 mg/m^2 depending on the number of prior cytotoxic chemotherapy regimens. No objective responses were observed. Plasma DAC concentrations approximated thresholds for gene induction in cultured cancer cells. Target gene induction was observed in 36% of patients. Posttreatment antibodies to NY-ESO-1 were detected in three patients exhibiting NY-ESO-1 induction in their tumor tissues. Complex, heterogeneous gene expression profiles were observed in pretreatment and posttreatment tissues.

Conclusion: Prolonged DAC infusions can modulate gene expression in primary thoracic malignancies. These findings support further evaluation of DNA-demethylating agents alone or in combination with other regimens targeting induced gene products for the treatment of these neoplasms.

Alterations in chromatin structure profoundly influence gene expression during normal cellular homeostasis and malignant transformation (1, 2). Methylation of cytosines within CpG islands located in promoter and proximal coding regions facilitates recruitment of chromatin-remodeling proteins, which inhibit gene expression. Posttranslational modifications, such as acetylation, methylation, and phosphorylation, of core histone proteins "mark" regions of chromatin for recognition by multiprotein complexes, which either promote chromatin relaxation and gene expression, or chromatin compaction and repression of gene expression (3, 4). Recent studies indicate that DNA methylation is mechanistically linked to the "histone code" (5, 6), and that the temporal sequence of chromatin remodeling events during activation or repression of transcription may be tissue and/or promoter specific (7, 8). For instance, DNA methylation triggers deacetylation and subsequent lysine 9 dimethylation of histone H3 in association with transcriptional silencing of *glutathione S-transferase* (9). In contrast, deacetylation and lysine 9 trimethylation of histone H3 coincide with transcriptional repression, which precedes methylation of the *RASSF1A* promoter (10).

Similar to other neoplasms, lung and esophageal cancers, and malignant pleural mesotheliomas, exhibit a "DNA methylation paradox"(11, 12). Site-specific DNA methylation silences a variety of tumor suppressors, such as *p16*, *RASSF1A*,

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and *TFPI-2* (13–15), in the context of genome-wide demethylation that facilitates loss of imprinting, derepression of parasitic DNA, and activation of a variety of germ cell–restricted genes encoding proteins recognized by CTLs from cancer patients (16–18). Of particular interest in this regard are *NY-ESO-1* and *MAGE-3*, which are aberrantly expressed in a wide variety of human malignancies, including 30% to 50% of non–small cell lung cancers, esophageal cancers, and malignant pleural mesotheliomas, and 75% of small-cell lung cancers (12, 19–22). Ironically, immune responses to these cancer-testis antigens (CTA) seem limited in thoracic oncology patients (23, 24), possibly due to insufficient antigen expression in their primary tumors. Conceivably, strategies to augment *NY-ESO-1* and/or *MAGE-3* expression in cancer cells may facilitate the development of efficacious immunotherapy regimens for thoracic malignancies.

Previously, we reported that the DNA demethylating agent 5-aza-2′-deoxycytidine (DAC) mediates robust, dose-dependent induction of *NY-ESO-1* and *MAGE-3* CTA expression under exposure conditions that restore *p16*, *RASSF1A*, or *TFPI-2* expression in cultured lung and esophageal cancer, and malignant pleural mesothelioma cells in which these tumor suppressor genes have been silenced by promoter methylation mechanisms (12, 18, 25). Furthermore, we reported that following exposure to DAC, cancer cells of various histologies, but not normal human bronchial epithelial cells, can be recognized by CTL specific for NY-ESO-1 (25). Collectively, these data provided the rationale for a phase I protocol designed to recapitulate in clinical settings DAC exposure conditions that simultaneously modulate CTA and tumor-suppressor gene expression in cultured thoracic malignancies. Primary and secondary end points for this trial included identification of the maximum tolerated dose (MTD) of DAC administered by continuous 72-hour infusion in thoracic oncology patients, clinical response at the MTD, and analysis of NY-ESO-1, MAGE-3, and p16 expression in tumor tissues before and after DAC treatment. Herein, we present data that establish proof of concept concerning the potential utilization of DNA-demethylating agents for modulating gene expression in primary thoracic malignancies.

Materials and Methods

DAC treatment regimen. Decitabine (DAC) was supplied through Cancer Therapy Evaluation Program from Supergen, Inc. (Dublin, CA). Lyophilized drug was reconstituted in 5% D5W, diluted in 0.9% normal saline, and administered by continuous central i.v. infusion over 72 hours on days 1 to 3 of each treatment cycle, which lasted ~34 days. Two cycles constituted one course of therapy. Because of the instability of DAC, fresh i.v. bags were prepared and hung every 2 hours.

Eligibility and response criteria. Patients ages ≥18 years with histologically or cytologically proven primary small-cell lung cancer, non–small cell lung cancer, esophageal cancer, or malignant pleural mesothelioma, as well as individuals with pleural or pulmonary metastases secondary to extrathoracic malignancies, were eligible for evaluation. All patients had Eastern Cooperative Oncology Group performance status of 0 to 2, and had not received chemotherapy, biological therapy, or radiation to target lesions within 30 days of commencing DAC treatment. All patients had FEV1 and DLCO values >30% predicted, $pCO_2 < 50$ mm Hg and $pO_2 > 60$ mm Hg on room air ABG, and no evidence of decompensated coronary artery disease. All patients had a platelet count >100,000, a hemoglobin count >10 g/dL, WBC >3,500/μL, a normal prothrombin time, total bilirubin <1.5 times upper limits of normal, and serum creatinine <1.6 mg/mL. Individuals excluded from study are those with primary and metastatic carcinomas that could not be readily biopsied by endoscopic or percutaneous fine needle aspirate (FNA) techniques, patients with untreated limited stage small-cell lung cancer or operable non–small cell lung cancer, patients with active intracranial or leptomeningeal metastases, individuals requiring anticonvulsant therapy, patients with prior Decitabine exposure, as well as those individuals with unstable angina, recent pulmonary embolism, or deep venous thrombosis requiring anticoagulation. Patients with life expectancy <6 months were also excluded from study. Response Evaluation Criteria in Solid Tumors criteria (26) were used to determine clinical response following two cycles of therapy. Patients who exhibited treatment response evidenced by either stabilization or regression of their disease were eligible to receive two additional cycles of DAC. Treatment continued in this manner until off-study criteria had been met.

Toxicity, treatment modification, and off-study criteria. Grade 4 or greater hematologic toxicities exceeding 5-day duration, or grade 3 or greater nonhematologic toxicities, excluding alopecia, as assessed by Common Toxicity Criteria for Adverse Events version 2 during cycle 1 of therapy, were considered dose limiting. Patients who exhibited response to treatment, yet experienced reversible dose-limiting toxicity (DLT) were eligible for additional treatment at the preceding dose level. Patients who exhibited response to therapy yet experienced reversible DLT at the entry dose level were eligible for additional therapy at 45 mg/m^2 (15 mg/m^2/d × 3 days). No further dose reduction was routinely considered irrespective of patient response. All patients who experienced disease progression, or exhibited either irreversible or unacceptable dose-limiting toxicities despite dose modification, were removed from study. Similarly, individuals who withdrew voluntarily for any reason were removed from study.

Pharmacokinetic studies. Plasma DAC concentrations were analyzed at 0, 30 minutes; 1, 3, 8, 24, 48, 72 hours; 72 hours 5 minutes; 72 hours 15 minutes; 72 hours 30 minutes; 72 hours 45 minutes; 73, 74, and 75 hours following the start of drug administration. Blood samples (3 mL) were obtained by venipuncture in heparinized tubes containing 8 μL tetrahydrouridine (500 μg/mL) to inhibit cytidine deaminase. Tubes were immediately transferred to the laboratory on ice, and centrifuged at 2,000 × *g* for 15 minutes; plasma samples were aliquoted and stored at −80°C. The average time from acquisition of blood samples to freeze down of plasma was <30 minutes. Plasma DAC concentrations were quantitated by a validated liquid chromatography-mass spectrometry method (27), with a lower limit of detection of 1 ng/mL. Data pertaining to 30 minutes and 1, 3, 8, 24, 48, and 72 hours were used to calculate mean steady-state DAC levels (±SD) for each patient. Mean (±SE) DAC steady-state values were determined for each cohort. A Jonckheere-Terpstra test (28) was used to ascertain if there was a trend between increasing DAC dose and plasma DAC concentration, with the trend considered statistically significant if the two-tailed *P* value was ≤0.05.

Tissue acquisition and molecular analyses. Biopsies of target lesions were obtained before and ~24 hours following completion of DAC infusions during the first two treatment cycles using endoscopic or computed tomography–directed percutaneous FNA techniques. For each patient, the same lesion was biopsied before and after DAC infusion. Considerable efforts were made to biopsy the same region of each tumor to minimize sampling artifact. Immediate cytopathologic review confirmed the presence of tumor cells in all biopsy specimens. Quantitative reverse transcription-PCR (RT-PCR) and methylation-specific PCR techniques analogous to those reported previously (15) were used to evaluate NY-ESO-1, MAGE-3, and p16 induction using RNA and DNA isolated from fresh FNA specimens. NY-ESO-1, MAGE-3, and p16 protein expression was evaluated in formalin-fixed, paraffin-embedded biopsy tissues using antibodies and immunoperoxidase protocols as previously described (18, 29, 30).

Laser capture microdissection and RNA amplification. Pretreatment and posttreatment tumor biopsies from four DAC-treated patients were processed for laser capture microdissection and comprehensive gene expression profiling using microarray techniques. Briefly, FNA or core needle biopsies were done under computed tomography guidance, and samples were immediately frozen in optimum cutting temperature compound on dry ice. Primary tumor tissues and adjacent histologically normal lung parenchyma from eight lung cancer patients undergoing definitive resections were processed in a similar manner. Serial 8-μm-thick frozen sections were prepared from each biopsy specimen (~100/case). Laser capture microdissection was done using the PixCell IIe laser capture microdissection System (Acturus, Mountain View, CA) according to the protocol of the manufacturer with several modifications. Sections were immersed in relevant fixatives and staining solutions using protocols and reagents contained in the HistoGene laser capture microdissection frozen section staining kit (Acturus), followed by dehydration through alcohol gradients and xylene for 2 minutes. Pathologic images were recorded for each case. Approximately 500 to 1,000 tumor cells or adjacent normal lung epithelial cells were microdissected from the sections, and total RNAs from these cells were isolated using the PicoPure RNA isolation kit (Acturus). The RNAs were stored at −80°C for subsequent analysis.

RNA amplification, labeling, and hybridization. Total RNA was amplified into antisense RNA (aRNA) via the Amino Allyl MessageAMP aRNA kit (Ambion, Austin, TX). Whereas the quantity of starting total RNA was in most cases insufficient to measure, aRNA was amplified successfully for microarray and Taqman analyses. Numerous correlative experiments using RNA from cell lines or primary lung cancer specimens confirmed the integrity, reliability, and reproducibility of the amplification and hybridization procedures (data not shown). Because the fidelity of aRNA hybridization was at least equal, if not superior, to that of total RNA for transcriptional profiling due to lack of contaminant ribosomal and tRNA, two-round amplified aRNA was routinely used to optimize consistency of results, particularly when low-quality total RNA was recovered from biopsy samples. After amplification, aRNA quality was confirmed with agarose gel electrophoresis and RT-PCR analysis of several housekeeping genes. Test and reference aRNAs were labeled with Cy5 and Cy3, and cohybridized to custom-made 23K long oligonucleotide microarrays, printed at the National Cancer Institute with the Human Operon Version 2.0 Genome Oligo Set. Hybridizations were done at 60°C overnight (10-16 hours). Thereafter, the slides were washed for 2 minutes in 2× SSC with 0.1% SDS, 1× SSC, and 0.2× SSC, respectively, and spun dry at 100 × *g* for 10 minutes. Fluorescence images were captured using a Genepix 4000B (Axon Instruments, Sunnyvale, CA) scanner. To establish reproducibility and minimize the effects of labeling bias, all arrays were repeated using reciprocal fluorescence techniques. Fluorescence intensities were normalized at 50% median ratio value and filtered at criteria of ≥200 (based on a scale of 1-68,000 units) and spot sizes ≥50 μm.

Data acquisition and analysis. The acquisition and initial quantification of array images were done using the GenePix Pro 6.0 (Axon Instruments). Subsequent data analysis was performed using DNA-Chip 1.3 (31), GeneSpring 7.2 (Silicon Genetics, Foster City, CA), Ingenuity Pathway Analysis 3.0[1], and PANTHER 6.0[2] software. Data were filtered initially by removing genes with signal intensity ≤ "median intensity of randomized negative controls + 1 SD" across all samples. The filtered data were then entered into GeneSpring 7.2 and normalized per chip and per gene. The thresholds for selecting significant genes were set at a relative difference >2-fold and/or statistical difference at $P < 0.05$. Cluster and linear discrimination analyses were done by using DNA-Chip Analyzer with default settings. Global functional, network, and pathway analyses were done using Ingenuity Pathway and PANTHER software.[6,7]

[6] www.ingenuity.com.
[7] http://www.pantherdb.org/.

Results

Patient accrual, toxicities, and clinical response. Demographic data pertaining to patients enrolled on the study are listed in Table 1. Thirty-four patients had primary thoracic malignancies, including non–small cell lung cancer (20 patients), small-cell lung cancer (2 patients), esophageal cancer (6 patients), or malignant pleural mesothelioma (6 patients). One individual with Ewing sarcoma metastatic to the pleura was also enrolled on study. The vast majority of patients had received prior chemotherapy; ~50% of the individuals had also received radiation therapy. The median age of the patients and the number of prior therapies reflected the histologies targeted in this study and the phase I nature of the protocol.

The first cohort of three patients received DAC at a total dose of 60 mg/m^2 without exhibiting DLT. The subsequent cohort of patients received DAC at a total dose of 75 mg/m^2; one of three patients experienced dose-limiting neutropenia. Consequently, three additional patients were accrued into the second cohort, the last of whom experienced dose-limiting neutropenia requiring hospitalization at an outside institution.

The two individuals that experienced DLT in the second cohort had been heavily pretreated, indicating that DAC might be tolerated differently in cancer patients based on prior treatment. Hence, with Institutional Review Board and Cancer

Table 1. Patient demographic data

Characteristic	No. patients
Total	35
Age, y	
Median	61
Range	21-75
Sex	
Male	30
Female	5
ECOG performance status	
0	21
1	14
2	
Tumor type	
Ewing sarcoma	1
NSCLC	20
SCLC	2
Malignant mesothelioma	6
Esophageal squamous carcinoma	
Esophageal adenocarcinoma	4
No. prior chemotherapy	
0	4
1	10
≥2	21
Median	2
Range	0-3
No. prior radiotherapy	
0	18
1	9
2	6
≥3	2
No. prior immunotherapy	
0	35
1	
3	

Abbreviations: NSCLC, non–small cell lung cancer; SCLC, small-cell lung cancer; ECOG, Eastern Cooperative Oncology Group.

Therapy Evaluation Program approval, the protocol was amended to stratify for prior treatment regimens; patients with two or less prior cytotoxic therapies were accrued separately from those with three or more prior regimens. Patients with three or more prior treatment regimens were accrued into cohort 1 to fully assess toxicities and molecular end points in these individuals. Two additional patients with limited prior treatment were accrued into cohort 2, tolerating DAC without DLT; hence, dose escalation continued in this group. One of three patients receiving DAC at a total dose of 90 mg/m^2 experienced dose-limiting neutropenia, necessitating that this cohort be expanded to six patients. Patient 4 in this cohort tolerated DAC well. Patient 5, who had received concurrent chemo-radiation therapy for an esophageal malignancy exhibited dose-limiting myelosuppression. As such, the MTD was 60 mg/m^2 for patients with three or more prior cytotoxic

Table 2. Summary of treatment response following DAC infusion

Patients	Diagnosis	No. prior chemotherapy	No. prior radiation therapy	No. cycles	Stage	Best response	Overall response
Cohort 1 (1.67 mg/m^2)							
1*	NSCLC:SCC	0	1	8	IIIB	SD	SD
2*	NSCLC:Adeno	1	0	2	IV	PD	PD
3*	NSCLC:Adeno	3	0	2	IV	SD	PD
4	NSCLC:Adeno	1	0	3	IV	SD	PD
9	NSCLC:SCC	5	1	2	IV	SD	SD
10*	MPM:epitheliod	1	0	2	III	PD	PD
11*	MPM:epitheliod	4	1	4	III	SD	PD
12*	SCLC	7	0	2	IV	SD	SD
13*	NSCLC: SCC	6	0	1	III	NE (DLT cycle 1)	NE
17*	EsC:Adeno	4	0	2	IV	PD	PD
20*	SCLC	3	1	2	IV	PD	PD
23*	NSCLC:Adeno	5	0	10	IV	SD	PD
24*,†	MPM:epitheliod	1	0	2	III	PD	PD
26	Ewing sarcoma	5	3	1	IV	NE (DLT cycle 1)	NE
27†	NSCLC: SCC	6	1	2	IV	PD	PD
28†	NSCLC:Adeno	4	2	2	IV	PD	PD
29	NSCLC:SCC	4	1	1	IV	NE (DLT cycle1)	NE
33	EsC:SCC	3	0	1	IV	NE (refused further treatment)	NE
34	NSCLC:Adeno	4	2	1	IV	NE (rapid deterioration of PS)	NE
Cohort 2 (2.08 mg/m^2)							
4*	NSCLC:Adeno	1	0	3	IV	SD (DLT cycle 1)	PD
5*	NSCLC:Adeno	1	1	2	IV	PD	PD
6*	NSCLC:Adeno	1	1	1	IV	PD	PD
7*	EsC:Adeno	3	2	1	IV	NE (voluntarily withdrew)	NE
8*	NSCLC:Adeno	0	0	1	IV	PD	PD
9*	NSCLC:SCC	5	1	2	IV	SD	SD
14*	MPM:epitheliod	2	0	4	III	SD	PD
15*	NSCLC:Adeno	1	0	1	IIIB	NE (occult brain metastasis detected before cycle 2)	PD
19	NSCLC:Adeno	2	1	2	IV	SD	PD
24	MPM: epitheliod	1	0	2	III	PD	PD
25*	MPM: epitheliod	1	0	2	III	PD	PD
30	SCLC	2	0	1	IV	NE (rapid spinal cord compression requiring surgery)	PD
31	EsC:Adeno	1	1	6	IV	SD	PD
32†	NSCLC:Adeno	2	1	1	IV	NE (occult brain metastasis detected before cycle 2)	PD
35	EsC:SCC	2	2	2	IV	PD	PD
Cohort 3 (2.5 mg/m^2)							
16*	MPM:epitheliod	1	0	2	I	PD	PD
18	NSCLC:Adeno	0	1	2	IV	PD	PD
19*	NSCLC:Adeno	2	1	2	IV	SD	PD
21	NSCLC:Adeno	0	0	2	IIIB	NE (refused to return for evaluation)	NE
22	EsC:SCC	2	1	1	IV	NE (death from rapid disease progression)	NE

Abbreviations: MPM, malignant pleural mesothelioma; EsC, esophageal cancer; SCC, squamous cell carcinoma; SCLC, small-cell lung carcinoma; Adeno, adenocarcinoma; SD, stable disease; PD, progressive disease; NE, not evaluable; PS, performance status.

*Tissue sufficient for immunohistochemical analysis; NY-ESO-1 induction was observed in patients 1, 4, 9, 11, and 12; MAGE-3 induction was observed in patients 1, 4, 9, 12, 13, and 14; p16 induction was observed in patients 4, 11, 13, and 20.

†Tissues used for laser capture microdissection microarray. Quantitative RT-PCR analysis of amplified RNA revealed 8-fold induction of MAGE-3 in patient 32, and 30-fold induction of MAGE-3 with 9-fold induction of p16 in patient 27. No tissue was available for confirmatory immunohistochemistry.

Table 3. Grade 3/4 toxicities during cycle 1 possibly, probably, or definitely attributable to DAC

	Grade	No. of patients
Hematology		
Anemia	3	3
Leukopenia	3	20
Leukopenia	4	7
Lymphocytopenia	3	3
Neutropenia	3	16
Neutropenia	4	15
Thrombocytopenia	3	2
Transfusion: pRBCs	3	1
Gastrointestinal		
Constipation	3	1
Constitutional		
Fatigue	4	1
Respiratory		
Hypoxia	3	1
Coagulation		
Increased PTT	3	1
Metabolic		
Increasing total billirubin	3	1
Infection		
Infected pleural effusion	3	1
Febrile neutropenia	3	1

chemotherapy regimens, and 75 mg/m^2 for individuals with two or less systemic cytotoxic regimens.

Ten of the patients enrolled on this trial were not evaluable for treatment response for a variety of reasons. Two individuals in cohort 1 developed dose-limiting neutropenia during the first cycle of treatment. One patient was noted to have an asymptomatic pulmonary embolism on computed tomography scan before cycle 2. Two patients who experienced no dose-limiting toxicities voluntarily withdrew from study after the first cycle of therapy. Three patients exhibited central nervous system metastases on scans obtained before commencing cycle 2. One individual with advanced esophageal cancer exhibited rapid disease progression, and died folloing completion of the first cycle; all hematologic toxicities attributable to DAC had resolved before his death. One individual from overseas withdrew from study after completing the second cycle without DLT, refusing to return to the National Cancer Institute for treatment evaluation.

No objective clinical responses were observed in the remaining 25 patients. Stabilization of disease was observed in four patients; one lung cancer patient (patient 1) remained on study for 10 months until being removed because of inability to image and biopsy residual disease in a previously radiated right hilum. An additional lung cancer patient (patient 23) remained on study for 1 year before exhibiting slow disease progression. Grade 3 leukopenia, thrombocytopenia, or anemia were observed in 20 of 35, 2 of 35, and 3 of 35 patients, respectively. Grade 4 neutropenia, which was observed in 15 patients, was dose limiting in four individuals. Two patients with extensive liver metastases experienced grade 3 hepatotoxicity. One individual developed an infected malignant pleural effusion. Clinical response and toxicities for all patients are summarized in Tables 2 and 3.

Pharmacokinetic analysis. Pharmacokinetic data were obtained from 16 patients, including six from cohort 1, six from cohort 2, and four from cohort 3. The steady-state concentration analysis is summarized in Table 4. Moderate interpatient heterogeneity was observed, particularly in cohort 1. Plasma concentrations achieved during cycle 1 were consistent with those observed during subsequent cycles (patients 23 and 25). Mean plasma DAC concentrations increased with increasing DAC dose administered: ($\pm$SE) 7.29 $\pm$ 0.86 ng/mL for cohort 1, 9.03 $\pm$ 0.67 ng/mL for cohort 2, and 9.56 $\pm$ 0.61 ng/mL for cohort 3 (Jonckheere-Terpstra trend two-tailed P = 0.058). These values indicate that steady-state plasma concentrations approximating 25 to 40 nmol/L were achieved during prolonged DAC infusion in patients with thoracic cancers. Our published preclinical studies indicated that DAC concentrations $\geq$50 nmol/L were sufficient for *NY-ESO-1* induction in cultured lung cancer cells (25). Identical exposure conditions mediated robust induction of *MAGE-3* as well as *p16 in vitro* (data available upon request).

Analysis of molecular end points in target tissues. A major goal of the present study was evaluation of target gene expression in tumor tissues, and assessment of immune response to NY-ESO-1 relative to plasma DAC concentrations in thoracic oncology patients. Nearly all patients accrued to the trial underwent four biopsies (pretreatment and posttreatment biopsies at cycles 1 and 2), without experiencing clinically significant sequellae; the vast majority of biopsies were obtained by computed tomography–guided FNA techniques. All specimens were reviewed immediately by cytopathology staff to confirm the presence of viable tumor cells. Quantitative RT-PCR and methylation-specific PCR analyses revealed induction of NY-ESO-1, MAGE-3 and p16 in only one of the first nine patients; these analyses were discontinued due to the presence of extensive stromal elements in the vast majority of FNA specimens (representative specimen depicted in Supplementary

Table 4. Steady-state plasma DAC levels during continuous 72-hour infusion

Cohort 1 (60 mg/m^2)			Cohort 2 (75 mg/m^2)			Cohort 3 (90 mg/m^2)		
Patient no.	Cycle	Drug level (mean $\pm$ SD)	Patient no.	Cycle	Drug Level (mean $\pm$ SD)	Patient no.	Cycle	Drug Level (mean $\pm$ SD)
1	1	10.56 $\pm$ 2.38	4	1	8.35 $\pm$ 1.84	18	1	10.1 $\pm$ 3.6
3	1	8.96 $\pm$ 1.41	8	1	9.33 $\pm$ 1.96	19	1	8.25 $\pm$ 2.55
10	1	7.31 $\pm$ 1.9	9	1	7.35 $\pm$ 2.25	21	1	10.97 $\pm$ 4.23
13	1	6.16 $\pm$ 2.1	14	1	11.05 $\pm$ 2.89	22	1	8.91 $\pm$ 1.82
23	1	5.49 $\pm$ 1.9	24	1	7.34 $\pm$ 2.07			
23	2	5.30 $\pm$ 1.76	25	1	9.37 $\pm$ 1.11			
23	3	6.47 $\pm$ 1.91	25	2	10.78 $\pm$ 2.13			
27	1	5.25 $\pm$ 1.1						

Data; Fig. 1). Immunohistochemistry analysis revealed apparent induction of NY-ESO-1, MAGE-3, and p16 in 5 of 13, 6 of 20, and 4 of 22 patients, respectively, whose tumor biopsies were sufficient for analysis. Virtually all of the non–small cell lung cancers that exhibited induction of NY-ESO-1, MAGE-3, and/or p16 had intense focal protein expression. In contrast, NY-ESO-1 or MAGE-3 protein expression was markedly uniform in one small-cell lung cancer specimen following DAC exposure (representative results of NY-ESO-1, MAGE-3, and p16 immunostains are depicted in Fig. 1A). Four patients exhibited simultaneous up-regulation of NY-ESO-1 and MAGE-3; two patients exhibited p16 with either NY-ESO-1 or MAGE-3 induction, and one lung cancer patient exhibited simultaneous induction of NY-ESO-1, MAGE-3, and p16, in tumor tissues following DAC infusion. Posttreatment antibodies to NY-ESO-1 were observed in three patients who exhibited NY-ESO-1 induction in biopsy specimens following DAC infusion. The lack of purified recombinant MAGE-3 protein precluded analysis of immune recognition of this CTA.

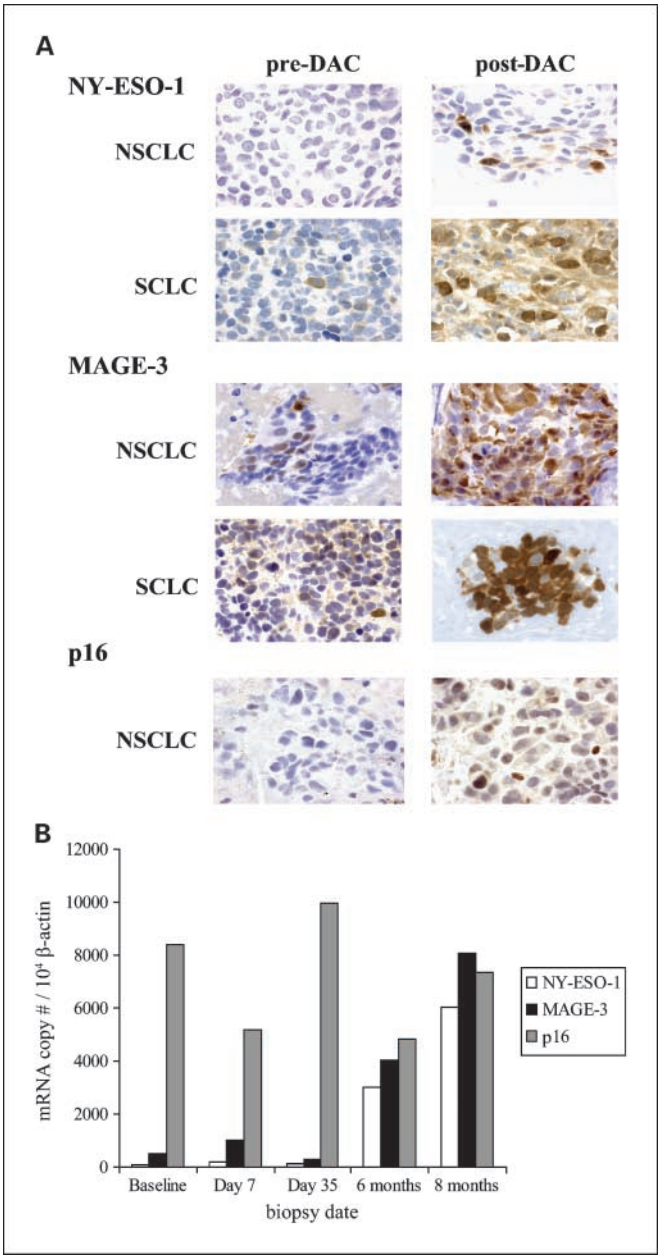

Fig. 1. Analysis of NY-ESO-1, MAGE-3, and p16 expression in tumor biopsies before and after DAC treatment. *A*, representative immunohistochemical analysis of NY-ESO-1, MAGE-3, and p16 expression in non – small cell lung cancer (*NSCLC*) and small-cell lung cancer (*SCLC*) biopsies before and after DAC treatment. CTA induction was more uniform in small-cell lung cancer in all likelihood due to higher basal levels of NY-ESO-1 and MAGE-3 before treatment. The results are consistent with our observations concerning induction of NY-ESO-1, MAGE-3, and p16 in cultured cancer lines. *B*, quantitative RT-PCR analysis of NY-ESO-1, MAGE-3, and p16 expression in lung cancer tissues from a patient exhibiting prolonged stabilization of disease following DAC treatment. A progressive increase in NY-ESO-1 and MAGE-3 mRNA copy numbers was observed; in contrast, p16 mRNA copy numbers remained relatively constant. Immunohistochemistry and serum ELISA done in a blinded manner revealed NY-ESO-1 protein expression and serum antibodies to NY-ESO-1 at the 6 months time point and thereafter.

Of particular interest was a lung cancer patient with recurrent endobronchial tumor that exhibited prolonged stabilization of disease following DAC treatment. This patient underwent numerous bronchoscopic biopsies, which were of sufficient quality to enable reliable quantitative RT-PCR analysis of target gene expression. NY-ESO-1 as well as MAGE-3 mRNA copy numbers increased dramatically, whereas p16 expression remained relatively stable over the course of DAC treatment (Fig. 1B). Interestingly, immunohistochemistry and serum ELISA (done in a blinded manner), revealed NY-ESO-1 expression as well as circulating NY-ESO-1 antibodies at the time of the fifth biopsy and thereafter. These data are consistent with a cumulative molecular treatment effect in this individual.

As this trial was nearing completion, we attempted to perform more comprehensive evaluation of gene expression in lung cancer cells mediated by DAC exposure. In particular, we sought to ascertain the effects of DAC treatment relative to gene expression profiles detected in laser-captured tumor cells and adjacent histologically normal bronchial epithelia from resected lung cancer specimens. Consistent with our observations regarding DAC treatment of lung cancer cells *in vitro* (data not shown), considerable interpatient heterogeneity was observed in baseline as well as posttreatment gene expression profiles. Data derived from eight resected specimens were sufficient for exploratory statistical analysis, allowing detection of induction/repression of gene expression with threshold of 1.2-fold and using $P < 0.05$ (two-tailed t test) to define potentially important modulation of gene expression. The limited number of arrays from DAC-treated patients precluded rigorous statistical analysis of gene modulation in these individuals. Seventy-five genes were induced, whereas 324 genes were repressed ≥2-fold following DAC treatment. Interestingly, those genes that were induced or repressed ≥2-fold by DAC seemed to be down-regulated or overexpressed, respectively, in resected primary lung cancers relative to adjacent, histologically normal bronchial epithelial cells (Fig. 2). Sixty of the 75 genes induced, and 287 of 324 genes repressed, by DAC were mapped to the PANTHER database. Subsequent Gene Ontology analysis identified several potentially enriched functional groups, including signal transduction and protein modification (Supplementary Data; Tables 1 and 2). Although limited and exploratory in nature, these data indicate that comprehensive gene expression profiling is feasible using RNA amplified from laser-captured tumor cells derived from FNAs of primary thoracic malignancies, and suggest that prolonged low-level DAC exposure can modulate global gene expression patterns in these neoplasms.

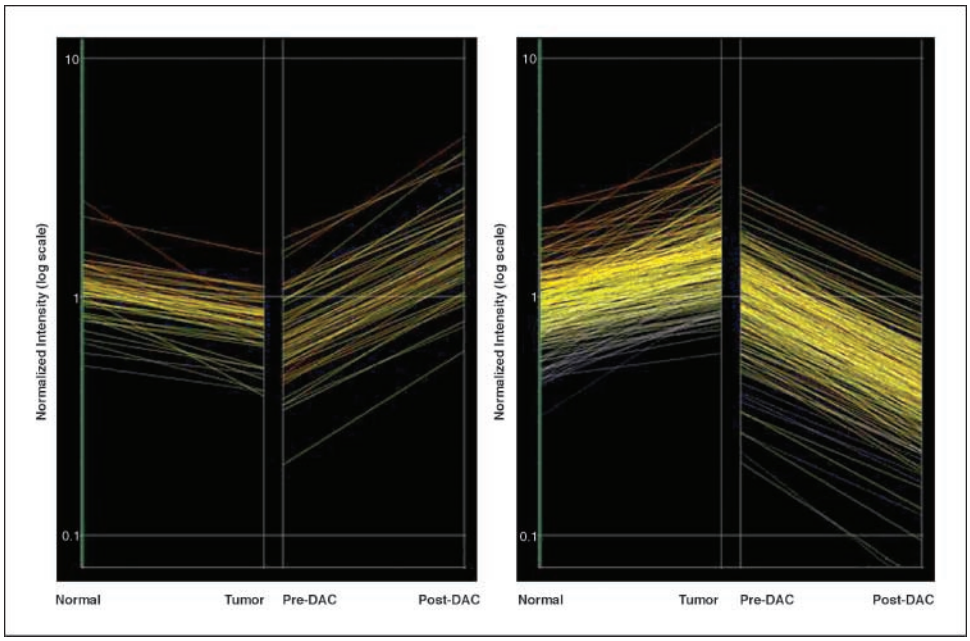

Fig. 2. Analysis of genes modulated ≥2-fold following DAC treatment in four patients, and corresponding expression of these genes in primary lung cancers relative to adjacent, histologically normal bronchial epithelia from eight patients undergoing potentially curative resections. Of note, the genes induced by DAC tended to be repressed in lung cancers relative to "normal" epithelial cells. In contrast, those genes that were repressed by DAC treatment tended to be overexpressed in lung cancer cells relative to adjacent normal bronchial epithelia. These data suggest that DAC can modulate gene expression profiles in cancer cells toward those observed in histologically normal bronchial epithelia from lung cancer patients.

Discussion

Whereas numerous clinical trials have shown activity of DAC in childhood and adult leukemias as well as myelodysplastic syndrome (32–34), relatively little data are available concerning the pharmacokinetics, toxicities, and activity of this DNA-demethylating agent in patients with thoracic malignancies. Momparler et al. (35) observed prolonged survival of one of nine lung cancer patients treated with eight hour DAC infusions.

Two recent trials have evaluated DAC infusions in patients with solid tumors using treatment schedules relevant to our current protocol. In a phase I study, Aparicio et al. (36) treated 19 patients with solid tumors (none of which were thoracic malignancies) using a 72-hour continuous DAC infusion administered on days 1 to 3 of a 28-day cycle. Twelve patients had metastatic melanoma, and the median age of all patients in this trial (49 years) was considerably less than that of individuals enrolled on our study (61 years). The MTD of DAC in these patients was 90 mg/m^2. The average plasma concentration of DAC in two patients treated at the MTD was ~140 nmol/L. Heterogeneous demethylation of 19 genes was observed in tumor tissues from five melanoma and two breast cancer patients following DAC treatment. No relationship was observed between DAC dose and genomic demethylation; no objective responses were observed in this study.

In an additional trial, Samlowski et al. (37) treated 10 patients with solid tumors refractory to standard therapy with DAC at a dose of 2 mg/kg/d × 7 days. Five of the 10 patients had renal cell carcinoma; none had a primary thoracic malignancy. Derepression of MAGE-A1 was detected in peripheral blood mononuclear cells. No data pertaining to DAC pharmacokinetics or target gene induction in tumor tissues were reported in this study. No clinical activity was observed with this treatment regimen.

The present trial was designed primarily to evaluate the feasibility and toxicity of prolonged DAC infusion in patients with lung and esophageal cancers, as well as malignant pleural mesotheliomas. The MTDs defined in our trial (60-75 mg/m^2) were slightly lower than that reported by Aparicio et al. (36), possibly reflecting the relatively advanced age, comorbidities, and cumulative toxicities of prior therapies of patients on our study. As with other trials, myelosupression was the major DLT in our study. Notably, plasma DAC levels consistently observed in our patients were considerably lower than those reported by Aparicio et al. (36). Discrepancies regarding systemic steady-state DAC concentrations detected in the two studies might be attributable, at least in part, to different cytidine deaminase levels in primary tumors or plasma from patients in the respective trials, protein binding, as well as technical issues pertaining to the acquisition, processing, or analysis of plasma samples (38).

Despite the relatively low steady-state plasma concentrations of DAC observed in thoracic oncology patients, an apparent molecular response was observed in approximately one third of patients with tumor biopsies sufficient for analysis. Specifically, 8 of 22 individuals exhibited induction of NY-ESO-1, MAGE-3, or p16 following DAC treatment, including four individuals from cohort 1, three from cohort 2, and one from cohort 3. Three patients who exhibited induction of NY-ESO-1 in tumor tissues seemed to have an increase in NY-ESO-1 serologic reactivity following DAC treatment. Although this reactivity was relatively low titered (<1:16), the presence of these autoantibodies suggests that NY-ESO-1 induced by DAC in tumor tissues was immunogenic in these individuals.

Several recent trials have used gene induction or DNA methylation/histone acetylation changes in peripheral blood mononuclear cells as surrogate markers of treatment response in cancer patients receiving chromatin remodeling agents (37, 39, 40). In our trial, we focused our efforts on examination of molecular end points in target tissues. As such, patients were not consented for acquisition of peripheral blood mononuclear cells or additional serum samples for DNA methylation analysis. In retrospect, it is unfortunate that these specimens

were not obtained in light of the fact that scant amounts of tumor cells in FNAs, and limitations concerning antigen retrieval and affinities of antibodies used for immunohistochemical analysis of CTA expression precluded more comprehensive evaluation of molecular end points in this study. In all likelihood, significant contamination of tumor aspirates with stromal elements contributed to the discordance between initial quantitative RT-PCR and methylation-specific PCR analyses relative to subsequent immunohistochemical data. Whereas all attempts were made to sequentially biopsy the same region of a target lesion before and after DAC treatment, it is possible that the apparent, focal induction of NY-ESO-1, MAGE-3, and p16 in cancer cells may have been due to sampling artifact, or different basal DNA methylation levels of various clones within tumor specimens, rather than pharmacologically mediated derepression of these target genes. On the other hand, the patterns of CTA induction detected *in vivo* are consistent with those observed in cultured lung cancer cells following exposure to DAC under comparable conditions (18, 29). Furthermore, our data regarding apparent up-regulation of target genes that are known to be modulated by DNA methylation mechanisms are consistent with recent studies by Rudek et al. (27), demonstrating inhibition of DNA methyltransferase activity in tumors from patients receiving 5-azacytidine in conjunction with phenylbutyrate.

Despite the limitations of the molecular analysis, data from this study as well as our published experiments (12, 18, 41) support additional clinical trials using chromatin remodeling agents for cancer immunotherapy, such as sequential administration of DAC with the HDAC inhibitor depsipeptide FK228 as means to enhance gene induction and apoptosis in target cells (25). In addition, our recent observations that a CTA induced *in vivo* by systemic DAC treatment can function as a bona fide target for adoptive immunotherapy in a murine tumor model (42) provide the rationale for evaluation of gene-induction regimens in conjunction with recombinant NY-ESO-1 or MAGE-3 protein vaccines (43, 44), and/or infusion of CTL recognizing these tumor antigens in cancer patients (45).

Although the microarray data must be viewed with caution due to the limited number of patients studied, it is intriguing that DAC seems to modulate expression of numerous genes, which are either up-regulated or repressed in primary lung cancer cells relative to adjacent histologically normal bronchial epithelial cells. Given the extremely low concordance between DAC responses in primary relative to cultured lung cancer cells,[8] the preliminary array data support further analysis of gene expression profiles in laser-captured tumor cells as a means to elucidate molecular mechanisms of treatment response, and to identify relevant translational end points for future protocols evaluating chromatin remodeling agents in thoracic oncology patients.

[8] M. Zhao et al., in preparation.

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